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Quinone-mediated extracellular electron transfer processes in *ex situ* biomethanation reactors

Paola A. Palacios [a], Hugo Kleikamp [b], Jeppe L. Nielsen [b], Mads B. Jensen [a], Anders Bentien [a], Michael V.W. Kofoed [a,*]

[a] Department of Biological and Chemical Engineering, Aarhus University, Aarhus 8200, Denmark
[b] Department of Chemistry and Bioscience, Aalborg University, Aalborg 9220, Denmark

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ABSTRACT

Redox mediators are used in a wide diversity of systems including biological ones. We investigated the effect of adding an artificial quinone (2,7-AQDS) as external redox molecule to an anaerobic digester system dominated by hydrogenotrophic methanogens. When oxidized AQDS was present, the methanogens diverted electrons from H_2 to reduce AQDS instead of CO_2. The AQDS reduction process was accompanied by a temporary CH_4 inhibition, which was re-established several days after the full reduction of AQDS to AH_2QDS. The presence of AQDS furthermore resulted in a community shift from *Methanobacterium* as the dominant methanogen to a more diverse community of methanogens. Protein expression profiles showed a shift in cofactor preference of the adapted community, as a potential response mechanism to AQDS inhibition. AH_2QDS was only used as electron donor to a limited extent. Stable isotope incorporation experiments here indicated that the acetogen *Acetoanaerobium* used AH_2QDS to reduce CO_2 into acetate.

1. Introduction

Current efforts concerning the climate and energy crisis aim towards the development of technologies that can help reduce greenhouse gas emissions, efficiently store renewable electricity, and produce green energy. From the biotechnological perspective, certain microbial groups such as methanogens and acetogens have the potential to become part of these multitask technologies by acting as biocatalysts, and thereby transforming and storing renewable electricity and CO_2 into biofuels and valuable products (De Vrieze et al., 2020), following the concept of Power-to-X.

As a promising technology, biomethanation aims to convert renewably sourced electricity *via* two steps: electrolysis and CO_2 reutilization (Schaaf et al., 2014). The H_2 produced *via* electrolysis can then be injected to conventional biogas reactors (*in situ*), or in reactors separated from the conventional biogas reactor (*ex situ*), so the biogas can be upgraded (Braga Nan et al., 2020). H_2-utilizing methanogens and acetogens can convert CO_2 from the biogas, and hereby increase its energy content and CH_4 composition, so the upgraded gas can be injected into the natural gas grid for further storage and use (Logroño et al., 2021).

On a volume basis, CH_4 has a higher calorific value compared to H_2, with CH_4 having a calorific value of 36 MJ m^{-3} and H_2 having a calorific value of 10.88 MJ m^{-3} (Thapa et al., 2023). Additionally, CH_4 can be directly injected into the existing natural gas grid without the need for technical modifications or stored in natural gas reservoirs (Logroño et al., 2020; Sun et al., 2015; Thapa et al., 2023; Kofoed et al., 2021). Hence, different strategies are being developed for the commercial application of biomethanation, as it also represents a promising alternative for CO_2 valorization.

Different reactors and system designs (Jensen et al., 2021), along with the addition of conductive particles (Martins et al., 2018), or redox mediators (Beckmann et al., 2016; Xu et al., 2022) account for some of the strategies that have been proposed to optimize biomethanation processes. Redox mediators have previously been used in microbial fuel cells (Gemünde et al., 2022; Rabaey et al., 2005; Watanabe et al., 2009) and in microbial electrosynthesis processes, where mediators assist in transferring electrons from cathodes, while in electro-fermentation, the presence of mediators is used to alter the redox potential and the product spectrum (Fruehauf et al., 2020). Key challenges in the use of mediators relate to their toxicity and long-term stability, which would require repeated dosing and aggravate toxicity issues (Fruehauf et al., 2020; Gemünde et al., 2022).

* Corresponding author.
E-mail address: mvk@bce.au.dk (M.V.W. Kofoed).

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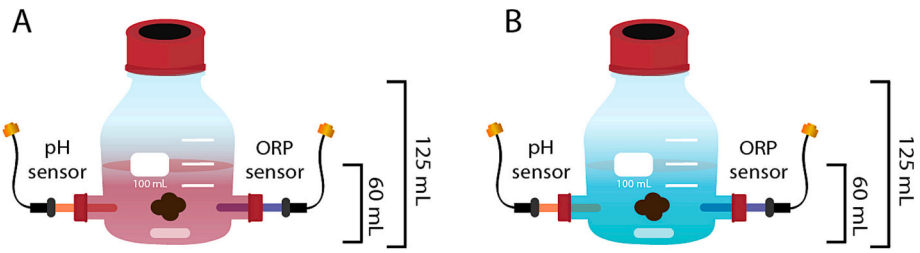

Fig. 1. Experimental set-up. Lab-scale reactors connected to an ORP and pH probes for the evaluation of (A) 2,7-AQDS as electron acceptor and electron donor in an autotrophic community during *ex situ* biomethanation processes, and (B) without AQDS (controls).

AQDS (9,10-anthraquinone-2,7-disulfonate disodium) could be an attractive redox mediator for industrial scale biomethanation, as it possesses good solubility and can be produced in large volumes at low cost (Leung et al., 2017). Its use as a humic analog in extracellular electron transfer has been explored with *Geobacter* (Lovley et al., 1999; Smith et al., 2015), *Shewanella* (Wu et al., 2014), as well as in dye-removal (Dos Santos et al., 2004). It has been shown that several electroactive methanogens can reduce AQDS (Bond and Lovley, 2002; Holmes et al., 2019), and under specific conditions they have shown favorable effects on methanogenesis (Liang et al., 2020; Xu et al., 2022). Yet, many questions remain on the utilization of AQDS, as its influence on different methanogenic systems can be perceived as contradictory.

Recent studies reported that AQDS enhanced the kinetics of H_2-fueled biological methanation, resulting in a 160 % increase in the H_2 consumption rates and up to 181 % increase in CH_4 rates (Tucci et al., 2023). It has also been suggested that AQDS serves as an electron shuttle to stimulate mediated interspecies electron transfer (MIET) among electroactive consortia, which accelerated acetate methanation and improved methanogenesis under ammonia stress (Xu et al., 2022). Nevertheless, electron transfer to humics in anoxic systems is considered to inhibit the reduction of other terminal electron acceptors, including CO_2 under methanogenic conditions (Klüpfel et al., 2014). In fact, the reduction of AQDS is more favorable than CO_2 reduction through methanogenesis (Cervantes et al., 2000), and different studies have reported that methanogenesis can be inhibited by the addition of AQDS (Cervantes et al., 2000; Garcia-Lopez et al., 1996; Ye et al., 2016). It is possible that the diverse microbial groups and metabolisms present in anaerobic digesters and biomethanation reactors can make it difficult to assess whether AQDS/AH_2QDS has a direct impact on methanogens and acetogens, which are the main microbial groups involved in CO_2 conversion.

In this study, we investigated the effect of exogenous AQDS addition on the H_2-based conversion of CO_2 to methane in *ex situ* biomethanation lab-scale reactors, and the microbial response and processes elicited by the added AQDS. By integrating different approaches such as metaproteomics, redox potential measurements, and conventional microbiology techniques, we studied the autotrophic microbial responses and processes influenced by AQDS in *ex situ* biomethanation reactors and whether it could mediate electron transfer processes *via* AQDS, as an exogenous redox molecule. We furthermore explored some of the microbial adaptations to high AQDS concentrations, and if reduced AQDS (AH_2QDS) could transfer electrons for microbial CO_2 reduction.

2. Materials and methods

2.1. Enrichment culture conditions

Mesophilic digestate sludge (inoculum) was obtained from an anaerobic digester fed with organic residues and manure, operated at 39 °C from Bånlev Biogas plant in Trige, Denmark. The sample was sieved through a 0.8 mm mesh and transferred to a 2L DURAN® glass reactor to enrich the inoculum under an anoxic atmosphere of H_2/CO_2 gas mix (80:20, v/v) at 1.8 bar. After one week of incubation at 37 °C and a daily flush with H_2/CO_2 gas mix, 10 % of the inoculum was transferred in a defined methanogenic medium. The mineral phosphate medium contained K_2HPO_4 (5.0 g/L) and KH_2PO_4 (2.5 g/L) to maintain a neutral pH (6.8–7.2) in the absence of $NaHCO_3$, as previously optimized (Chen et al., 2019). The medium also contained NH_4Cl (1 g/L), KCl (0.05 g/L), NaCl (1 g/L), $CaCl_2{\cdot}2H_2O$ (0.05 g/L), $MgCl_2{\cdot}6H_2O$ (0.16 g/L), 10 mL of modified Wolin's mineral solution 141 (DSMZ), and 10 mL of 141 Wolin's vitamin solution (DSMZ). An autotrophic microbial community for biogas production was enriched after five consecutive transfers (10 % inoculum was transferred every two weeks) using the defined methanogenic medium, and by supplementing H_2 as sole electron donor, and CO_2 as the only electron acceptor and carbon source (H_2/CO_2 gas mix, 80:20).

Anthraquinone disulfonate disodium salt 98 % (2,7-AQDS) was purchased from TCI ($E^0_{SHE} = \sim -200\,mV@pH = 7$) (Huskinson et al., 2014; Wedege et al., 2016). The effect of 9,10-anthraquinone-2,7-disulfonate disodium (2,7-AQDS) was initially tested at different concentrations (see Supplementary material) using digestate that was not enriched with H_2/CO_2. The digestate was incubated with 0–250 mM AQDS in 120 mL chlorobutyl-rubber stoppered glass vials with a headspace of H_2/CO_2 gas mix (80:20). Pressure values were continuously monitored, and gas analyses were performed to confirm the effect of AQDS on methane yields. The concentration of AQDS has a dose-dependent effect on CH_4 yields from the consumed H_2. Based on these experiments, the concentration of 25 mM AQDS was selected.

2.2. ORP/pH reactors set-up

The experimental set-up for the continuous monitoring of the microbial culture's redox potential (transfer No.5), and the redox state (oxidized/reduced) of the supplemented AQDS was performed *via* an oxidation reduction potential (ORP) probe (Atlas Scientific LLC, Long-Island City, USA), and a pH probe (Atlas Scientific LLC, LongIsland City, USA). Each sensor (ORP and pH) was tightly attached to a GL25 glass port assembled into a 100 mL Duran glass bottle (see Fig. 1). The reactors were also sealed with a black butyl rubber stopper, and a screw cap to maintain the cultures under anoxic conditions. The working volume of the reactors was 60 mL, with 50 % of fresh methanogenic defined medium and 50 % of inoculum (transfer No.5). Three replicates of the autotrophic cultures were exposed to 25 mM AQDS (in oxidized state) and three replicates without AQDS were used as controls. All reactors were daily flushed with H_2/CO_2 gas mix (80:20, v/v), and the initial pressures were adjusted to 1.8 bar for 18 days. On day 18 and 19, the headspace of all reactors was changed to N_2/CO_2 gas mix (80:20, v/v) at 1.3 bar to test if the microbially reduced AQDS (AH_2QDS) could act as an electron donor for the autotrophic community. In day 20, the headspace was exchanged back to H_2/CO_2 to allow the full microbial reduction of AQDS, and in day 21 and 22 N_2/CO_2 gas mix were re-used to corroborate if AH_2QDS could act as sole electron donor during CO_2 conversion by supplementing all the cultures with 7.5 mM of $NaHCO_3$ mixed with 2.5 mM of ^{13}C-labelled $NaHCO_3$ (98 atom % 13C, 99 %, Sigma-Aldrich) for proteomic analyses. The final concentration of added $NaHCO_3$ was 10 mM. Pressure measurements along with gas and liquid

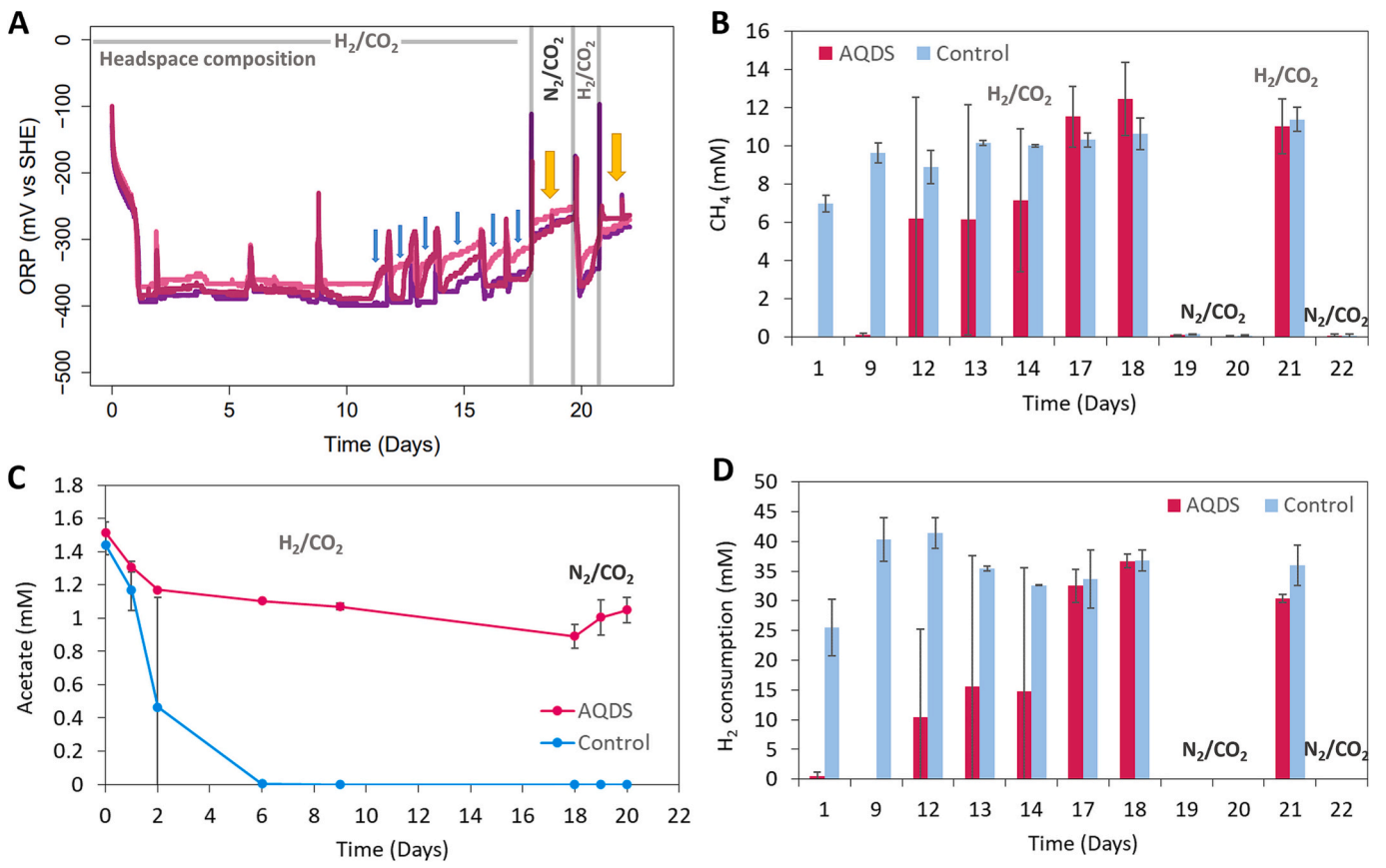

Fig. 2. Evaluation of 2,7-AQDS as electron acceptor and electron donor in an autotrophic community during *ex situ* biomethanation processes. (A) ORP values obtained from microbial reactors supplemented with AQDS. The blue arrows represent the oxidation of AH_2QDS due to small O_2 leakages, and the yellow arrows represent the potential microbial oxidation of AH_2QDS. (B) Daily methane production, (C) accumulated acetate production, and (D) daily hydrogen consumption, when AQDS was used as electron acceptor (under a H_2/CO_2 headspace), and as electron donor (AH_2QDS, under a N_2/CO_2 headspace). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

samples were constantly taken from all reactors to monitor H_2, CO_2, CH_4, N_2 and VFAs.

2.3. 16S rRNA amplicon sequencing

Microbial community analyses of Bånlev digester sludge, and transfer No. 4 of the autotrophic enrichment culture were performed *via* gene amplicon sequencing targeting the bacteria/archaea 16S rRNA gene variable region 4 (abV4-C) in combination with taxonomic classification against the MiDAS 4.8.1 database. DNA extraction, QC, sequencing library preparation, DNA sequencing (Illumina MiSeq 2 × 300 PE, scaled for 50k raw reads/sample) and data analysis with taxonomic classification were performed by DNASense ApS (Aalborg, Denmark).

2.4. Metaproteomics

For protein extraction, lysis of cell pellets was performed with a Covaris Tissue homogenizer. Subsequent digestion, reduction and alkylation and purification were done using the PreOmics Its kit. Analysis of peptides was done using ESI LC-MS on a QE Orbitrap system with a 2-h gradient (García-Moreno et al., 2020).

2.5. Metaproteomics data analysis

Metagenome-free annotation was performed against GTDB r207 with the CHEW pipeline (https://github.com/hbckleikamp/CHEW), which involves an initial search against UniRef50 with MSFragger (Kong et al., 2017) followed by homology search with DIAMOND (Buchfink et al., 2015) against an unclustered GTDB protein reference database (Parks et al., 2022), and refinement cycles to construct a sample-specific focused database. Final database searching was performed against the focused database with variable modifications: oxidation, phosphorylation, acetylation and pyroglutamate formation. The filtering cutoff was set to a false discovery rate of 0.05. Functions of identified proteins were annotated with KEGG orthologies GhostKOALA (Kanehisa et al., 2016; Kanehisa and Goto, 2000). To analyze the shift in redox mediators, iron-sulfur proteins and cytochromes, identified proteins were aligned to UniprotKB to obtain EC numbers, which were then mapped to BRENDA (Schomburg et al., 2002) and narrowed down to the nearest taxonomy. The BRENDA enzyme database was used to annotate expressed hydrogenases to check the shift in cofactor preference, as well as the use of iron-sulfur clusters and cytochromes. Stable isotope incorporation was tracked using Calis-P (Kleiner et al., 2023).

2.6. Chemical analyses

Prior to gas sampling, pressure values from the culture's headspace were obtained with a gas pressure sensor. The gas composition of the cultures was then monitored with a gas chromatography system (GC-2014, Shimadzu, Japan) equipped with a thermal conductivity detector (TCD) and two different columns. For H_2 analyses, a ShinCarbon ST Packed Column (Restek Cat#80486-800, Bellafonte, Pennsylvania, USA) was used with argon as the carrier gas, whereas for CO_2 and CH_4 analyses a Porapak Q column (CS-Chromatographie Service GmbH, Manufacturer Item No.: 662, Langerwehe, Germany) was used with

helium as carrier gas.

Volatile fatty acids (VFA) analyses from the liquid samples were also determined by gas chromatography. The sample preparation involved 1 mL of liquid sample dissolved in a 4 mL solution of 0.3 M oxalic acid containing an internal standard of pivalic acid (trimethylacetic acid). The solution was homogenized for 10 min on a rotation mixer and centrifuged for 10 min at 4700 rpm. The supernatant was then filtered (0.45 μM) and transferred to a sampling vial to be analyzed on a gas chromatograph system (6890N Agilent Technologies, United States) with an HP-INNOWAX column (30-m length, 0.25-mm i.d., and 0.25-μm film unit) connected to a flame ionization detector (FID) (Agilent Technologies, United States) using helium as a carrier gas.

3. Results and discussion

3.1. Community composition of the autotrophic enrichment

To properly address the effect and role of AQDS as electron mediator during microbial CO_2 reduction processes (including *ex situ* biomethanation), the microbial community from Bånlev digestate was enriched under autotrophic conditions with 80 % H_2 and 20 % CO_2 as feed gas, using 10 % of inoculum and four consecutive transfers. 16S rRNA gene amplicon sequencing data from the initial Bånlev sludge samples showed that the most abundant genera in relative abundance were *Clostridium sensu_stricto_1* (21.6 %), and *Gallicola* (15.5 %). *Methanosarcina* was the most abundant methanogen with a relative abundance of 7.5 % (see Supplementary material). After the 4th transfer, a clear difference in the microbial composition was observed, as hydrogenotrophic methanogens from the genera *Methanobrevibacter* (28.95 %), *Methanobacterium* (28.42 %), and *Methanoculleus* (3 %) were dominating the community along with homoacetogenic bacteria from the genus *Sporomusa* (15.04 %).

3.2. Role of 2,7-AQDS as electron mediator in the autotrophic community

Once the microbial community was enriched, the role of AQDS as electron mediator in CO_2 reduction processes was evaluated in lab-scale batch reactors. The AQDS microbial reactors were supplemented with 25 mM of the oxidized redox mediator, while being absent in the control reactors. With this set-up (see Fig. 1), AQDS was tested on its ability to act as: (i) electron acceptor by keeping a headspace composition of H_2/CO_2 (80:20, v/v) in all reactors, and (ii) as electron donor by fully flushing and exchanging the headspace composition of all reactors to a N_2/CO_2 gas mix (80:20, v/v), so the sole electron donor available would be the microbially reduced AH_2QDS.

ORP results showed that during the first 18 days, the autotrophic community was able to use AQDS as electron acceptor. Once the reactors were flushed and fed with H_2/CO_2, the ORP values started to decrease. In <2 days, the lowest reduction state of AQDS was achieved with a value of −381 ± 13.8 mV (see Fig. 2A), and no changes in pH were induced (see Supplementary material). During these 2 days of initial microbial AQDS reduction, there was no CH_4 production, opposite to what was observed in the controls without AQDS (see Fig. 2B). All reactors were fed with H_2 as electron donor, and CO_2 as electron acceptor (daily), but in incubations with AQDS no CH_4 was produced. At the same time, the ORP of the incubations with AQDS decreased to −381 ± 13.8 mV (see Fig. 2A). In broad terms, oxidation-reduction potential (ORP) is a measurement of the redox state of a solution in millivolts (mV). AQDS has a standard redox potential around −200 mV at neutral, depending on the exact pH and electrolyte composition (Huskinson et al., 2014; Khataee et al., 2017). By definition, if the ORP measured is the same as the standard potential, the molecule is 50/50 in its reduced/oxidized state. Despite H_2 having a lower redox potential than AQDS, it cannot be reduced without a catalyst (*e.g.* Pt) (Preger et al., 2020). For this reason, any change in the ORP must be microbially mediated. If the ORP becomes more positive AQDS is oxidized, and if it becomes more negative AQDS is reduced. Therefore, the combined decrease in ORP values, and absence of methane production showed that AQDS was microbially reduced (see Fig. 2A and B). The presence of AQDS also inhibited acetate consumption, which could indicate an inhibition not only of hydrogenotrophic methanogenesis but also of acetoclastic methanogenesis (see Fig. 2C). Similar outcomes are also reported in literature, but using 20 mM of anthraquinone-2,6- disulfonate (2,6-AQDS), and anaerobic microbial consortia from sludge, sediments, and paddy soils (Cervantes et al., 2000). In the presence of H_2 ($E\,0' = -414$ mV), this phenomenon could be explained by the fact that AQDS (2,6) respiration ($E\,0' = -184$ mV) is thermodynamically more favorable than methanogenesis ($E\,0'$ of $CO_2/CH_4 = -240$ mV). CO_2 became the less favorable electron acceptor due to its lower redox potential, and thus, the flux of electrons got diverted towards the reduction of AQDS. However, the standard potentials are under standard conditions *i.e.* if the reaction quotient deviates far from unity, the actual potential can decrease/increase as much as 0.1 V. Because of this, it is speculated that the reason why AQDS is preferred as electron acceptor is that it is catalyzed better by the microbes, potentially due to higher activation barrier or slower reaction kinetics of CO_2 reduction. It is here noted that AQDS can only be reduced chemically by H_2 in the presence of a Pt or other PGM catalyst (Preger et al., 2020). While CO_2 can only be reduced chemically by H_2 in the presence of a nickel catalyst and elevated temperatures (>300 °C) and pressure (Sabatier reaction). Thus, demonstrating that the AQDS reduction is microbially mediated.

Surprisingly, methane production in the AQDS reactors was not detected until day nine, even though AQDS reached its maximum reduced state (AH_2QDS) before day 2. With the Nernst equation it was estimated that the ORP of −375 mV corresponded to >99.9 % of AQDS in its reduced state. After reaching full reduction, the methane rates were not enhanced by the presence of AH_2QDS compared to the controls without mediator. It has been reported that methane inhibition is alleviated once the AQDS is fully reduced (Xu et al., 2013). Yet, it took around 17 days to reach similar methane rates in all reactors compared to the controls without AQDS (see Fig. 2B), suggesting that the microbial community was going through a secondary adaptation phase to enrich autotrophic microorganisms that could tolerate and grow in the presence of this higher energy yielding electron acceptor.

After the methanogenic community adapted to AQDS (mainly present in a reduced state), and methane production was restored, oxygen leakages became more frequent due to negative pressure values caused by consumption of H_2 in the headspace by the hydrogenotrophic methanogenesis process ($4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$). This chemical oxidation (O_2 leakages) would normally oxidize AH_2QDS and increase the ORP values. However, the ORP values were continuously decreased towards −381 mV, confirming that the autotrophic community could keep the redox mediator in a reduced state by using H_2 as electron donor (see Fig. 2A and D).

Furthermore, in this study the presence of acetate in the reactors was a result of the microbial metabolism (acetate was not added as a co-substrate), and it was clear that acetate consumption was affected through all the experiments even when AQDS was present in a reduced state. The negligible capacity of reducing AQDS with acetate as electron donor has been reported in granular sludge consortia (Cervantes et al., 2000), as observed in our AQDS reactors.

After concluding that AQDS could be used as an electron acceptor by the adapted microbial community, we investigated whether AH_2QDS could be used as an electron donor for the reduction of CO_2. On day 19 and 20, all reactors were flushed with a N_2:CO_2 gas mix to make sure that AH_2QDS was the sole electron donor, while the controls did not contain any H_2 as energy source. The pressure of all reactors remained constant under a N_2/CO_2 headspace at 1.3 bar to avoid oxygen leakages. Under these conditions the controls and the AQDS reactors produced very small amounts of methane (0.11 ± 0.04 mM and 0.08 ± 0.04 mM, respectively). If all electrons of the 25 mM AQDS were utilized, it would result in a headspace concentration of 6.25 mM CH_4, however, the AQDS

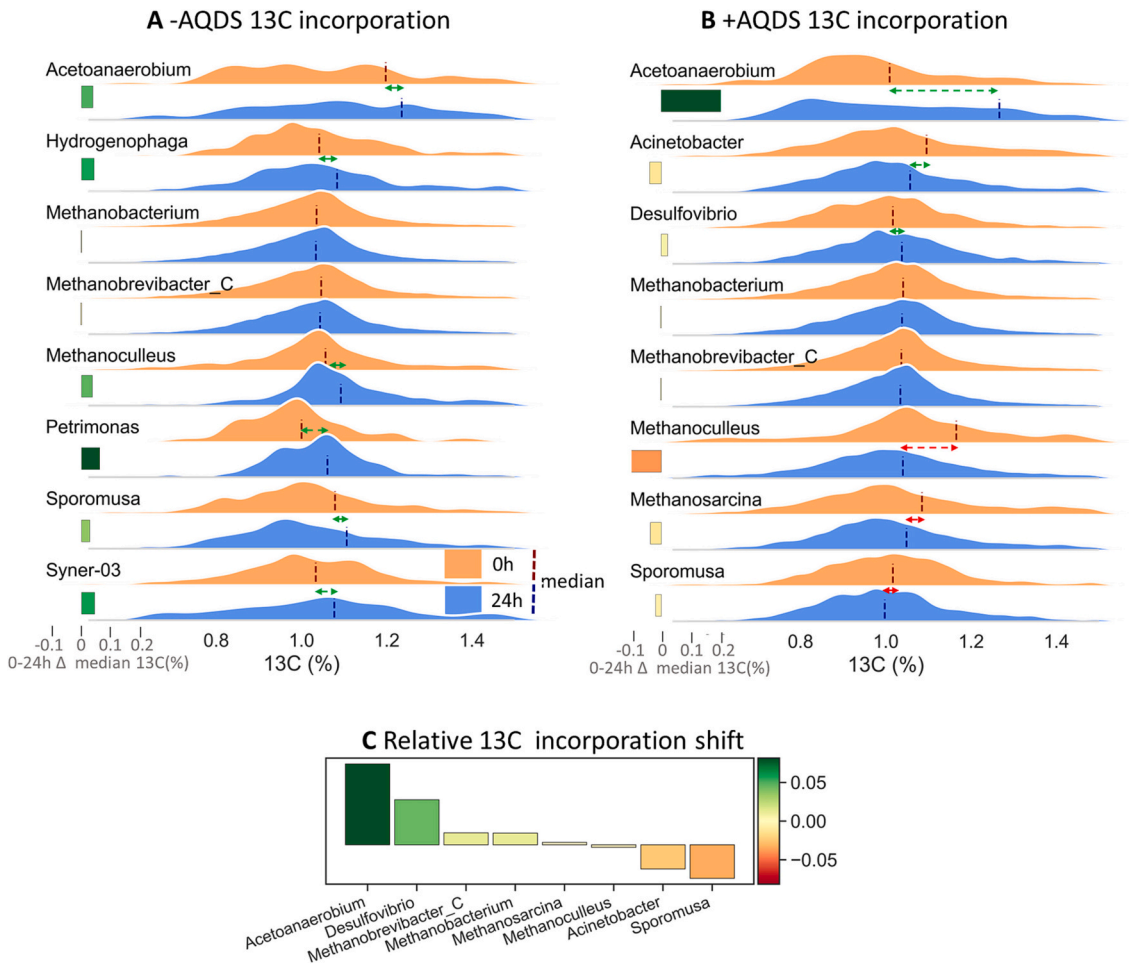

Fig. 3. Stable isotope incorporation. C13(%) abundance patterns before (orange, 0 h) and after (blue, 24 h) incubation with 2.5 mM labelled sodium bicarbonate of controls as (A) AQDS- and (B) AQDS+ cultures with delta median C13(%) incorporation. C: describes the shift of C12/13 ratios normalized to total biomass contributions between AQDS+ and AQDS- cultures. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

enriched methanogens were not capable of utilizing electrons from the AH_2QDS to reduce CO_2 (see Fig. 2B). Yet, as observed in Fig. 2A, there is another factor causing the oxidation of AH_2QDS on day 19 and 20. A similar trend of oxidation was observed for a second time on day 22. During these two periods of AH_2QDS oxidation, the ORP values went approximately from -366 ± 16.5 mV to -271 ± 9 mV (see Fig. 2A), but no methane production was observed in days 19 and 20 nor in day 22 (see Fig. 2B). This increase in ORP was accompanied by a slight increase in the acetate production (from 0.8 ± 0.07 mM to 1 ± 0.07 mM) (see Fig. 2C), which suggests that few electrons from AH_2QDS might have been used to reduce CO_2 into acetate.

3.3. *Acetoanaerobium oxidizes AH_2QDS to reduce CO_2 into acetate*

To investigate if acetogens or other microorganisms could have utilized the reduced AH_2QDS, stable isotope incorporation was performed with ^{13}C-labelled $NaHCO_3$ in all reactors, without any H_2 in the headspace on day 22. After 24 h, small amounts of labeling were observed in all the samples, with median labeling ratios centering mostly around the natural ^{13}C abundance of 1.1 %. Controls without AQDS showed slight ^{13}C-incorporation for a range of acetogens, such as *Petrimonas* and *Sporomusa*, and commamox genus *Hydrogenophaga*, but comparatively less incorporation in methanogens *Methanobacterium* and *Methanobrevibacter*, which showed highly stable isotopic profiles (see Fig. 3). The largest delta 13C was observed for proteins affiliated with *Acetoanaerobium*, which showed a more pronounced incorporation compared to other organisms. Comparing genera that were abundant in all samples, the shift in ^{13}C allocation normalized to the total number of carbon atoms, highlighted that *Acetoanaerobium* accounted for a larger fraction of ^{13}C-incorporation, relative to their biomass in the AQDS culture (see Fig. 3C). These results indicate that *Acetoanaerobium* was the CO_2-reducer responsible for the oxidation of AH_2QDS and the small acetate increase. Utilization (oxidation) of AH_2QDS was accompanied by an increase in ORP values and acetate production which could imply that *Acetoanaerobium* was using electrons from AH_2QDS to reduce CO_2 into acetate (see Fig. 2A and C). This is not the first time that this homoacetogen is related to extracellular electron uptake processes as this genus has been enriched on metallic iron (Fe^0), and negative poised cathodes (Jourdin et al., 2016; Philips, 2020; Philips et al., 2019; Xafenias and Mapelli, 2014).

3.4. *Metaproteomics reveal details on the community adaptive response to AQDS*

Metaproteomics were used to investigate changes in the microbial community composition and expressed functions of the incubations supplemented with AQDS and the controls without AQDS. This showed a clear change in the taxonomic abundance of various methanogens. Methanogens were predominant in both communities, with and without AQDS, particularly the genera *Methanobacterium* and *Methanobrevibacter* (see Fig. 4). While the community without AQDS was dominated by *Methanobacterium*, the AQDS community showed an increase in

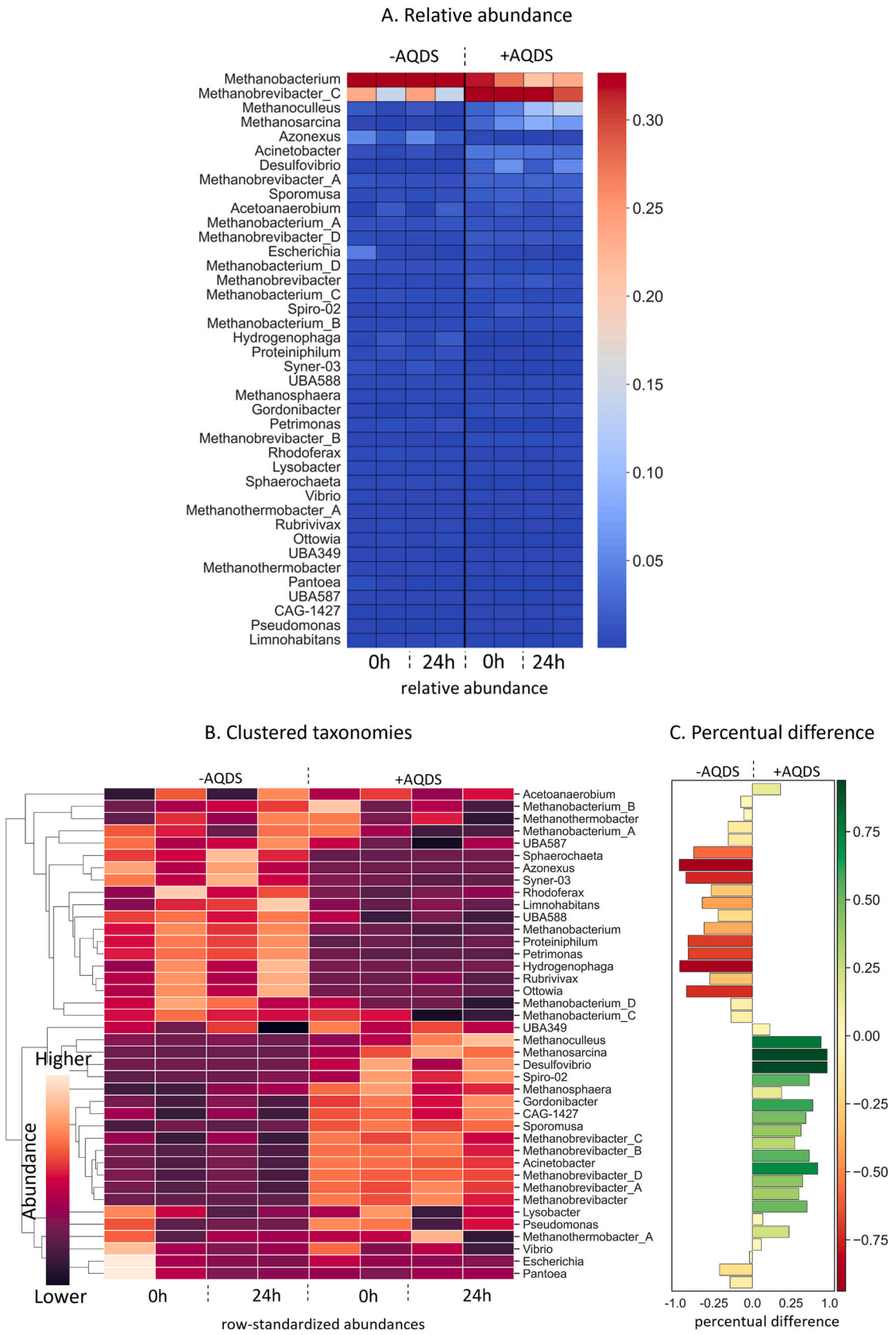

Fig. 4. Analysis of taxonomic shift between controls without AQDS (AQDS-) and AQDS reactors (AQDS+) including two samples at 0 h and 24 h of the SIP experiment. (A) Relative metaproteomic abundance of peptide spectrum counts normalized to the top 40 most abundant genera. (B) row-clustering of standardized abundances, showing a shift from higher (bright) to lower (dark) abundance, and C: the percentual difference of median abundances between AQDS+ and AQDS- samples.

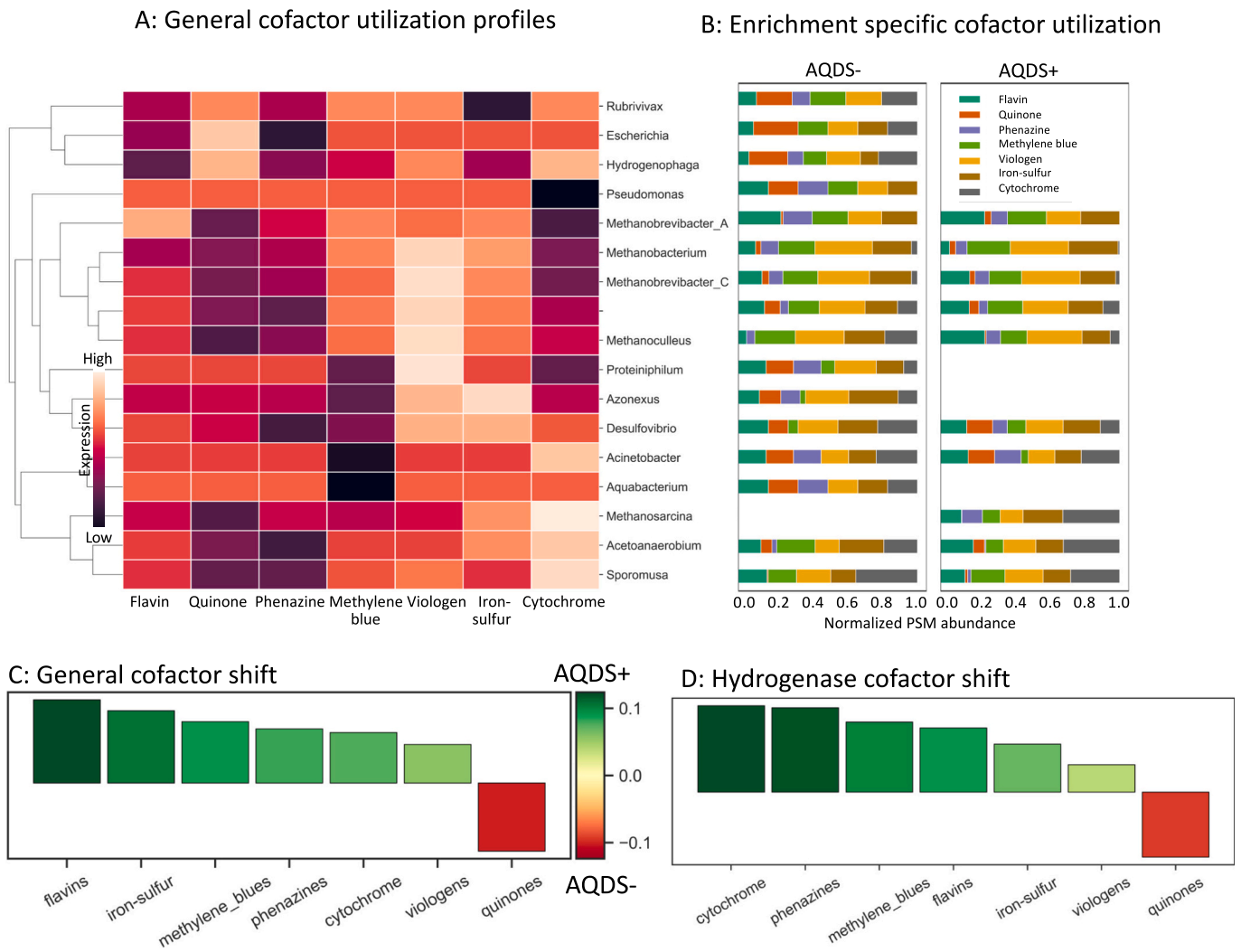

Fig. 5. Analysis of cofactor preference. Metadata from the BRENDA enzyme database was used to map cofactor preference of expressed enzymes, which was targeted specifically towards redox mediators (flavin, quinone, phenazine, methylene blue, viologen) as well as annotating usage of iron-sulfur clusters and cytochromes. A: shows the mean expression patterns for each category over combined controls as AQDS- and AQDS+ samples, clustered among genera using Euclidian distance between normalized peptide spectrum matches attributed to each category. B shows normalized PSM distribution of each category for each genus of A. C & D show median percentual differences of cofactor categories between AQDS+ and AQDS- samples for combined genera, with C for all enzymes, and D specifically for hydrogenases.

methanogenic archaea diversity, including the genera *Methanoculleus*, *Methanosarcina*, as well as *Methanosphaera* and *Methanothermobacter*. Other community shifts in the AQDS reactors included an increase in the bacteria *Sporomusa* and *Desulfovibrio*, both of which have been implicated with extracellular electron transfer (Walker et al., 2009), and a decrease in acetogens *Proteiniphilum* and *Petrimonas* (Hahnke et al., 2016) and commamox genera *Hydrogenophaga* and *Ottowia*. Methanogens and *Desulfovibrio* have been previously shown to participate in syntrophic relations through extracellular electron transfer with AQDS (Cervantes et al., 2002; Stams and Plugge, 2009).

Compared to a clear taxonomic shift, the distribution of expressed functions remained highly similar, for both general KEGG modules and methanogenesis specific enzymes (see Supplementary material). Distribution of global KEGG modules showed methane metabolism as the most abundant module, with a slight increase in the AQDS enrichment. Metabolism of cysteine, which lowers the redox potential and is a key amino acid in methanogenic enzymes, was less abundant in the AQDS enrichment. Shifts in less abundant modules include a shift from nitrogen to sulfur metabolism, which coincides with a taxonomic shift of *Hydrogenophaga* and *Ottowia* to *Desulfovibrio* (Cai et al., 2021).

Since functional profiles proved highly similar, a more in-depth look was given to the cofactor preference in expressed enzymes. Using the BRENDA enzyme database, enzymes could be grouped by their preferred class of redox mediator: flavin, quinone, viologen, phenazine, methylene blue, as well as the usage of cytochrome or iron-sulfur clusters (see Fig. 5A). The general cofactor preference of expressed enzymes was compared with and without AQDS, which showed that methanogens mostly preferred viologen utilizing enzymes over quinone-like cofactors, which could explain the lack of methane production when AQDS acted as sole electron donor. *Methanosarcina* expressed more cytochromes and showed greater similarity in cofactor preference to *Acetoanaerobium* and *Sporomusa*, which are known to be electroactive (Aryal et al., 2017; Jourdin et al., 2016; Philips, 2020; Philips et al., 2019; Xafenias and Mapelli, 2014). Cytochromes have often been implicated in extracellular electron transfer, though the exact mechanisms remain largely unknown (Gemünde et al., 2022; Smith et al., 2015; Wu et al., 2014). Several genera that showed higher quinone utilization, such as *Rubrivivax*, *Escherichia* and *Hydrogenophaga* were less abundant in the AQDS enrichment, which could suggest that AQDS inhibits a key enzyme (see Fig. 5B). Looking specifically at hydrogenases (see Fig. 5D), which are the prime candidates to utilize AQDS, quinone-preferring hydrogenases were expressed less in the AQDS enrichment, while cytochromes and

phenazines were expressed more. This implies that the addition of AQDS was ineffective at enriching quinone-utilizing microorganisms. Though BRENDA annotations provide an indication of cofactor preference, the analysis of natural redox mediator usage is challenging, as they are produced in small amounts, and synthetic routes often overlap with aromatic amino acids, or are poorly characterized. Investigation of cofactor preference by use of molecular dynamics and docking simulations could be an effective strategy to identify targets of inhibition, and preferred combinations of methanogens and redox mediators (Beeckman et al., 2022; Hoshino and Gaucher, 2018; Pang et al., 2021; Schomburg et al., 2002; Wong et al., 2022).

4. Conclusions

Mediated extracellular electron transfer using AQDS as redox mediator, was studied in biomethanation reactors. The H_2/CO_2 enriched community used AQDS as main electron acceptor, and AQDS reduction was accompanied by a temporary CH_4 inhibition, low acetate accumulation, and a shift in the community composition from *Methanobacterium* to a more diverse group of archaea. CH_4 production was re-established after the reduction of AQDS, but CH_4 production was not enhanced. Metaproteomics revealed a shift in cofactor preference within the community, which moved away from quinone-like substrates in the AQDS community. AH_2QDS was also tested as a sole electron donor. Stable isotope incorporation analysis showed that *Acetoanaerobium* converted CO_2 into acetate *via* AH_2QDS oxidation.

CRediT authorship contribution statement

Paola A. Palacios: Conceptualization, Methodology, Investigation, Writing- Original draft preparation. Hugo Kleikamp: Conceptualization, Methodology, Investigation, Writing- Original draft preparation. Jeppe L. Nielsen: Conceptualization, Supervision, Funding acquisition. Anders Bentien: Conceptualization, Supervision, Funding acquisition. Mads B. Jensen: Investigation. Michael V. W. Kofoed: Conceptualization, Supervision, Writing - Review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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