Journal of Visualized Experiments

Extraction of cofactor F420 for analysis of polyglutamate tail length from methanogenic pure cultures and environmental samples

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE62737R2
Full Title:	Extraction of cofactor F420 for analysis of polyglutamate tail length from methanogenic pure cultures and environmental samples
Corresponding Author:	Andreas Otto Wagner Universitat Innsbruck, Institute of Microbiology Innsbruck, Tyrol AUSTRIA
Corresponding Author's Institution:	Universitat Innsbruck, Institute of Microbiology
Corresponding Author E-Mail:	Andreas.Wagner@uibk.ac.at
Order of Authors:	Andreas Otto Wagner
	Rudolf Markt
	Mathias Wunderer
	Eva Maria Prem
	Mira Mutschlechner
	Nina Lackner
Additional Information:	
Question	**Response**
Please specify the section of the submitted manuscript.	Environment
Please indicate whether this article will be Standard Access or Open Access.	Standard Access ($1400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Innsbruck, Tyrol, Austria
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please provide any comments to the journal here.	
Please confirm that you have read and agree to the terms and conditions of the video release that applies below:	I agree to the Video Release

TITLE:

Extraction of Cofactor F_{420} for Analysis of Polyglutamate Tail Length from Methanogenic Pure Cultures and Environmental Samples

AUTHORS AND AFFILIATIONS:

Rudolf Markt[1], Mathias Wunderer[1], Eva Maria Prem[1], Mira Mutschlechner[1], Nina Lackner[2], Andreas Otto Wagner[1]

[1]Department of Microbiology, Universität Innsbruck, Innsbruck, Austria
[2]Department of Hygiene and Medical Microbiology, Medical University of Innsbruck, Innsbruck Austria

Email addresses of co-authors:

Rudolf Markt	(rudolf.markt@uibk.ac.at)
Mathias Wunderer	(mathias.wunderer@uibk.ac.at)
Eva Maria Prem	(eva.prem@uibk.ac.at)
Mira Mutschlechner	(mira.mutschlechner@uibk.ac.at)
Nina Lackner	(nina.lackner@i-med.ac.at)
Andreas Otto Wagner	(andreas.wagner@uibk.ac.at)

Corresponding author:

Rudolf Markt	(rudolf.markt@uibk.ac.at)

KEYWORDS:

F_{420}, coenzyme F_{420}, cofactor F_{420}, redox coenzyme, methanogenesis, solid-phase extraction, F_{420} tail length, polyglutamate tail

SUMMARY:

A method for the extraction of cofactor F_{420} from pure cultures was optimized for the liquid chromatographic separation and analysis of F_{420} tail length in pure culture and environmental samples.

ABSTRACT:

The cofactor F_{420} plays a central role as a hydride carrier in the primary and secondary metabolism of many bacterial and archaeal taxa. The cofactor is best known for its role in methanogenesis, where it facilitates thermodynamically difficult reactions. As the polyglutamate tail varies in length between different organisms, length profile analyses might be a powerful tool for distinguishing and characterizing different groups and pathways in various habitats. Here, the protocol describes the extraction and optimization of cofactor F_{420} detection by applying solid-phase extraction combined with high-performance liquid chromatography analysis independent of cultural or molecular biological approaches. The method was applied to gain additional information on the expression of cofactor F_{420} from microbial communities in soils, anaerobic sludge, and pure cultures and was evaluated by spiking experiments. Thereby, the study succeeded in generating different F_{420} tail-length profiles for hydrogenotrophic and acetoclastic

methanogens in controlled methanogenic pure cultures as well as from environmental samples such as anaerobic digester sludge and soils.

INTRODUCTION:

F_{420} is a widespread but often neglected cofactor, which functions as an obligate two-electron hydride carrier in primary and secondary metabolic processes of both *Archaea* and *Bacteria*[1,2]. F_{420} is a 5-deazaflavin and structurally similar to flavins, whereby its chemical and biological properties are more comparable with those of NAD^+ or $NADP^+$. Due to the substitution of nitrogen with carbon at position 5 of the isoalloxazine ring, it is a strong reductant, thus exhibiting a low standard redox potential of -340 mV[1,3]. F_{420} comprises a 5-deazaflavin ring and a 2-phospho-L-lactate linker (F_{420}-0). An oligoglutamate tail containing n+1 glutamate monomers can be attached to the molecule (F_{420}-n+1)[4].

For a long time, the cofactor F_{420} has been solely associated with *Archaea* and *Actinobacteria*. This has largely been overturned. Recent analyses revealed that F_{420} is distributed among diverse anaerobic and aerobic organisms of the phyla *Proteobacteria*, *Chloroflexi*, and potentially *Firmicutes* inhabiting a myriad of habitats like soils, lakes, and the human gut[1,5]. In 2019, Braga et al.[6], showed that the proteobacterium *Paraburkholderia rhizoxinica* produces a unique F_{420} derivative, containing a 3-phosphoglycerate instead of a 2-phospholactate tail, which might be widespread in various habitats. Within the domain *Archaea*, F_{420} has been found in several lineages, including methanogenic[7], methanotrophic[8,9], and sulfate-reducing orders[10], and is supposed to be produced in *Thaumarchaeota*[11]. F_{420} is best known as an essential redox coenzyme in hydrogenotrophic and methylotrophic methanogenesis. The reduced form of F_{420} ($F_{420}H_2$) functions as an electron donor for the reduction of methylenetetrahydromethanopterin (methylene-H_4MPT, Mer) and methenyl-H_4MPT[12,13]. It can also be used as an electron carrier in H_2-independent electron transport pathways of methanogens containing cytochromes[12,14]. Moreover, the oxidized form of F_{420} has a characteristic blue-green fluorescence upon excitation at 420 nm, which facilitates the detection of methanogens microscopically (**Figure 1**). Due to its low redox potential, F_{420} facilitates (i) the exogenous reduction of a broad spectrum of otherwise recalcitrant or toxic organic compounds, (ii) synthesis of tetracycline and lincosamide antibiotics or phytotoxins in streptomycetes (phylum *Actinobacteria*), and (iii) resistance to oxidative or nitrosative stress or other unfavorable conditions in mycobacteria (phylum *Actinobacteria*)[1,5,15–22]. Consequently, F_{420}-dependent oxidoreductases are promising biocatalysts for industrial and pharmaceutical purposes as well as for bioremediation of contaminated environments[1,23]. Despite these recent findings, the exact roles of the cofactor F_{420} are still marginally known in *Actinobacteria* or other bacterial phyla.

There are at least three pathways for F_{420} biosynthesis[2,6,24]. In the beginning, the biosynthesis pathway is split into a 5-deazaflavin biosynthesis and a 2-phospholactate metabolism branch. The reactive part of the F_{420} molecule is synthesized via F_O-synthase using the substrates tyrosine and 5-amino-6-ribitylamino-2,4(1*H*, 3*H*)-pyrimidinedione. The result is the riboflavin level chromophore F_O. Within the currently accepted lactate metabolism branch, L-lactate is phosphorylated to 2-phospho-L-lactate by an L-lactate kinase (CofB); 2-phosphoL-lactate, in turn, is guanylated to L-lactyl-2-diphospho-5'-guanosine by 2-phospho-L-lactate guanylyltransferase

(CofC). In the next step, L-lactyl-2-diphospho-5'-guanosine is linked to F_O by a 2-phospho-L-lactate transferase (CofD) to form F_{420}-0[2]. Finally, the enzyme F_{420}-0:γ-glutamyl ligase (CofE) ligates glutamate monomers to F_{420}-0, forming the final cofactor[6] in varying numbers[23,25]. Different organisms show different patterns in the number of attached glutamate residues, with shorter tails found for methanogens than in mycobacteria[2,25,26]. Generally, methanogens show tail lengths from two to three, with up to five in the acetoclastic methanogen, *Methanosarcina* sp., while tail lengths found in *Mycobacterium* sp. ranged from five to seven glutamate residues[2,25–27]. However, recent findings showed that long-chain F_{420} binds to F_{420}-dependent oxidoreductases with a higher affinity than short-chain F_{420}; moreover, bound long-chain F_{420} increases the substrate affinity but decreases the turnover rate of respective enzymes[23].

Detection of cofactor F_{420} is often based on its fluorescence. Thereby, its oligo glutamate derivates were separated using RP-HPLC[27,28]. Recently, Ney et al. used tetrabutylammonium hydroxide as an ion-pairing reagent for the negatively charged glutamate tail to enhance separation on RP-HLPC successfully[5]. Here, we present a method for the preparation of samples, subsequent lysis, extraction, purification, separation, and quantification of cofactor F_{420} not only from pure cultures but also from different environmental samples (i.e., soils and digester sludge).

PROTOCOL:

NOTE: Extraction and analysis of cofactor F_{420} is a three-step process including sample lysis, cofactor pre-purification via solid-phase extraction (SPE), and cofactor detection via ion-paired-reversed-phase HPLC (IP-RP-HPLC) with fluorescence detection. Prior to starting, prepare the materials and reagents as stated in **Table 1**.

1. Sample lysis

1.1. Add up to 5 g of sample to appropriate tubes (e.g., 50 mL conical tubes).

1.2. Add 5 mL of the lysis buffer (2x stock solution, **Table 1**) to the samples.

1.3. Bring to a final volume of 10 mL with distilled water to reach a final concentration of 0.5 $g \cdot mL^{-1}$.

1.4. Vortex the diluted samples for 20 s.

1.5. Autoclave for 30 min at 121 °C.

1.6. For dry samples like forest soil, bring to a final volume of 20 mL with distilled water after autoclaving and vortex the diluted sample.

CAUTION: Temperature increase during autoclaving might cause tubes to burst.

2. Pre-purification of the cofactor F_{420} via solid-phase extraction (SPE)

NOTE: All steps of SPE are conducted at room temperature

2.1. Cooldown the samples to room temperature.

2.2. Centrifuge the autoclaved samples for 5 min at 11,000 x *g*.

2.3. Prepare 5 mL SPE columns filled with 100 mg of weak anion mixed-mode polymeric sorbent.

2.4. Condition the anion exchanger with 3 mL of methanol (condition solution, **Table 1**).

2.5. Equilibrate the anion exchanger with 3 mL of distilled water (equilibration solution, **Table 1**).

2.6. Load up to 9.0 mL of the supernatant from the centrifuged lysate onto the SPE column.

2.7. Wash the impurities with 5 mL of 25 mM ammonium acetate (SPE wash solution 1, **Table 1**).

2.8. Wash the impurities with 5 mL of methanol (SPE wash solution 2, **Table 1**).

2.9. Elute the cofactor F_{420} in 1.0 mL of elution buffer (**Table 1**).

NOTE: Prepare fresh elution buffer. Due to the applied vacuum and the high vapor pressure of the elution buffer, elution volumes might differ from sample to sample. In order to secure the same final volume in all the samples, it is recommended to weigh the collection vessels before and after elution and calculate the effective elution volume. Balance the differences by the addition of elution buffer.

3. Detection of the cofactor F_{420}

3.1. Set the oven to 40 °C and fluorescence detector to 420 nm extinction-wavelength and 470 nm emission-wavelength. Achieve separation via gradient mode using mobile phases A and B (**Table 1)**: 0–3 min: 26% B; 3–24 min: 26%–50% B; 24–25 min: 50% B; 25–27 min: 50%–26% B; 27–35 min: 26% B at a flow rate of 0.75 mL·min^{-1}.

NOTE: Guarantee equilibration of column conditions prior to injecting samples by flushing the column at least with 3 column volumes of 74% mobile phase A and 26% mobile phase B (**Table 1**).

3.1.1. Filter the eluted samples from the SPE into HPLC vials using a PTFE filter with a pore size of 0.22 µm.

NOTE: PTFE filters with a pore size of 0.22 µm are recommended.

3.1.2. Inject 50 µL of the eluted sample onto the HPLC system to analyze the cofactor F_{420} composition and concentration.

NOTE: As no quantitative standard is used in this protocol, samples and variants have to be compared by peak area.

REPRESENTATIVE RESULTS:

Pure cultures of *Methanosarcina thermophila* and *Methanoculleus thermophilus*, both thermophilic methanogenic *Archaea*, were grown in suitable media as described previously[29,30]. For *Methanosarcina thermophila,* methanol was used as an energy source, whereas *Methanoculleus thermophilus* was grown on H_2/CO_2. Growth was checked by microscopic evaluation, while activity was examined via measurement of methane (CH_4) by gas chromatography as described previously[31]. Pure cultures were used for the extraction of cofactor F_{420} according to the presented protocol. In addition, soil and samples from a mesophilic biogas reactor sludge (wastewater treatment plant, Zirl, Austria; for details regarding sludge parameters, please refer to[32]), an agriculturally used meadow (Innsbruck, Austria), and forest soil (Lans, Austria) were taken in autumn 2020 for the extraction and analysis of cofactor F_{420}.

The growth of pure cultures has been verified by microscopy (**Figure 1**), with the produced CH_4 analyzed via gas chromatography during 14 days of incubation (data not shown). The efficiency of cofactor F_{420} extraction from pure cultures was tested by applying different disintegration strategies: beat-beating using 0.5–1.0 mm ceramic beats, ultrasonic treatment, and pressure-temperature disintegration using 121°C and 1.2 bar pressure (autoclaving). Maximum extraction efficiency became apparent using pressure-temperature treatment applying a buffer as described in the protocol section and was thus further applied for all subsequent experiments (**Figure 2**). Extraction efficiency tests were performed via standard addition of different volumes of a well-growing *Methanoculleus thermophilus* culture. Furthermore, the comparison of different samples and variants was based on peak area from chromatograms.

Subsequently, cell extracts were subjected to a solid phase extraction (SPE) procedure. For this purpose, different ion exchangers were tested. It turned out that a weak anion mixed-mode polymeric sorbent yielded the highest amount of cofactor F420 after elution. In addition, different elution buffers and wash solutions were tested and showed the best results for 25 mM ammonium acetate as a wash buffer and a mixture of NH_3 in methanol as an elution buffer. Methanol from the elution step could be exchanged after elution with water via a vacuum-temperature treatment.

HPLC analysis of cofactor F_{420} was tested with different C18 columns with the best results for the system configuration achieved during the presented study with an NX C18 column. A standard containing a known distribution of F_{420} derivates with varying glutamate tail length was used for reference purposes. This standard was kindly provided by Prof. Colin Jackson from the Australian National University. Analysis of glutamate tail length revealed differences in the overall

concentration of cofactor F_{420} and the distribution of F_{420} tail length of methanogenic pure cultures and environmental samples (**Figure 3**).

FIGURE AND TABLE LEGENDS:

Table 1: Buffer and mobile phase composition for solid-phase extraction (SPE) and HPLC analysis.

Figure 1: Fluorescing methanogens. Methanogens pure culture of *Methanosarcina thermophila* in (**A**) phase-contrast microscopy emit a blueish light in (**B**) fluorescence microscopy when cofactor F_{420} is excited with UV light (excitation at 395–440 nm and emission at 475–495 nm). Scale bar: 10 µm.

Figure 2: Standard addition. Peak area of recovered cofactor F_{420} after SPE from 1.0 g of matrix spiked with different volumes of *M. thermophilus* cultures. Matrix was amended with 0 µL, 250 µL, 500 µL, 750 µL, and 1000 µL of culture and subjected to different disintegration strategies: beat-beating, ultrasonic treatment, and pressure-temperature disintegration (autoclaving).

Figure 3: Glutamate tail length distribution. Cofactor F_{420} tail length distribution of pure cultures and environmental samples. From top to bottom: agriculturally used meadow (soil), forest (soil), mesophilic biogas reactor, pure culture of *M. thermophilus,* and pure culture of *M. thermophila.* Relative absorbance was calculated by normalization on the highest peak within the shown chromatogram.

DISCUSSION:

For the evaluation of cofactor F_{420} from methanogenic pure cultures, a microscopic evaluation can be performed to visualize the growth and activity (fluorescence microscopy) of the involved microorganisms (**Figure 1**). For samples deriving from natural environments, the use of microscopy to detect or quantify F_{420} is limited due to interferences with other fluorescent microorganisms, organic and inorganic particles. In this context, extraction of F_{420} and subsequent fluorometric analysis using HPLC, as described previously[5], can not only provide information on the overall concentration of cofactor F_{420} but also on polygutamate tail length distribution.

For extraction of cofactor F_{420}, a pressure-temperature treatment was shown to be highly effective (**Figure 2**) and is in accordance with the previous findings[5,27,33]. Via this method and applying a phosphate buffer lysis system including EDTA and polysorbate, the highest concentrations of cofactor F_{420} were obtained from methanogenic pure cultures containing high concentrations of the factor. Moreover (and in comparison with the other tested cell disruption methods), the pressure-temperature treatment is easily applicable and material-saving.

A solid-phase extraction (SPE) was performed to enable a downstream HPLC analysis aiming at the determination of cofactor F_{420} polyglutamate tail length distribution within a sample. Among various ion exchangers, a weak anion mixed-mode polymeric sorbent showed the best performance allowing for effective binding of the cofactor F_{420} to the matrix for washing purposes

as well as its subsequent removal from the extraction matrix after washing away undesired by-products. For this purpose, basic methanol proved best.

Via the presented method, various pure cultures and environmental samples could be analyzed reproducibly regarding cofactor F_{420} (**Figure 3**). Even samples such as soils or sludges containing high proportions of undesired by-products could be analyzed by the presented procedure. Therefore, downstream analysis via HPLC was successfully implemented for analyzing the total concentration of F_{420} and the length distribution of the polyglutamate tails of F_{420} derivatives. The detection of high levels of F_{420} in soil and other samples supports Ney et el.[5], who proposed that the cofactor is widespread in aerobic soil bacteria based on genomic and metagenomics analysis.

To sum up, this is the first protocol aiming at extracting and analyzing cofactor F_{420} not only from pure cultures but also from environmental samples like soil or sludge. The most critical step in extracting F_{420} from environmental samples is the solid phase extraction (SPE) needed for the pre-clean-up of lysates for subsequent HPLC analysis. The presented protocol will be helpful for future projects to unveil the role of F_{420} in various environments and bioprocesses.

ACKNOWLEDGMENTS:

We gratefully thank Prof. Colin Jackson for the support with purified cofactor F_{420}. This research was supported by Tyrolean science fund (TWF) and the Universität Innsbruck (Publikationsfonds). We greatly acknowledge the support of GPS, HK, SB, GG, and HB.

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Greening, C. et al. Physiology, biochemistry, and applications of F420- and Fo-dependent redox reactions. *Microbiology and Molecular Biology Reviews: MMBR.* **80** (2), 451–493 (2016).
2. Bashiri, G. et al. A revised biosynthetic pathway for the cofactor F420 in prokaryotes. *Nature Communications.* **10** (1), 451 (2019).
3. Grinter, R., Greening, C. Cofactor F420: an expanded view of its distribution, biosynthesis, and roles in bacteria and archaea. *FEMS Microbiology Reviews,* 10.1093/femsre/fuab021 (2021).
4. Eirich, L. D., Vogels, G. D., Wolfe, R. S. Proposed structure for coenzyme F 420 from methanobacterium. *Biochemistry.* **17** (22), 4583–4593 (1978).
5. Ney, B. et al. The methanogenic redox cofactor F420 is widely synthesized by aerobic soil bacteria. *The ISME Journal.* **11** (1), 125–137 (2017).
6. Braga, D. et al. Metabolic pathway rerouting in Paraburkholderia rhizoxinica evolved long-overlooked derivatives of coenzyme F420. *ACS Chemical Biology.* **14** (9), 2088–2094 (2019).
7. Eirich, L. D., Vogels, G. D., Wolfe, R. S. Distribution of coenzyme F420 and properties of its hydrolytic fragments. *Journal of Bacteriology.* **140** (1), 20–27 (1979).
8. Michaelis, W. et al. Microbial reefs in the Black Sea fueled by anaerobic oxidation of methane. *Science.* **297** (5583), 1013–1015 (2002).
9. Knittel, K., Lösekann, T., Boetius, A., Kort, R., Amann, R. Diversity and distribution of methanotrophic archaea at cold seeps. *Applied and Environmental Microbiology.* **71** (1), 467–

479, (2005).
10. Lin, X. -L., White, R. H. Occurrence of Coenzyme F420 and Its y-Monoglutamyl derivative in nonmethanogenic archaebacteria. *Journal of Bacteriology.* **168** (1), 444–448 (1986).
11. Spang, A. et al. The genome of the ammonia-oxidizing Candidatus Nitrososphaera gargensis: insights into metabolic versatility and environmental adaptations. *Environmental Microbiology.* **14** (12), 3122–3145 (2012).
12. Mand, T. D., Metcalf, W. W. Energy conservation and hydrogenase function in methanogenic archaea, in particular the genus Methanosarcina. *Microbiology and Molecular Biology Reviews: MMBR.* **83** (4) (2019).
13. Lupa, B., Hendrickson, E. L., Leigh, J. A., Whitman, W. B. Formate-dependent H2 production by the mesophilic methanogen Methanococcus maripaludis. *Applied and Environmental Microbiology.* **74** (21), 6584–6590 (2008).
14. Kulkarni, G., Mand, T. D., Metcalf, W. W. Energy conservation via hydrogen cycling in the methanogenic archaeon Methanosarcina barkeri. *mBio.* **9** (4) (2018).
15. Purwantini, E., Gillis, T. P., Daniels, L. Presence of F420-dependent glucose-6-phosphate dehydrogenase in Mycobacterium and Nocardia species, but absence from Streptomyces and Corynebacterium species and methanogenic Archaea. *FEMS Microbiology Letters.* **146** (1), 129–134 (1997).
16. Purwantini, E., Daniels, L. Purification of a novel coenzyme F420-dependent glucose-6-phosphate dehydrogenase from Mycobacterium smegmatis. *Journal of Bacteriology.* **178** (10), 2861–2866 (1996).
17. McCormick, J. R. D., Morton, G. O. Identity of cosynthetic factor I of Streptomyces aureofaciens and fragment FO from coenzyme F420 of Methanobacterium species. *Journal of the American Chemical Society.* **104** (14), 4014–4015 (1982).
18. Coats, J. H., Li, G. P., Kuo, M. -S. T., Yurek, D. A. Discovery, production, and biological assay of an unusual flavenoid cofactor involved in lincomycin biosynthesis. *The Journal of Antibiotics.* **42** (3), 472–474 (1989).
19. Bown, L., Altowairish, M. S., Fyans, J. K., Bignell, D. R. D. Production of the Streptomyces scabies coronafacoyl phytotoxins involves a novel biosynthetic pathway with an F420 -dependent oxidoreductase and a short-chain dehydrogenase/reductase. *Molecular Microbiology.* **101** (1), 122–135 (2016).
20. Gurumurthy, M. et al. A novel F(420) -dependent anti-oxidant mechanism protects Mycobacterium tuberculosis against oxidative stress and bactericidal agents. *Molecular microbiology.* **87** (4), 744–755 (2013).
21. Greening, C. et al. Mycobacterial F420H2-dependent reductases promiscuously reduce diverse compounds through a common mechanism. *Frontiers in Microbiology.* **8**, 1000 (2017).
22. Mathew, S., Trajkovic, M., Kumar, H., Nguyen, Q. -T., Fraaije, M. W. Enantio- and regioselective ene-reductions using F420H2-dependent enzymes. *Chemical Communications (Cambridge, England).* **54** (79), 11208–11211 (2018).
23. Ney, B. et al. Cofactor tail length modulates catalysis of bacterial F420-dependent oxidoreductases. *Frontiers in Microbiology.* **8**, 1902 (2017).
24. Grinter, R. et al. Cellular and structural basis of synthesis of the unique intermediate dehydro-F420-0 in mycobacteria. *mSystems.* **5** (3) (2020).
25. Peck, M. W. Changes in concentrations of coenzyme F420 analogs during batch growth of

Methanosarcina barkeri and Methanosarcina mazei. *Applied and Environmental Microbiology.* **55** (4) (1989).
26. Gorris, L. G., van der Drift, C. Cofactor contents of methanogenic bacteria reviewed. *BioFactors (Oxford, England).* **4** (3-4), 139–145 (1994).
27. Bair, T. B., Isabelle, D. W., Daniels, L. Structures of coenzyme F(420) in Mycobacterium species. *Archives of Microbiology.* **176** (1-2), 37–43 (2001).
28. Portillo, M. C., Gonzalez, J. M. Moonmilk deposits originate from specific bacterial communities in Altamira Cave (Spain). *Microbial Ecology.* **61** (2011).
29. Wagner, A. O. et al. Medium preparation for the cultivation of microorganisms under strictly anaerobic/anoxic conditions. *Journal of Visualized Experiments: JoVE.* **150**, 60155 (2019).
30. Lackner, N., Hintersonnleitner, A., Wagner, A. O., Illmer, P. Hydrogenotrophic methanogenesis and autotrophic growth of Methanosarcina thermophila. *Archaea.* **2018** (5), 1–7 (2018).
31. Wagner, A. O., Reitschuler, C., Illmer, P. Effect of different acetate: Propionate ratios on the methanogenic community during thermophilic anaerobic digestion in batch experiments. *Biochemical Engineering Journal.* **90**, 154–161 (2014).
32. Wagner, A. O. et al. Sample preparation, preservation, and storage for volatile fatty acid quantification in biogas plants. *Engineering in Life Sciences.* **17** (2), 132–139 (2017).
33. Bashiri, G., Rehan, A. M., Greenwood, D. R., Dickson, J. M. J., Baker, E. N. Metabolic engineering of cofactor F420 production in Mycobacterium smegmatis. *PloS one.* **5** (12), e15803 (2010).

Figure 1

Click here to access/download;Figure;Figure 1.tif

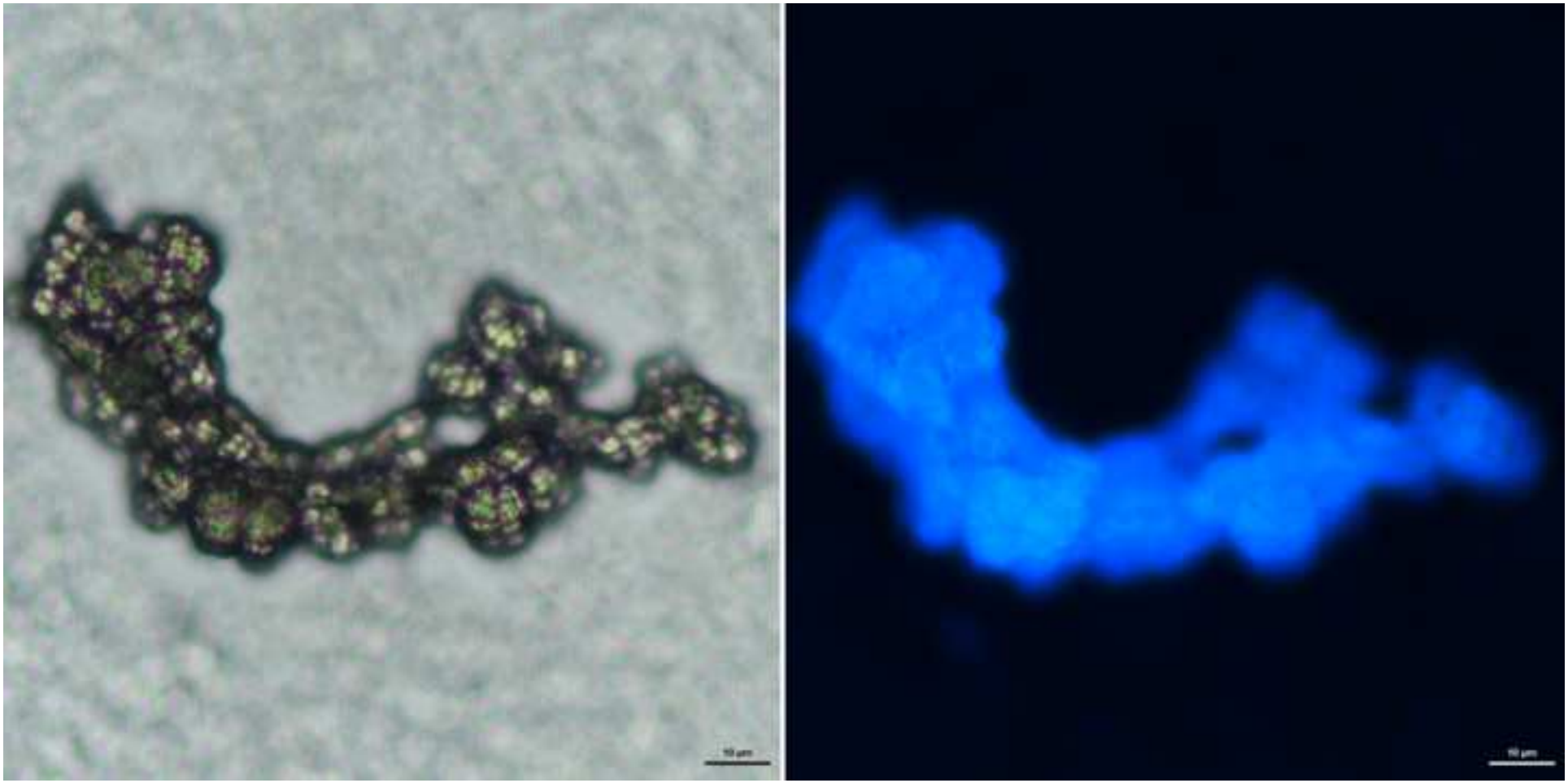

Figure 1A

Click here to access/download;Figure;Figure 1A.tif

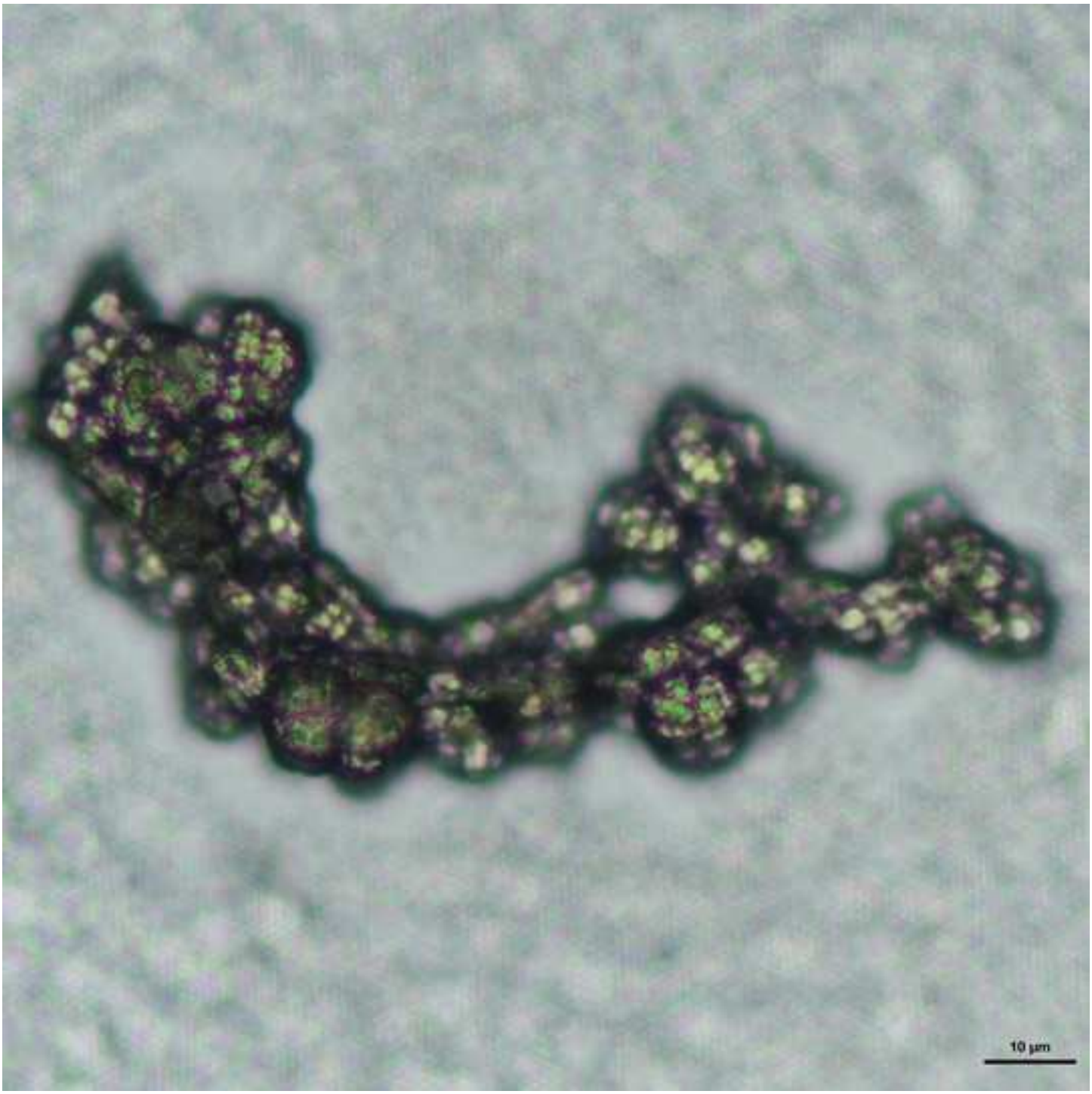

Figure 1B

Click here to access/download;Figure;Figure 1B.tif

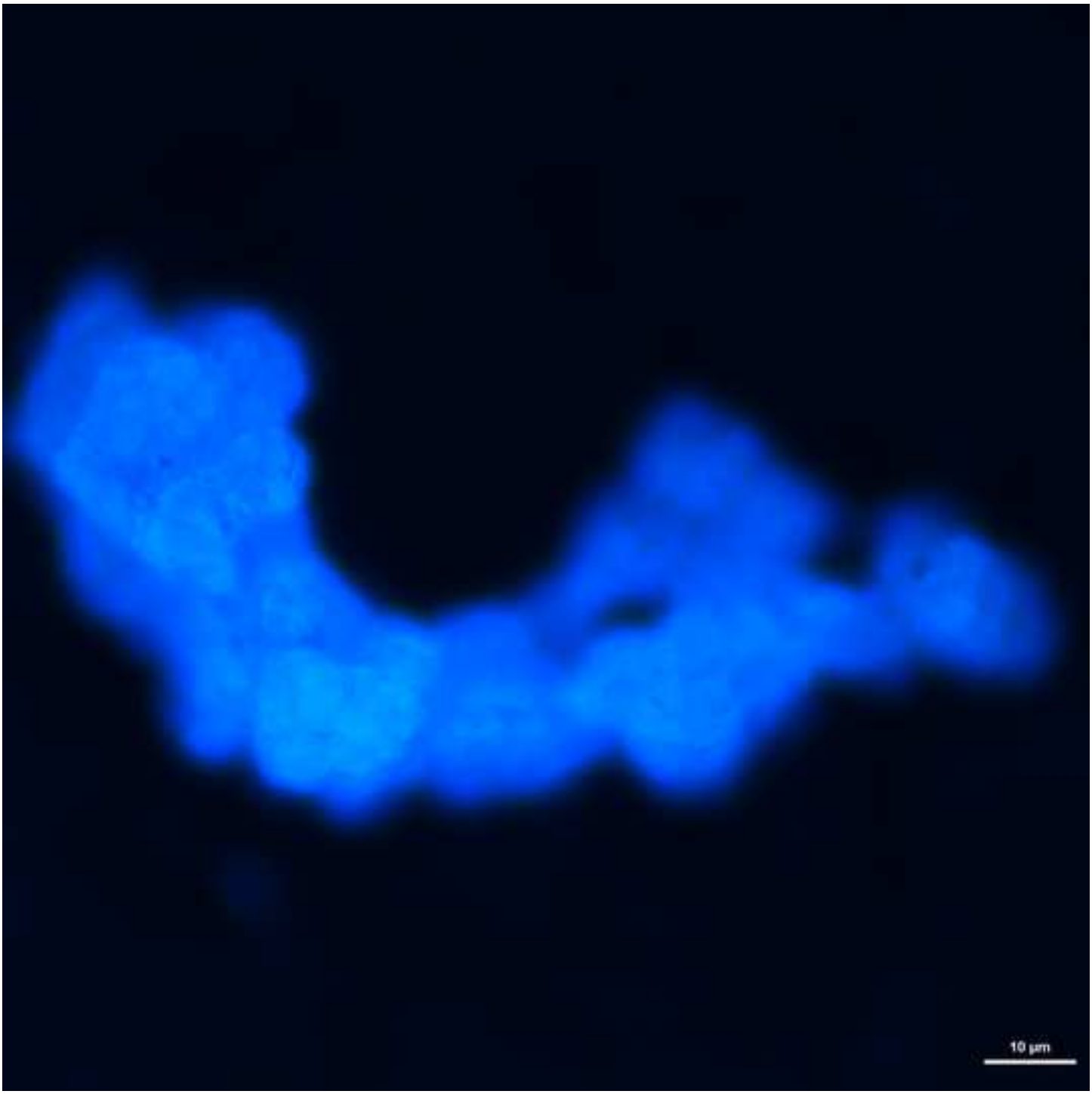

Figure 2

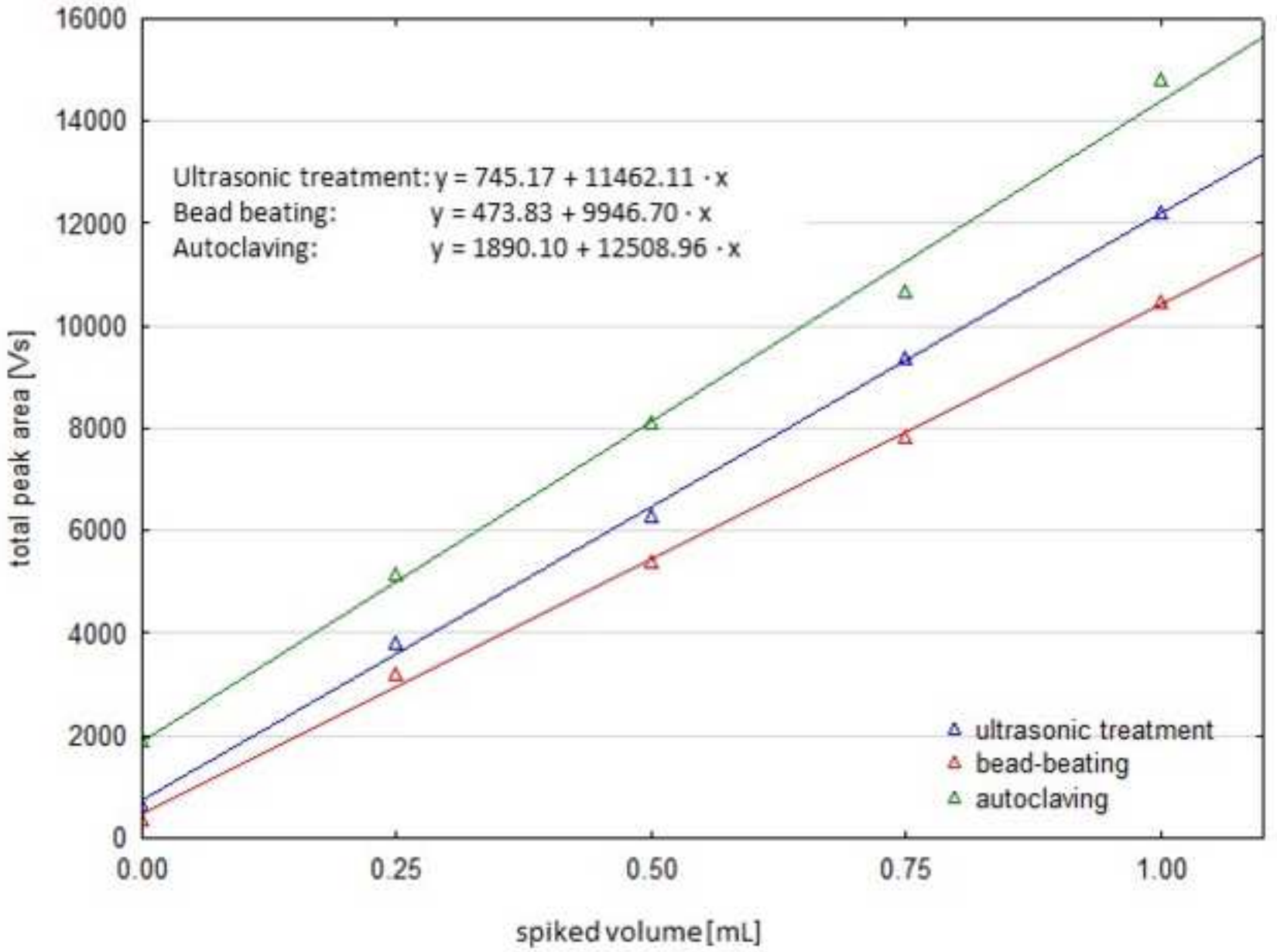

Figure 3

Click here to access/download;Figure;Figure 3_high res.png

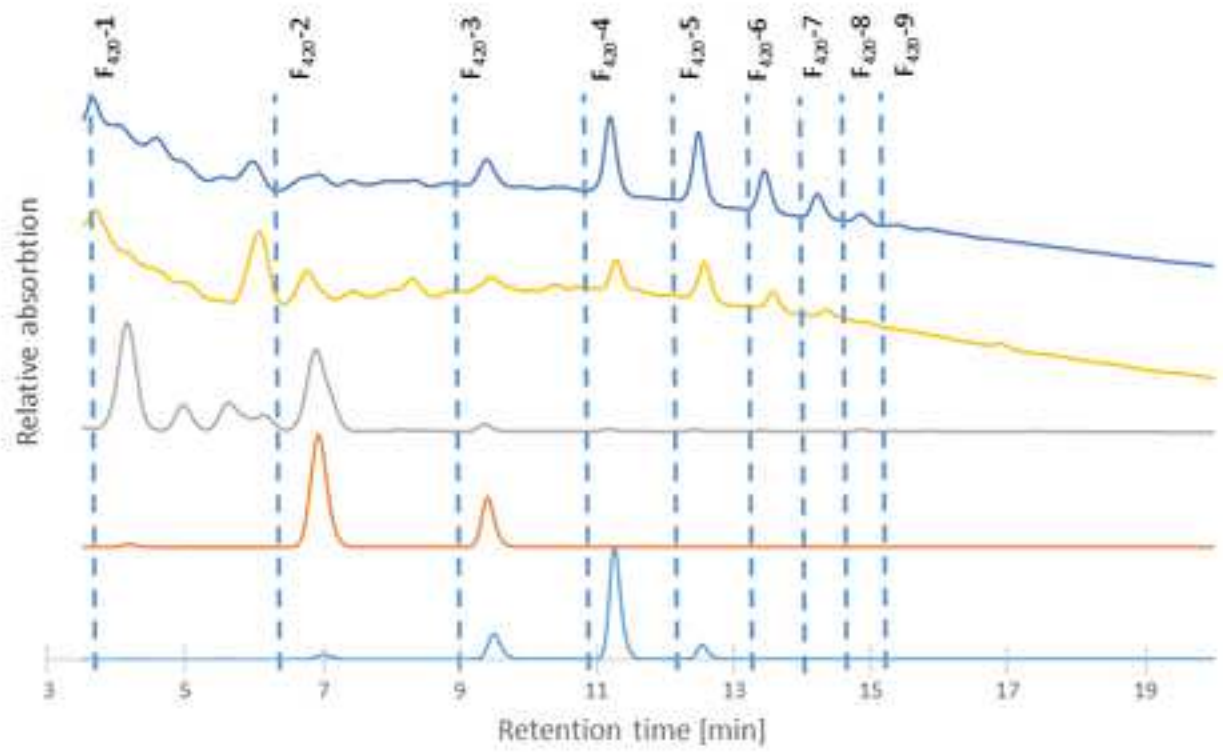

Table 1:

Buffer	Composition
Lysis buffer (2x stock solution)	200 mM potassium dihydrogenphosphate (KH_2PO_4) 50 mM ethylenediaminetetraacetic acid (EDTA) 1% (w/v) polysorbate 80 (Tween 80) adjusted to pH 7.0 with 5 M sodium hydroxide solution
SPE conditioning solution	Methanol (HPLC grade)
SPE equilibration solution	distilled water 0.2 µm filtered
SPE wash solution 1	25 mM ammonium acetate
SPE wash solution 2	Methanol (HPLC grade)
SPE elution buffer	2% (v/v) ammonia in methanol by diluting 20%–25% aqueous ammonia solution in methanol
HPLC mobile phase A	10 mM tetrabutylammoniumhydroxide (TBAH) 20 mM di-ammonium hydrogenphosphate ($(NH_4)_2HPO_4$) adjusted to pH 7.0 with 85% phosphoric acid
HPLC mobile phase B	Acetonitrile (HPLC grade)

Please note that the reviewers raised some significant concerns regarding your method and your manuscript. Please revise the manuscript to thoroughly address these concerns. Additionally, please describe the changes that have been made or provide explanations if the comment is not addressed in a rebuttal letter. We may send the revised manuscript and the rebuttal letter back to peer review.

Editorial comments:

1. Please make the changes/revisions in the attached manuscript file, which has been formatted to fit the journal standard.

Changes / revisions were done accordingly.

2. Please revise the following lines to avoid previously published work: 35-36, 92-94

We rephrased the sentences.

3. Line 125: Vortex for how long?

20 sec, changed in the ms.

4. Line 271-274: Please rephrase the lines to add more clarity.

Sentence was rephrased.

5. Please ensure that all the comments addressed in the rebuttal are included in the appropriate section of the manuscript.

All comments of the reviewer / editor are addressed in the revised manuscript.

Reviewers' comments:

Reviewer #1:

The authors much improved the manuscript during the revision, but there remains typos and other issues that should be addressed before publication:

L27: "ploy**g**lutamate" to "poly**g**lutamate"

Changed accordingly

L30: "towards the" to "for the"

Changed accordingly

L47: "like" to "such as"

Changed accordingly

L55: -310 mV is too high. -340 or -350 mV is consistent with other reviews, e.g. Greening et al. MMBR 2016, Grinter et al., FEMS Microbiol Rev 2021.

Thank you! The ms was changed accordingly.

L55: ", in its plainest form," can be removed

Changed accordingly

L61: Add 'potentially' before Firmicutes as this hasn't been proven experimentally

Changed accordingly

L63: Explain that the derivative contains a 3-phosphoglycerate not 2-phospholactate tail.

Changed accordingly

L63: "derivate" to "derivative"

Changed accordingly

L73: "potentials" to "potential"

Changed accordingly

L76: "mycobacterial taxa" to "mycobacteria"

Changed accordingly

L77: "phylum Actinomycetes" to "phylum Actinobacteria"

Changed accordingly

L78: Better citations here would be Greening et al., Front Microbiol 2017 and Mathew et al., Chem Comm 2018.

Thank you! The ms was changed accordingly and the references added.

L82: Remove "whereby they are not fully elucidated yet". Only the archaeal pathway remains unresolved.

Changed accordingly

L83: This refers to the archaeal pathway only. I recommend referencing Grinter et al., FEMS Microbiol 2021 for the other pathways.

Thank you! The ms was changed accordingly.

L83: Modify "flavin biosynthesis" to "5-deazaflavin biosynthesis" and "lactate" to "2-phospholactate"

Changed accordingly

L84: It should be Fo not F0 in all instances

Changed accordingly

L87: Protein names shouldn't be italicised in all instances

Changed accordingly

L100: "fluorescent character" to "fluorescence" or "fluorescent characteristics"

Changed accordingly

L100: The second sentence isn't well explained and needs some expansion.

We are sorry and rephrased and extended the sentence.

L118: "the appropriate tubes" to "appropriate tubes"

Changed accordingly

L120: Change x to multiply sign × in all instances

Changed accordingly

L142: Remove the hyphen after mL

Changed accordingly

L193: "gas-chromatography" to "gas chromatography"

Changed accordingly

L246: This is a vague and repetitive sentence. What does "might be interesting" mean? An issue with microscopy is that it doesn't directly confirm the presence of F420 in contrast to a HPLC-based method. Given other molecules might cause similar fluorescence.

Thank you for the input. We changed the sentence to be more precise.

L254: This inference isn't statistically supported given you didn't perform replicates. Extraction efficiency looks high between all three methods. Perhaps change "most effective" to "highly effective".

The reviewer is right. Changed accordingly.

L273: Amend to "The detection of high levels of F420 in soil and other samples supports Ney et al., who proposed that the cofactor is widespread in aerobic soil bacteria based on genomic and metagenomic analysis."

Thank you! The ms was changed accordingly.

Figure 3: The axis titles are too small, axis labels lack ticks, subscripts should be used for 420 in all cases, and underscores should be replaced with emdashes. F420-1 not F420_1.

Axis titles were changed, ticks added, subscripts for 420 used and F420_1/2/3 etc changed to F_{420}-1 etc.

Table 1: Add chemical abbreviation for potassium dihydrogenphosphate. EDTA is misspelt in multiple ways. Polysorbate is misspelt. What is ammoniac?
We are sorry! Abbreviation was added, misspellings corrected (incl. ammonia).

Table 2: Equipment is misspelt
Thank you! The Table was changed accordingly.

Reviewer #3:
Manuscript Summary:
A protocol is described that can detect the F420 content with is characteristic polyglutamate tail in various samples

Major Concerns:
The actions taken by the authors to accommodate the reviewers' comments are appreciated but there are still issues to be resolved.

What is still missing is:
=> a better framing of this protocol within the current related protocols
The text: Detection of cofactor F420 is often based on its fluorescent character. Thereby, its oligo glutamate derivates were separated using RP-HPLC24,25. Recently Ney et al. used

tetrabutylammoniumhydroxid as an ion-pairing reagent for the negatively charged glutamate tail to enhance separation on RP-HLPC successfully4 might not be efficient to substantiate why your protocol is 'better'.

Thank you for this suggestion. We highlighted the extraction from environmental samples, which is the most important innovation of the described method, in comparison to pure culture-samples. This protocol yields reproducible amounts of cofactor F420 from environmental samples, as visible by excellent linear regression after analysis from spiked samples.

Furthermore:
"It turned out that a weak anion mixed-mode polymeric sorbent was most suitable

=> which criteria were used to determine that this sorbent was 'most suitable'

Thank you for this note, we rephrased the sentence in line 219 – 221 to: "Subsequently, cell extracts were subjected to a solid phase extraction (SPE) procedure. For this purpose, different ion exchangers were tested. It turned out that a weak anion mixed-mode polymeric sorbent yielded the highest amount of cofactor F420 after elution"

"can not only provide information on the overall concentration of cofactor F420 but also on polygutamate tail length distribution."
=> explain a bit more here (again) why knowledge of this polyglutamate tail length would be so interesting to know
Thank you for this information, we rephrased the sentence from line 97 – 100 in the introduction to highlight the importance of different tail lengths.

Finally:
=> The discussion does not really read as discusion but more as a summary.

The focus of this journal is the clear presentation of methods to facilitate their use. Therefore, our discussion aimed to explain the advantages and to outline possible future applications of the presented method. However, we carefully revised the discussion section and hope, that we could improve the quality of the ms.

Minor Concerns:
Line 72 which facilitates the "identification" of methanogens microscopically => 'detection' is better than 'identification'

Changed accordingly