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Novel organic contaminants in recycled organic waste and their fate in a mushroom production system

Forekomst av aktuelle organiske forurensninger i resirkulert organisk avfall

Astrid Solvåg Nesse

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Astrid Solvåg Nesse

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PhD Supervisors

Main supervisor:
Professor Trine A. Sogn
Faculty of Environmental Sciences and Natural Resource Management
Norwegian University of Life Sciences

Co-supervisors:
Professor Roland Kallenborn
Faculty of Chemistry, Biotechnology and Food Science
Norwegian University of Life Sciences

Associate Professor Susanne Eich-Greatorex
Faculty of Environmental Sciences and Natural Resource Management
Norwegian University of Life Sciences

Professor Sissel Torre
Faculty of Biosciences
Norwegian University of Life Sciences

Evaluation committee

Prof. Dr. Rolf-Alexander Düring
Institute of Soil Science and Soil Conservation
Research Centre for BioSystems, Land Use and Nutrition (iFZ)
Justus Liebig University
Giessen, Germany

Professor Hans Peter Arp
Norwegian Geotechnical Institute
Oslo, Norway
and
Professor II in Environmental Chemistry
Norwegian University of Science and Technology
Trondheim, Norway

Committee administrator:
Professor Gunnhild Riise,
Faculty of Environmental Sciences and Natural Resource Management
Norwegian University of Life Sciences

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List of papers

Paper I

Ali AM, Nesse AS, Eich-Greatorex S, Sogn TA, Aanrud, SG, Bunæs JAA, Lyche JL, Kallenborn R (2019) Organic contaminants of emerging concern in Norwegian digestates from biogas production. Environmental Science Processes and Impacts 21: 1498 – 1508, DOI: 10.1039/c9em00175a

Paper II

Nesse AS, Aanrud SG, Lyche JL, Sogn T, Kallenborn R (2022) Confirming the presence of selected antibiotics and steroids in Norwegian biogas digestate. Environmental Science and Pollution Research 29: 88595 – 86605, DOI: 10.1007/s11356-022-21479-1

Paper III

Nesse AS, Jasinska A, Ali AM, Sandblom O, Sogn TA, Benskin JP (2023) Uptake of ultrashort chain, emerging, and legacy per- and polyfluoroalkyl substances (PFAS) in edible mushrooms (*Agaricus spp.*) grown in a polluted substrate. Journal of Agricultural and Food Chemistry 71: 4458-4465. DOI: 10.1021/acs.jafc.2c03790

Paper IV

Nesse AS, Jasinska A, Stoknes K, Aanrud SG, Risinggård KO, Kallenborn, R., Sogn, TA, Ali, AM, Low uptake of pharmaceuticals in edible mushrooms grown in contaminated biogas digestate. Manuscript accepted with revisions in Chemosphere.

Abbreviations

ANOVA	Analysis of variance
BAF	Bioaccumulation Factor
CAS	Chemical Abstracts Service
DEET	*N,N*-Diethyl-*meta*-toluamide
diPAP	Polyfluorinated phosphoric acid diesters
DONA	Dodecafluoro-3H-4,8-dioxanonanoic acid
EFSA	European Food Safety Authority
EU	European Union
FASAA	Perfluoroalkyl sulfonamido derivative
FCM	Food Contact Material
FTCA	Fluorotelomer carboxylic acid
FTS	Fluorotelomer sulfonic acid
FW	Food waste
K_{ow}	Octanol – water partition coefficient
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
MeFOSAA	*N*-methyl perfluorooctane sulfonamidoacetic acid
NMBU	Norges Miljø- og Biovitenskaplige Universitet / Norwegian University of Life Sciences
NGI	Norwegian Geotechnical Institute
OECD	Organisation for Economic Co-operation and Development
PAH	Polyaromatic hydrocarbons
PBT	Persistent, Bioaccumulative, Toxic
PC	Principal component
PCA	Principal component analysis
PCB	Polychlorinated biphenyls
PCP	Personal care product
PEC	Predicted Environmental Concentration
PFAA	Perfluoroalkyl acids
PFAS	Per- and polyfluoroalkyl substances
PFBA	Perfluorobutanoic acid
PFCA	Perfluoroalkyl carboxylic acids
PFDA	Perfluorodecanoic acid
PFDoDA	Perfluorododecanoic acid
PFHpA	Perfluoroheptanoic acid
PFHxA	Perfluorohexanoic acid
PFNA	Perfluorononanoic acid
PFOA	Perfluorooctanoic acid
PFOcDA	Perfluorooctadecanoic acid

PFOS	Perfluorooctanesulfonic acid
PFPrA	Perfluoropropanoic acid
PFSA	Perfluoroalkane sulfonic acids
PFTeDA	Perfluorotetradecanoic acid
PFUnDA	Perfluoroundecanoic acid
PNEC	Predicted No Effect Concentration
POSF	Perfluorooctanesulfonyl fluoride
PPCPs	Pharmaceuticals and personal care products
PTFE	Polytetrafluoroethylene
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals.
SS	Sewage sludge
TCPP	Tris(2-chloroethyl) phosphate
TFA	Trifluoroacetic acid
TWI	Tolerable Weekly Intake
UNEP	United Nations Environment Programme
UV-filter	Ultra-Violet filter
WWTP	Wastewater treatment plant

Summary (English)

Biogas production is a common treatment method for organic waste in Norway. The waste is anaerobically degraded, to stabilize the waste and to produce renewable energy (biogas). Most of the nutrients in the waste are preserved in the organic residue after biogas production, called biogas digestate. Food waste and sewage sludge are the most common feedstocks for biogas production in Norway, in addition to some livestock manure and residual products from the fishing industry. While these materials contain a lot of nutrients, they can also contain both heavy metals and organic contaminants. Organic contaminants include for example pharmaceuticals, such as antibiotics, personal care products, and per- and polyfluoroalkyl substances (PFAS).

To increase nutrient recovery and to add organic matter to the soil, biogas digestate is often used as fertiliser. In this case, any contaminants in biogas digestate could be spread on agricultural land. Risks linked to the spread of pollutants on agricultural land include uptake in food and fodder plants, leakage to groundwater, runoff to surface waters and harm towards soil and water organisms, which can ultimately affect biogeochemical cycles. For antibiotics, there is also a risk associated with promoting antibiotic resistance in the environment.

Since many species of fungi can break down various organic pollutants, in some cases also pollutants that are otherwise difficult to decompose, using biogas digestate as a nutrient source in substrate for (commercial) mushroom production can be a way to remediate organic waste materials. One can expect that the concentration of contaminants in such a mushroom growth medium decreases with time. After mushroom production, the medium can be used further as fertilizer in plant production.

This research project was carried out as part of the project NovelPol: "Novel organic pollutants from recycling of organic waste as risk factors for human exposure", which was funded by the Norwegian Research Council. The framework for the PhD project was therefore limited to emerging organic contaminants that can be found in biogas digestate. The PhD project had two parts. The first part addressed organic pollutants in Norwegian biogas

digestates. The main objective was to investigate the occurrence of pharmaceuticals and personal care products (PPCP) **[Articles I and II]**, as well as per and polyfluoroalkyl substances (PFAS) **[Synopsis]** in biogas digestates. Biogas digestates from all municipal large-scale biogas plants in Norway were collected for analysis. In the second part of the PhD project, a mushroom production system with a substrate based on biogas digestate was investigated, with the overall aim of estimating the uptake of selected PFASs **[Article III]** and PPCPs **[Article IV]** in the edible mushrooms, as well as to follow the concentration of PPCPs and PFAS in the growth medium **[Article III and IV]**.

In Part I, the screening of Norwegian biogas digestates, the UV filter octocrylene, the insecticide DEET and the drugs acetaminophen (paracetamol) and ibuprofen were found in concentrations of up to a few mg kg^{-1} dry biogas digestates. Several other pharmaceuticals which are used in high quantities, including losartan and metoprolol, were found in many biogas digestates in several hundred µg kg^{-1} dm **[Article I]**. Antibiotics, on the other hand, were infrequently detected **[Article I and II]**. Some of the antibiotics that were found were, however, quantified in several hundred µg kg^{-1} dm in 1 – 2 biogas digestates, for example ciprofloxacin, amoxicillin, and sulfadiazine. All of these are on the World Health Organization (WHO) list of highly important or critically important antimicrobial agents. A risk analysis showed that some of these contaminants can pose a risk to soil organisms when the biogas digestates are used as fertilisers.

The total concentration of pharmaceuticals in the biogas digestates did not depend on whether it was sewage sludge, food waste or a mixture which were used as feedstock. Nor did the number of contaminants detected in the biogas digestates depend on the content of sewage sludge in the feedstock for biogas production. Biogas digestates based on food waste can therefore, like biogas digestates based on sewage sludge, be an important source of contamination in the environment.

In contrast, PFASs, were generally quantified in higher concentrations in biogas digestates where sewage sludge formed part or all of the substrate, than biogas digestates based on food waste. Most of the PFASs were found in a somewhat higher concentration in biogas digestates based (partially) on sewage sludge than food waste, and some PFASs in much higher

concentrations. The only PFAS found in all biogas digestates was perfluorooctanoic acid (PFOA), despite international regulations for phasing out the substance. However, the concentration of PFOA was low in all biogas digestates.

Neither PFASs **[Article III]** nor PPCPs **[Article IV]** were accumulated in the mushroom fruiting bodies. For perfluoro carboxylic acids with a carbon chain length of 3 to 7 (PFPrA to PFHpA), the bioaccumulation factors (BAF) decreased sharply with increasing chain length. A similar relationship was observed for perfluoalkane sulfonic acids. In contrast to plants, no relationship between BAF and chain length was observed for long-chain perfluorinated carboxylic acids **[Article III]**. The PPCPs were either not observed, or were found only in very low concentrations, in the mushroom fruiting bodies **[Article IV]**.

Most of the pharmaceuticals, but not all, were largely removed from the mushrooms growth medium **[Article IV]**. One of the mushroom species, *Agaricus subrufescens*, generally had a lower uptake of both pharmaceuticals and PFAS than *Agaricus bisporus* **[Articles III and IV]**. The pharmaceutical residues were also removed to a greater extent from the growth medium of *A. subrufescens* than from the growth medium of *A. bisporus*.

These studies show that many organic contaminants can be found in biogas digestates used for food production, in some cases in concentrations that can harm soil organisms when biogas digestates are used as fertiliser. Both biogas digestate based on food waste and sewage sludge can be a source of contamination in the environment. Contaminated biogas digestate can be partially remedied by cultivating mushrooms in the biogas digestate, with only a very low uptake of PFASs and PPCPs. However, as PFAS are not degraded in the growth medium, mushroom production is not suitable for remediation of waste contaminated with PFAS.

Sammendrag (Norsk)

Biogassproduksjon er en vanlig behandlingsmetode for organisk avfall i Norge, hvor avfallet brytes ned anaerobt, for å stabilisere avfallet og for å produsere fornybar energi (biogass). Det meste av næringsstoffene i avfallet bevares i den organiske resten etter biogassproduksjon, kalt biorest. Matavfall og avløpsslam er de vanligste substratene for biogassproduksjon i Norge, i tillegg til noe husdyrmøkk og restprodukter fra fiskeindustrien. I tillegg til at disse materialene inneholder mye næring, kan de også inneholde både tungmetaller og organiske forurensninger. Organiske forurensninger kan bestå av legemidler, som antibiotika, personlig pleie produkter, og perfluorerte stoffer (PFAS).

For å øke gjenvinning av næringsstoff og for å tilføre organisk materiale til jord, brukes biorest ofte som gjødsel. I dette tilfelle vil eventuelle skadestoffer i biorest kunne spres på landbruksjord. Risikoer knyttet til spredning av forurensninger på landbruksjord inkluderer opptak i mat- og fôrplanter, lekking til grunnvann, avrenning til overflatevann og skade mot jord- og vannorganismer, som i ytterste konsekvens kan påvirke biogeokjemiske sykluser. For antibiotika er det i tillegg en risiko knyttet til å fremme antibiotika-resistens i miljøet.

Siden mange sopparter kan bryte ned ulike organiske forurensninger, i noen tilfeller også forurensninger som ellers er tungt nedbrytbare, kan bruk av biorest som næringskilde i substrat for (kommersiell) sopproduksjon være en måte å redusere organiske forurensninger på. Man kan forvente at konsentrasjonen av forurensninger i et slikt soppvekstmediet synker med tid. Etter sopproduksjon kan mediet eventuelt brukes videre som gjødsel i planteproduksjon.

Dette forskningsprosjektet ble utført som en del av prosjektet NovelPol: «Novel organic pollutants from recycling of organic waste as risk factors for human exposure», som ble finansiert av det norske forskningsrådet. Rammen for ph.d.-prosjektet ble derfor begrenset til organiske forurensninger som kan finnes i biorest. Ph.d.-prosjektet hadde to deler. Den første delen adresserte organiske forurensninger i norske biorest**.** Hovedmålet var å undersøke forekomsten av legemidler og personlige pleieprodukter (PPCP) **[Artikkel I**

og II], samt perfluorerte stoffer (PFAS) **[Synopsis]**. Biorest fra alle offentlige storskala-biogassanlegg i Norge ble samlet inn for analyse. I den andre delen av ph.d.-prosjektet ble et sopproduksjonssystem med substrat basert på biorest undersøkt, med det overordnete målet å estimere opptaket av PFAS-forbindelser **[Artikkel III]** og utvalgte PPCP-stoffer **[Artikkel IV]** i matsoppen, samt å følge konsentrasjonen av PFAS- og PPCP-stoffer i vekstmediet **[Artikkel III og IV]**.

I Del I, screeningen av norske biorester, ble UV-filteret octocrylene, innsektsmiddelet DEET og legemidlene acetaminophen (paracetamol) og ibuprofen funnet i konsentrasjoner opp til noen få mg kg^{-1} tørr biorest. Flere andre legemidler, bl.a. losartan og metoprolol, som brukes i høye kvanta ble funnet i mange biorester i flere hundre µg kg^{-1} tørrstoff **(Artikkel I)**. Antibiotika ble derimot sjeldent funnet **(Artikkel II)**. Noen av de antibiotika som ble funnet, ble imidlertid funnet i flere hundre µg kg^{-1} tørrstoff i 1 – 2 biorester, for eksempel ciprofloxacin, amoxicillin og sulfadiazine. Alle disse står på Verdens helseorganisasjons (WHO) liste over veldig viktige eller kritisk viktige antimikrobielle midler. En risikoanalyse viste at enkelt av disse stoffene kan utgjøre en risiko mot jordorganismer når biorestene brukes som gjødselmidler.

Den totale konsentrasjonen av legemidler i biorestene var ikke avhengig av om det var kloakkslam, matavfall eller en blanding som ble brukt som substrat. Heller ikke antallet detekterte stoffer i biorestene viste noen sammenheng med innhold av kloakkslam i substratet for biogassproduksjonen. Biorester basert på matavfall kan derfor, i likhet med biorester basert på kloakkslam, være en viktig kilde til forurensninger i miljøet.

PFAS-forbindelser ble derimot generelt kvantifisert i høyere konsentrasjoner i biorester hvor kloakkslam utgjorde en del av eller hele substratet, enn biorester laget fra matavfall. De fleste PFAS-forbindelsene ble funnet i en noe høyere konsentrasjon i biorester basert (delvis) på kloakkslam enn matavfall, og enkelte PFAS i mye høyere konsentrasjoner. Den eneste PFAS-forbindelsen som ble funnet i samtlige biorester var perfluorert oktansyre (PFOA), på tross av internasjonale reguleringer for utfasing av stoffet. Konsentrasjonen av PFOA var imidlertid lav i alle biorestene.

Verken PFAS-er **(Artikkel III)** eller PPCP-er **(Artikkel IV)** ble akkumulert i sopphattene. For perfluorerte karboksylsyrer med en karbon-kjedelengde f.o.m. 3 t.o.m. 7 (PFPrA til PFHpA), sank bioakkumuleringsfaktorene (BAF) skarp med økende kjedelenge. En lignende sammenheng ble observert for perfluorerte sulfonylsyrer. I motsetning til planter ble det ikke observert noen sammenheng mellom BAF og kjedelengde for langkjedete perfluorerte karboksylsyrer **(Artikkel III)**. PPCP-ene ble enten ikke observert, eller ble funnet i kun veldig lave konsentrasjoner, i sopphattene **(Artikkel IV)**.

De fleste legemidlene, men ikke alle, ble i stor grad fjernet fra soppenes vekstmedie ila. sopp-produksjonen **(Artikkel IV)**. Den ene sopparten, *Agaricus subrufescens*, hadde generelt et lavere opptak av både legemidler og PFAS enn *Agaricus bisporus* **(Artikkel III og IV).** Legemiddel-restene ble også i høyere grad fjernet fra vekstmediet til *A. subrufescens* enn i vekstmediet til *A. bisporus*.

Disse studiene viser at mange organiske forurensninger kan finnes i biorester som brukes i matproduksjon, i noen tilfeller i konsentrasjoner som kan skade jordorganismer når biorest brukes som gjødsel. Både biorest basert på matavfall og på kloakkslam kan være en kilde til forurensninger i miljøet. Forurensede biorester kan delvis remedieres ved at sopp dyrkes i bioresten, med kun et veldig lavt opptak av PFAS og PPCP-er. Ettersom PFAS ikke fjernes ved nedbrytning i vekstmediet er imidlertid ikke sopproduksjon egnet for å remediere avfall forurenset med PFAS.

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1. Introduction

Contaminants in the food-chain; a side-effect of the circular economy

In 2009, the concept of Planetary Boundaries was introduced. The aim was to define the limits within which the environment can continue to be stable (Rockström et al., 2009). To sustain life, nine interrelated Earth system processes must stay within their respective planetary boundaries. Six out of nine boundaries have already been exceeded (Richardson et al., 2023). Amongst those are novel entities (Persson et al., 2022), earlier called chemical pollution.

At least 69,000 chemicals are in commerce globally (Z. Wang et al., 2020). Many of these compounds can be found in various waste streams. For example, sewage sludge can contain per- and polyfluoroalkyl substances (PFAS, Stahl et al., 2018), pharmaceuticals and personal care products (PPCPs, Verlicchi & Zambello, 2015), phthalates, polybrominated diphenyl ethers, synthetic musks (Clarke & Smith, 2011) and many more. Food waste is less acknowledged as a source for contaminants, but both PFAS and other persistent organic pollutants including polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) have been found in composts of food waste and green waste (Brändli et al., 2005; O'Connor et al., 2022).

The planetary boundary of biogeochemical flows, including nitrogen and phosphorus, has also been exceeded. The main reason is the large input of mineral fertilizers to croplands (Steffen et al., 2015). To close nutrient cycles and thereby reduce the input of mineral fertilisers, the EU have "Food, water, and nutrients" included as one of the key product value chains in the Circular Economy Action Plan (European Commission, 2020). Biogas production has a great potential to be a commercial driver for closing the nitrogen and phosphorus loops by producing fertiliser from organic wastes, with the additional benefit of renewable energy production (Rosemarin et al., 2020).

However, a major challenge in agriculture is the simultaneous recycling of contaminants via waste streams. Contaminants in soil may accumulate in agricultural plants (Lesmeister et al., 2021), harm soil organisms (Roose-

Amsaleg & Laverman, 2016), build up in the soil (Dalkmann et al., 2012), leach to groundwater (Lapworth et al., 2012), or drain into nearby streams (Gottschall et al., 2012). Figure 1 shows some aspects of the coupling of nutrient cycles and contaminant flows, with focus on compound groups included in this thesis, namely PPCPs and PFAS.

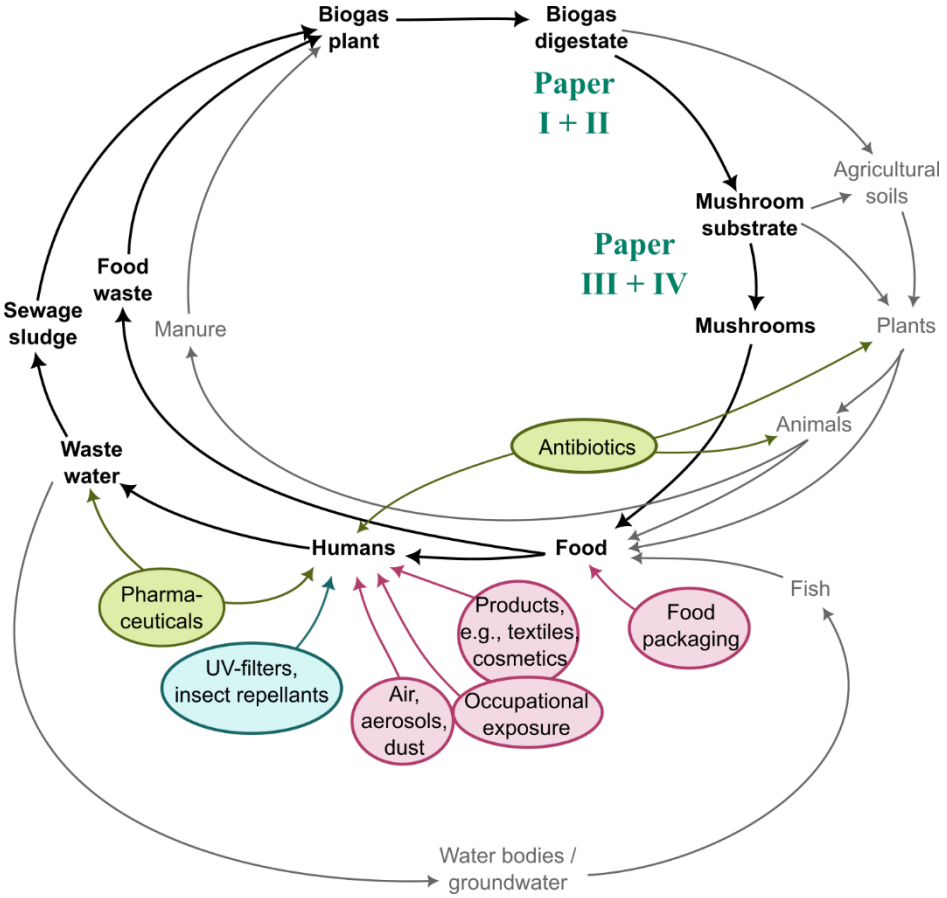

Figure 1. Coupling of contaminant and nutrient cycles in the circular economy. The figure shows relevant streams, where nutrients and contaminants are cycled together through the recirculation of organic residues. Input of pharmaceuticals are coloured green, personal care products are blue, and per- and polyfluoroalkyl substances (PFAS) are coloured pink. In Paper I and II, the levels of organic contaminants in biogas digestates were examined. In Paper III and IV, the fate of organic contaminants in a mushroom production system was investigated. The figure is not exhaustive.

2. Background

2.1 Per- and polyfluoroalkyl substances

One specific group of contaminants for which it is argued that the planetary boundary has been transgressed is per- and polyfluoroalkyl substances (PFAS, Cousins et al., 2022). PFAS are a large group with thousands of synthetic compounds, which all have perfluorinated alkyl or alkene moieties in their molecular structure (–CF3 or –CF2–, OECD, 2021). Due to the strength of the C–F bond, the majority of PFASs are either extremely persistent or are expected to degrade into persistent compounds (Cousins et al., 2020).

Many PFASs are used as surfactants, and are both hydrophobic and oleophobic, i.e., they repel both water and oil/stains (Buck et al., 2011). Fluorinated surfactants are very effective at lowering the aqueous surface tension at very low concentrations, are surface active in organic solutions, and are extremely resistant to chemical attack (Kissa, 2001). Fluorosurfactants are therefore used in a variety of applications including stain-resistant coatings for textiles (including furniture and carpets), floor polishes, as levelling agents in paints, and are used to lower the surface tension of aqueous firefighting foams (Kovalchuk et al., 2014). The first commercially available fluorosurfactants were the perfluoroalkane sulfonates (PFSAs) and the perfluoroalkyl carboxylic acids (PFCAs) (Buck et al., 2012), both of which belong to the perfluoroalkyl acids (PFAAs). The most investigated PFSAs and PFCAs are perfluorooctanesulfonic acid (PFOS) and perfluorooctane carboxylic acid (PFOA), respectively.

PFAS also include polymers. Polytetrafluoroethylene (PTFE) is the most common fluoropolymer, and makes up about 60% of the global fluoropolymer market (Puts et al., 2019). PTFE is widely used e.g., as a non-stick coating, insulator of electrical cables and lubricant, and can be marketed as Teflon. Expanded PTFE, amongst others marketed as GoreTex®, is used as a coating on textiles for waterproofing (Puts et al., 2019). Historically, the ammonium salt of PFOA was the main dispersant applied in the manufacture of PTFE (Z. Wang et al., 2013).

The high persistence of PFASs makes them ubiquitous environmental contaminants found e.g. in soils, surface- and groundwater, wildlife, humans (incl. breastmilk) and in food (Buck et al., 2011; Pasecnaja et al., 2022; Sims et al., 2022). In the early 2000s, long-chained PFCAs ($C_nF_{2n+1}COOH$, $n \geq 7$) and PFSAs ($C_nF_{2n+1}SO_3H$, $n \geq 6$) became recognised as global contaminants (Buck et al., 2011; Z. Wang et al., 2013). In addition to their persistence, the long-chained PFCAs an PFSAs are bioaccumulative and have been detected ubiquitously in various environmental compartments, in wildlife, and in humans (Giesy & Kannan, 2001; Olsen et al., 2003; Z. Wang et al., 2013).

Consequently, PFOS, PFOA, perfluorohexanoic acid (PFHxA) and their related compounds became listed under the Stockholm convention in 2009, 2019, and 2022, respectively (UNEP, 2023c). PFOS became listed under Annex B, which restricts production and use, whereas PFOA and PFHxA became listed under Annex A which aims at elimination of the compounds (UNEP, 2023b). Long-chained PFCAs as a group have been proposed to be listed under the Stockholm convention as well (UNEP, 2023a). In parallel, several long-chained PFAAs have been included also on the Candidate List of Substances of Very High Concern under the EU chemicals regulation REACH, due to being persistent, bioaccumulative and toxic (PBT-compounds) and/or being toxic for reproduction (Brendel et al., 2018).

As a result, long-chained PFAAs and their precursors are being replaced by fluorinated and non-fluorinated alternatives by the industry (Z. Wang et al., 2015). The most important fluorinated substitutes for the long-chained PFAAs are short-chained PFAAs and perfluoroether acids (Z. Wang et al., 2015). The short-chained PFAAs are assumed to have a lower bioaccumulation potential than the long-chained PFAAs (Brendel et al., 2018). However, the short-chained PFAAs are equally persistent (Brendel et al., 2018), and plants strongly favour uptake of shorter-chained PFAAs (Lesmeister et al., 2021).

Perfluoroether acids include for example the chlorinated polyfluoroalkyl ether sulfonic acid F-53B, which is used as a replacement of PFOS (He et al., 2022), and the polyfluoroalkyl ether carboxylic acid ADONA. The perfluoroether acids are structurally similar to PFAAs, but oxygen atoms have been inserted into the fluorinated carbon chain to increase degradability (Baker & Knappe, 2022; Z. Wang et al., 2015). Under environmental

conditions, the perfluoroether chains are however similarly persistent as the perfluoroalkyl chains (summarized by Wang et al. (2015)).

Perfluoroalkyl acids (PFAAs) are degradation products of many other PFASs. Fluorotelomer phosphate diesters (diPAPs), fluorotelomer carboxylates (FTCAs), and fluorotelomer sulfonates (FTSs) may all degrade into PFCAs (Buck et al., 2011; Butt et al., 2014; Lee et al., 2010; Liu & Liu, 2016). Both PFCAs and PFSAs may be formed from perfluorooctane sulfonamide (FOSA) and perfluoroalkane sulfonamido acetic acids (Buck et al., 2011; Maldonado et al., 2022). Many of these precursors are found in higher concentrations in various environmental matrices than the PFAAs. For instance, diPAPs are often found in high concentrations in biosolids (D'Eon et al., 2009; Kärrman et al., 2019). An overview over the structures of the compounds included in this PhD project are shown in Figure 2. The compound lists include several PFAAs, alternatives to PFAAs, and precursors to PFAAs.

PFASs are manufactured by two principal processes; electrochemical fluorination and telomerization (Buck et al., 2011). Electrochemical fluorination is used to produce perfluorooctanesulfonyl fluoride, which is the starting material of PFOS, FOSA and perfluoroalkane sulfonamidoacetic acids (FASAAs, Buck et al., 2011). Electrochemical fluorination is a crude process which yields both linear, branched, and cyclic structures, and other byproducts (Buck et al., 2012). Hence, both linear and branched isomers of the above-mentioned compounds were included in the screening of PFAS in the biogas digestates. Also PFCAs can be produced by electrochemical fluorination (Buck et al., 2011)

The second main PFAS manufacturing process is telomerization, during which a perfluoroalkyl iodide is reacted with tetrafluoroethylene (Buck et al., 2011). Telomerization can be used to produce PFCAs, FTSAs, FTCAs, and diPAPs (Buck et al., 2011). Unlike ECF, telomerization yields mainly linear isomers with an even number of carbon atoms, like linear PFHxA, PFOA, perfluorodecanoic acid (PFDA) etc.

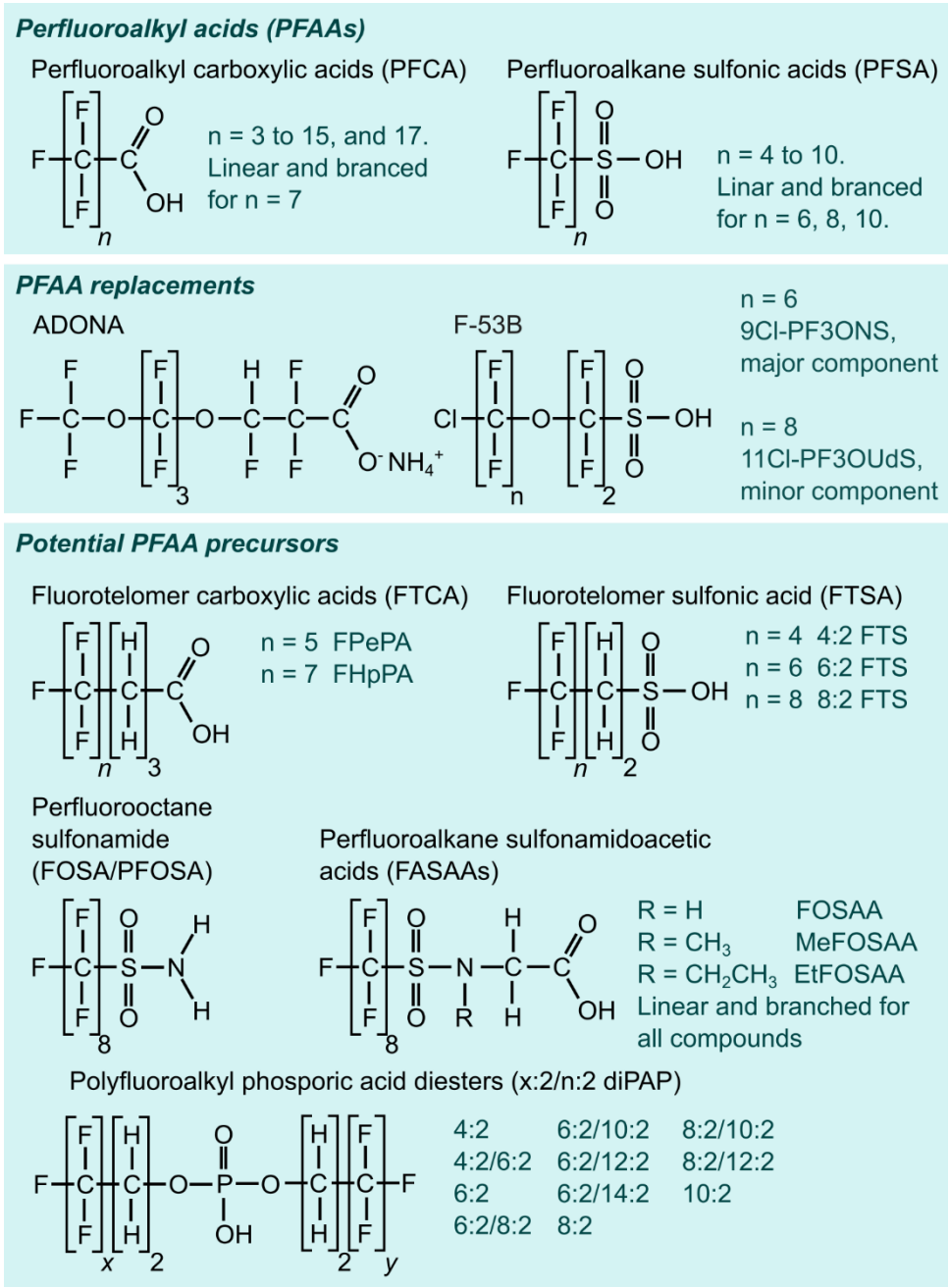

Figure 2: Groups of PFAS, and individual compounds, which are included in the PhD research project. For groups, it is noted which compounds are included. See Table 3 for a complete list of PFASs screened for. ADONA: ammonium salt of dodecafluoro-3H-4,8-dioxanonanoic acid, 9Cl-PF3ONS: 9-chlorohexadecafluoro-3-oxanonane-1-sulfonic acid, 11Cl-PF3OUdS: 11-chloroeicosafluoro-3-oxaundecane-1-sulfonic acid

2.2 Pharmaceuticals and personal care products

Unlike PFAS, which are classified as a group based on common chemical structure, pharmaceuticals and personal care products (PPCPs) are classified as a group based on their usage profile. The term was first introduced by Daughton and Ternes (1999), who defined PPCPs to include pharmaceuticals and the active ingredients in personal care products. Personal care products include UV-filters used in e.g., sunscreens, antiseptics used in e.g., creams and shoes, synthetic musks used in perfumes, X-ray contrast media and numerous others (Daughton & Ternes, 1999). As such, the physiochemical properties, environmental fate, and toxicity of PPCPs are diverse.

The border between PFAS and PPCPs are not strict. Certain PFASs which are used in cosmetics or other personal care products can also be included in the PPCP group. For example, PFCAs are found in many cosmetic products in low concentrations, and diPAPs are found at high concentrations in especially foundations (Harris et al., 2022; Schultes et al., 2018). Some pharmaceuticals which contain a trifluoromethyl group, such as fluoxetine and norfluoxetine, can be classified as PFAS according to the definition by OECD (2021) mentioned earlier. In the following, however, all non-pharmaceuticals PFASs will be discussed as PFAS, and all pharmaceuticals will be discussed as PPCPs.

The following background information is mainly concerned with pharmaceuticals, especially antibiotics, as these were the main PPCPs included in this PhD project.

When pharmaceuticals are administered, they are partly excreted unmetabolized or as conjugated metabolites which can transform back to the parent compound (Daughton & Ternes, 1999). Hence, pharmaceuticals are commonly quantified in wastewater treatment plant (WWTP) influents (Gao et al., 2012; Jelic et al., 2011; Y. Wang et al., 2018) and in animal manure (Ghirardini et al., 2020). Many pharmaceuticals are small (<500 g mol^{-1}), polar, have an octanol-water partitioning constant (logK_{OW} or logP) smaller than 5, and are highly water soluble (Daughton & Ternes, 1999; Walters & Murcko, 2002). Thus, many pharmaceuticals partition mainly into the aqueous phase at WWTPs and are discharged with the treated wastewater (Gao et al., 2012; Jelic et al., 2011; Y. Wang et al., 2018). However, other

pharmaceuticals, such as the fluoroquinolone antibiotics, partition mainly in the sewage sludge (Golet et al., 2003; Y. Wang et al., 2018).

Pharmaceuticals also need to have a long enough half-life to give the intended effect before they are degraded and/or excreted. Low metabolic stability is a challenge in drug discovery, but the half-life can be extended by substitution of a fluorine atom or a trifluoromethyl group (Shah & Westwell, 2007). The addition of fluorine may however enhance the environmental recalcitrance as well (Murphy, 2016). Fluoroquinolone antibiotics for example are recalcitrant towards degradation in e.g., WWTPs and soils (Dalkmann et al., 2012; Y. Wang et al., 2018). Pharmaceuticals which contain a trifluoromethyl group are potential precursors of the ultra-short-chain PFCA trifluoroacetic acid (TFA) (Freeling & Björnsdotter, 2023). Fluorinated pharmaceuticals have become more common since they first became synthesised 60 years ago, and now comprise about 25% of pharmaceuticals on the market or in the pipeline (Murphy, 2016).

The environmental effects of many PPCPs are subtle and may be difficult to separate from natural occurring events (Daughton & Ternes, 1999). There are however a few infamous examples of acute environmental effects of PPCPs. In the 1990s, a decline of over 95% in the vulture population in India and Pakistan began. The decline was probably caused by the vultures feeding on livestock carcasses which had been treated with the nonsteroidal anti-inflammatory drug diclofenac (Oaks et al., 2004). More recently, several holiday locations with tropical coral reefs have banned the use of sunscreens containing certain ultraviolet (UV) filters, including octocrylene, as the UV-filters are suspected to cause bleaching and enhanced morality of coral reefs (Miller et al., 2021). Two groups of pharmaceuticals which are of special importance due to their biological effects are the steroidal pharmaceuticals, which are designed to modulate the endocrine system, and antibiotics (Daughton & Ternes, 1999).

Antibiotics, antibiotic resistant bacteria, and antibiotic resistance genes are released to the environment by e.g., fertilisation of soils with manure and sewage sludge and by irrigation with treated wastewater (Chow et al., 2021; Ghirardini et al., 2020; Menz et al., 2019). The resulting environmental concentration of antibiotics can be in a range that can promote antibiotic resistance (Andersson & Hughes, 2014; Chow et al., 2021). When bacteria are

exposed to sublethal concentrations of antibiotics, antibiotic resistance may increase by enrichment of pre-existing resistant bacteria or by selection of *de novo* resistance (Andersson & Hughes, 2014). Transmission of new antibiotic resistant genes from environmental biota to humans or livestock is rare and difficult to quantify, but the consequences may be large and irreversible (Larsson & Flach, 2022). It has also been suggested that antibiotics in the environment may disrupt biogeochemical processes due to shifts in community structure. In a review by Roose-Amsaleg and Laverman (2016) it was found that sulfonamides and fluoroquinolones can partially inhibit denitrification at environmentally relevant concentrations.

Antibiotic consumption is expected to increase and the development pace of therapeutics with new mode of action is slow (Klein et al., 2018; Miethke et al., 2021). It is therefore important to reduce antibiotic resistance as much as possible, as seen by the increased use of last-resort antibiotics which can be used on multi-resistant bacterial strains (Klein et al., 2018). Several of the antibiotics reported about in this PhD project are on the World Health Organization (WHO) list of critically important antimicrobials for Human Medicine. Both the fluoroquinolones ciprofloxacin, norfloxacin and the β-lactam amoxicillin are categorized as critically important, whereas the sulfonamides sulfadiazine and sulfamethazine are listed as highly important (WHO, 2018).

2.3 Anaerobic digestion of organic wastes

Anaerobic digestion and composting are the two most common biological treatment methods for organic wastes in Norway (Ålund & Gentile, 2020). In Norway, biogas production is organised by the waste management sector (Lyng et al., 2018), and is commonly used to stabilize organic wastes, primarily sewage sludge and food waste (Ålund & Gentile, 2020). Other, less common feedstocks in municipal biogas plants, include manure and fish silage.

Anaerobic digestion is a complex and sensitive process performed by microorganisms in several consecutive steps (Schnürer & Jarvis, 2010). Briefly, the feedstock is fed into a closed container where it is degraded into biogas (mainly composed of methane and carbon dioxide), which can be used

for energy purposes (Lyng, 2018). Prior to the digestion, the feedstock is usually pre-treated by a combination of mechanical, thermal, or biological treatments (Montgomery & Bochmann, 2014).

The residual organic matter after anaerobic digestion is called the biogas digestate, which is a nutrient rich slurry. The digestate can be applied to soils directly, or it can be separated into a liquid and solid fraction (Lyng, 2018). In Norway, the digestate is mainly applied to agricultural areas, this include the digestate based on sewage sludge (Ålund & Gentile, 2020). A life cycle assessment of the Norwegian biogas value chain showed that use of the digestate as fertilizer substantially improved the climate budged for the biogas production, mainly due to the replacement of mineral fertilizers (Lyng et al., 2018).

Norwegian sewage sludges have been screened for contaminants approximately every fifth year since 1997 (Blytt & Stang, 2018). A wide range of compound groups have been included in the screenings; however, pharmaceutical residues has been included only once (in 2017/18) and only a few antibiotics was included on the target list. PFAS has been included in three screenings, but the target list has mainly been limited to PFSAs and PFCAs (Blytt & Stang, 2018). For food waste, there are fewer studies. Two studies investigated the presence of PAHs, PCBs, phthalates, and other compounds in food waste prior to digestion (Amundsen, 2012) and in digestates (Govasmark et al., 2011) from two and three biogas plants, respectively. Neither included PFAS or PPCPs, except for caffeine (Amundsen, 2012).

2.4 Fungi and organic contaminants

Lignocellulose is a complex and recalcitrant assembly of the polymers lignin, hemicellulose, and cellulose (Zoghlami & Paës, 2019). Lignocellulose is primarily degraded by fungi, especially basidiomycetes. To break down the lignocellulose and other macromolecules such as proteins and lipids, the fungi utilize a range of enzymes, both extracellular and intracellular (Griffin, 1994; Harms et al., 2011). After external degradation, nutrients are absorbed through the plasmalemma/cell wall as low-molecular-weight water soluble monomers (Griffin, 1994). The cell wall does not limit the transport of small

molecules, but may contain enzymes which change the chemicals (Griffin, 1994).

The enzymatic battery of fungi is considered as non-specific, due to the adaption to the random structure of lignin, which allows the fungi to co-metabolize structurally diverse organic contaminants (Harms et al., 2011). The most notable example is *Phanerochaete chrysosporium*, which can degrade benzene, toluene, ethylbenzene, xylenes, explosives, organochlorines, polyaromatic hydrocarbons, hydrocarbons, pesticides, synthetic dyes, and synthetic polymers (Harms et al., 2011). The ability to attack the contaminant with extracellular oxidoreductases is unique to fungi (Harms et al., 2011).

Based on their degradation patterns, fungi can be classified as e.g., the wood-decaying white-rots and brown-rots, or as the litter-decaying litter-rots (Doddapaneni et al., 2013), where the ability to degrade lignin is most pronounced for the white-rots (Griffin, 1994). The white-rot fungi have the enzymatic machinery to completely degrade all components of the plant cell wall including lignin, whereas brown-rot fungi can modify lignin (Doddapaneni et al., 2013).

A schematic overview of the mechanisms of fungal degradation of organic contaminants is provided in Figure 3. Outside the fungal cell, the contaminant can be transformed by extracellular enzymes including laccases and peroxidases (Harms et al., 2011). However, for non-ligninolytic fungi, transformations usually require the contaminant to pass through the cell wall, where they can be internally transformed by e.g., cytochrome P_{450} monooxygenases and epoxide hydrolases (Marco-Urrea et al., 2015).

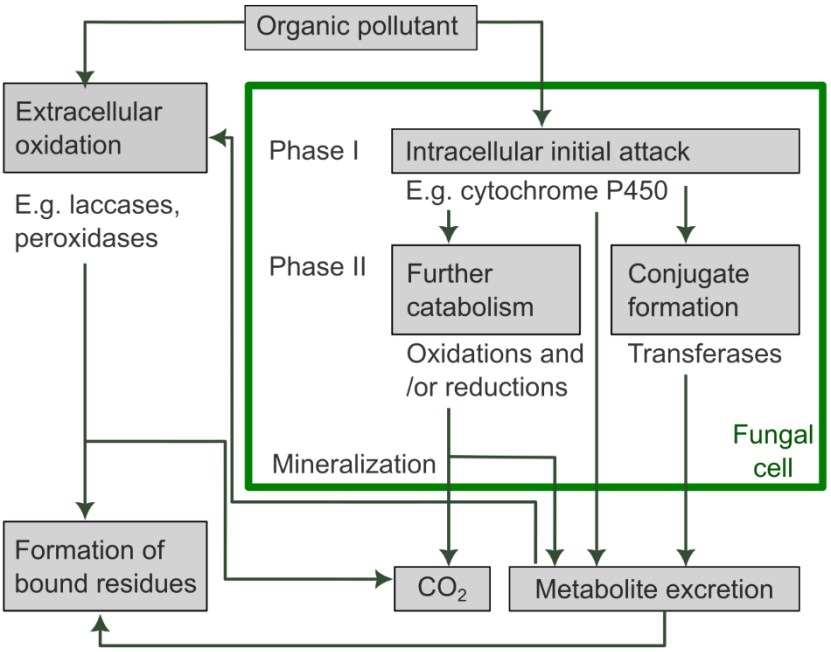

Figure 3. The main methods used to degrade organic contaminants by non-ligninolytic fungi. The figure is adapted from Harms et al. (2011) and Marco-Urrea et al. (2015).

Many studies on fungal degradation of organic contaminants are performed in laboratories with sterile synthetic mediums and cultures of white-rot fungi. These controlled laboratory studies have revealed transformation pathways for several organic contaminants in detail, including carbamazepine (Golan-Rozen et al., 2011, 2015).

More recently, a few studies also cultivated edible mushrooms in various substrates containing organic contaminants (Carrasco-Cabrera et al., 2019; Gbylik-Sikorska et al., 2020; Golovko et al., 2021; Moeder et al., 2005; Schildt et al., 2021). These studies revealed a low mushroom uptake of organic contaminants, with very low bioaccumulation factors for almost all compounds. Metabolites were in some instances also detected in the mushroom fruiting bodies, but it was unclear whether these were formed inside the fruiting bodies or if they had been transported there.

2.5 *Agaricus bisporus* and *A. subrufescens*

The button mushroom, *Agaricus bisporus*, is historically the most cultivated mushroom globally and is still the most important mushroom crop in the USA (Singh et al., 2021). *A. bisporus* belongs to the litter-rots, and are adapted to live on partially decomposed organic matter (Morin et al., 2012). *A. bisporus* is mainly cultivated on agricultural residues, often hay and wheat straw in combination with nitrogen rich materials such as chicken manure or inorganic nitrogen (Munshi et al., 2010; Suwannarach et al., 2022).

Agaricus subrufescens is best known for its medicinal properties, but is also considered a nutritious mushroom (Wisitrassameewong et al., 2012). The cultivation process of *A. subrufescens* have been adapted from *A. bisporus* (Zied et al., 2010) and the mushroom can be grown on a wide range of agricultural wastes (Wisitrassameewong et al., 2012). In nature, *A. subrufescens* grows on rotten leaves (Wisitrassameewong et al., 2012).

While *Agaricus spp.* is not known for their ability to degrade organic contaminants, genomic analysis of *A. bisporus* reveals that also this fungus has genes encoding for the enzymes required to decompose lignocellulosic materials (Morin et al., 2012). However, the number of Cytochrome P_{450} genes was less numerous than in white-rot fungi (Doddapaneni et al., 2013). Two studies have earlier investigated the uptake of organic contaminants into *Agaricus bisporus* fruiting bodies.

Gbylik-Sikorska et al. (2020) studied the concentration dependent uptake of the antibiotic doxycycline into the fruiting bodies of *A. bisporus*. Up to a substrate concentration of 250 µg kg^{-1} fresh weight, doxycycline was not detected in the mushrooms. From 500 to 5000 µg kg^{-1} fw, the bioaccumulation factors were consistently below 0.011. Removal of doxycycline from the substrate was not studied, hence, the bioconcentration factors were related to the nominal concentration of doxycycline. Removal of doxycycline in the substrate would have led to higher bioaccumulation factors.

Schildt et al. (2021) developed a method for the quantification of 21 pharmaceuticals in mushrooms. In a screening of commercial button mushrooms, only traces of chloramphenicol were detected in two out of twenty samples. In the same study, the fate of the pharmaceuticals

ciprofloxacin, chloramphenicol and praziquantel during cultivation of *A. bisporus* was investigated. Ciprofloxacin and chloramphenicol were rapidly removed from the growth substrate and not taken up in the mushrooms, whereas praziquantel remained in the substrate and was quantified in concentrations close to 100 µg kg^{-1} DW at the second harvest.

A few studies also investigated the removal and degradation of organic contaminants by the white-rot fungi oyster mushroom (*Pleurotus ostreatus*), with similar results. Clarithromycin, clindamycin, sertraline and fenbendazole were rapidly removed from the mushroom substrate (Golovko et al., 2021) and were not detected in the mushroom hats, whereas clarithromycin and caffeine remained in the substrates and were taken up (Carrasco-Cabrera et al., 2019; Golovko et al., 2021). Also, PFAS remained in the substrate of *P. ostreatus*, as the compounds were neither degraded nor accumulated into the mushrooms (Golovko et al., 2021).

At Lindum waste management facility in Drammen (Norway), a cultivation system of *A. bisporus* and *A. subrufescens* where the mushrooms are grown on a substrate composed of mainly digestate and straw, has been developed. The aim of using digestate in the compost was to make a system where organic wastes were efficiently recycled to new food with a low demand on energy and land, for production in an urban environment. The biogas produced from the food waste provided energy (i.e., methane) and carbon dioxide for the greenhouse production (Stoknes et al., 2013, 2016). In later experiments, it was shown that *A. subrufescens* could extract over 80% of the cadmium in the growth substrate (Stoknes et al., 2019), but the uptake of organic contaminants into the mushroom fruiting bodies was not investigated.

3. Aims and hypothesis

This PhD-project consisted of two parts. The aim of the first part was to investigate the contaminant profiles of Norwegian biogas digestates. The compound groups PPCPs and PFASs were chosen for this investigation. It was expected to find an increasing number of contaminants, and in an increasing concentration with an increase in the amount of sewage sludge in the feedstock.

The aim of the second part was to determine the fate of selected contaminants in a mushroom production system. The production system did mimic commercial mushroom production, and the emphasis was on the uptake of contaminants into the mushroom hats (with regards to food safety) and the concentration of contaminants in the growth media after production (with regards to remediation for further use of the spent mushroom substrate).

Hypotheses of the screening studies

1. The number of contaminants present in digestates are higher in digestates based on from sewage sludge than in digestates based on from food waste.
2. The concentration of pharmaceuticals is higher in digestates based on sewage sludge than in digestates based on food waste.
3. The concentration of PFASs is higher in digestates based on sewage sludge than in digestates based on food waste.

Hypotheses of the mushroom cultivation study

1. The studied organic contaminants are not bioaccumulative in mushrooms.
2. The mushroom uptake of PFAAs is chain-length dependent.
3. The presence of the mushrooms increases the removal of pharmaceuticals from the growth medium.

4. Materials and methods

Figures presenting data were prepared in R version 4.3.1, and were edited in Inkscape version 1.3. All other figures were prepared in Inkscape version 1.3.

4.1 Screening of biogas digestates (Papers I and II)

Sampling campaign

All municipal biogas plants in Norway were contacted in the autumn 2017, and all agreed to send their digestate for analysis. Personnel at the plants sampled about one litre digestate, sent it to NMBU, and provided information on the operating conditions at the plant, including feedstock composition, reactor temperature, retention time, which kinds of pre-treatments that were applied. Most digestate samples were taken as grab samples and represent thus only a snapshot at a particular time of which compounds could be present in the digestate. A few of the plants prepared composite samples of digestate sampled every day during a week. Selected information about the different biogas digestates can be found in Table 1.

Compounds analysed in the digestates

The analysed PPCPs are listed in Table 2 along with compound abbreviations and a description of the compound (see also Papers I and II). Some compounds were validated for quantification in solid digestates only, some for liquid digestates only and some for both. This is also listed in Table 2. PFASs analysed in the digestates are listed in Table 3.

Table 1. Dry matter and feedstock composition of the digestates from Norwegian municipal biogas plants. The digestate codes are composed of two letters where the first is the station code and the second describes if it is a solid (S) or liquid (L) digestate. The station codes equal the codes used in Paper I, where details on the operating conditions of the biogas plants can be found. For those digestates where the station codes differ between Paper I and II, the code used in Paper II are shown in parenthesis.

		Feedstock composition (%)			
Code	**Dry matter (%)**	**Food waste**	**Sewage sludge**	**Manure**	**Fish silage**
Digestates based on food waste					
EL	3.0	100	0	0	0
ES	26.6	100	0	0	0
GL	3.5	100	0	0	0
LL (KL)	2.5	100	0	0	0
LS (KS)	34.8	100	0	0	0
Digestates based on a mix of food waste and sewage sludge					
FL	1.6	45	55	0	0
FS	21.2	45	55	0	0
IL (LL)	4.8	28	72	0	0
IS (LS)	38.9	28	72	0	0
BS	26.2	15	85	0	0
Digestates based on sewage sludge					
DS	47.4	0	100	0	0
HS	26.1	0	100	0	0
JS (IS)	31.6	0	100	0	0
KS (JS)	33.1	0	100	0	0
Digestates based on other feedstocks in addition to food waste and sewage sludge					
CL	4.8	72	0	27	0
AL	1.0	45	53	0	2
AS	27.0	45	53	0	2

Table 2. PPCPs screened for in Norwegian biogas digestates. Abr.: abbreviation. S: validated for solid digestates. L: validated for liquid digestates. See Paper I and II for CAS-numbers and structures. P: Which paper the compound was included in, I = Paper I, II = Paper II.

Compound	Abr.	Description	S	L	P
Antibiotics					
Amoxicillin	AMX	β-lactam antibacterials, penicillins	X	X	II
Penicillin G (Benzylpenicillin)	PENG	β-lactam antibacterials, penicillins	X	X	II
Sulfadiazine	SDZ	Sulfonamide antibiotic	X	X	II
Sulfamethazine (Sulfadimidine)	SMZ	Sulfonamide antibiotic	X	X	II
Sulfadoxine	SDX	Sulfonamide antibiotic	X	X	I+II
Sulfamethoxazole	SMX	Sulfonamide antibiotic	X		I
Ciprofloxacin	CIP	Fluoroquinolone antibiotic	X	X	II
Norfloxacin	NOR	Fluoroquinolone antibiotic	X	X	II
Difloxacin	DFX	Fluoroquinolone antibiotic	X	X	II
Enrofloxacin	ENR	Fluoroquinolone antibiotic	X	X	II
Sarafloxacin	SFX	Fluoroquinolone antibiotic	X	X	II
Tiamulin	TIA	Pleuromutilin antibiotic	X	X	II
Monensin	MON	Pyranes and hydropyranes, antiprotozoal		X	I
Cephalexin	CPX	β-lactam antibacterials, first-generation cephalosporins	X		I
Trimethoprim	TMP	Trimethoprim antibiotic	X	X	I
Narasin	NAR	Pyranes and hydropyranes, antiprotozoal		X	I
Ipronidazole	IPRO	Nitroimidazole derivative, antiprotozoal	X	X	II
Metronidazole	MET	Nitroimidazole derivative, antiprotozoal	X	X	II
Ronidazole	RAN	Nitroimidazole derivative, antiprotozoal	X	X	II
Salinomycin	SLM	Pyranes and hydropyranes, antiprotozoal	X	X	I+II
Other pharmaceuticals					
Prednisolone	PRE	Glucocorticoid (steroid)	X	X	I+II
Dexamethasone	DEXA	Glucocorticoid (steroid)	X	X	II
Ibuprofen	IBP	Nonsteroidal anti-inflammatory drug		X	I
Diclofenac	DCF	Nonsteroidal anti-inflammatory drug		X	I
Acetaminophen (paracetamol)	ACE	Analgesics and antipyretics		X	I
Atorvastatin	ATO	Cholesterol-lowering	X		I

Table 2. Continued

Compound	Abr.	Description	S	L	P
Carbamazepine	CBZ	Anticonvulsant	X	X	I
Amitriptyline	AMT	Tricyclic antidepressant	X	X	I
Atenolol	ATN	β-blocker		X	I
Ranitidine	RAN	Stomach acid lowering	X		I
Losartan	LOS	High blood pressure treatment	X	X	I
Metoprolol	MTP	High blood pressure treatment	X	X	I
Metformin	MEF	Antidiabetic	X		I
Norfluoxetine	NFX	Antidepressant	X	X	I
Warfarin	WAR	Anticoagulant		X	I
Caffeine	CAF	Central nervous system stimulant	X	X	I
Transformation products of pharmaceuticals					
5-hydroxy-DCF	5OH_DCF	TP of diclofenac	X	X	I
Carboxy-ibuprofen	CAR_IBP	TP of ibuprofen	X		I
2-hydroxy atorvastatin	2OH_ATO	TP of atorvastatin	X	X	I
Acridine	ACR	TP of carbamazepine	X	X	I
3-hydroxy-carbamazepine	3OH_CBZ	TP of carbamazepine		X	I
N-acetyl SDZ	NAcSDZ	TP of sulfadiazine	X		I
Carbamazepine-10,11-epoxide	CBZ-EP	TP of carbamazepine	X	X	I
2-hydroxy IBP	2OH-IBP	TP of ibuprofen		X	I
N-acetyl SMX	NAcSMX	TP of sulfamethoxazole	X		I
Personal care products					
Tris(2-chloroprolyl) phosphate	TCPP	E.g. flame retardant, plasticizer	X	X	I
N,N-Dimethyl-*meta*-toluamide	DEET	Insect repellent	X	X	I
Octocrylene	OCT	UV – filter	X	X	I

Table 3. Compounds included in the PFAS-screening.

Compound	Acronym / trade name	Structure	CAS-number
Perfluoroalkyl carboxylic acids (PFCAs)			
Perfluorobutanoic acid	PFBA	$C_3F_7CO_2H$	375-22-4
Perfluoropentanoic acid	PFPeA	C_4F_9COOH	2706-90-3
Perfluorohexanoic acid	PFHxA	$C_5F_{11}COOH$	307-24-4
Perfluoroheptanoic acid	PFHpA	$C_6F_{13}COOH$	375-85-9
Perfluorooctanoic acid	PFOA	$C_7F_{15}COOH$	335-67-1
PFOA branched isomer	Br-PFOA	$C_7F_{15}COOH$	
Perfluorononaoic acid	PFNA	$C_8F_{17}COOH$	375-95-1
Perfluorodecanoic acid	PFDA	$C_9F_{19}COOH$	335-76-2
Perfluoroundecanoic acid	PFUnDA	$C_{10}F_{21}COOH$	2058-94-8
Perfluorododecanoic acid	PFDoDA	$C_{11}F_{23}COOH$	307-55-1
Perfluorotridecanoic acid	PFTriDA	$C_{12}F_{25}COOH$	72629-94-8
Perfluorotetradecanoic acid	PFTeDA	$C_{13}F_{27}COOH$	376-06-7
Perfluoropentadecanoic acid	PFPeDA	$C_{14}F_{29}COOH$	141074-63-7
Perfluorohexadecanoic acid	PFHxDA	$C_{15}F_{31}COOH$	6905-19-5
Perfluorooctadecanoic acid	PFOcDA	$C_{17}F_{34}COOH$	16517-11-6
Perfluoroalkane sulfonic acid (PFSAs)			
Perfluorobutanesulfonic aicd	PFBS	$C_4F_9SO_3H$	375-73-5
Perfluoropentanesulfonic acid	PFPeS	$C_5F_{11}SO_3H$	2706-91-4
Perfluorohexanesufonic acid	PFHxS	$C_6F_{13}SO_3H$	355-46-4
PFHxS branched isomer	Br-PFHxS	$C_6F_{13}SO_3H$	
Perfluoroheptanesulfonic acid	PFHpS	$C_7F_{15}SO_3H$	375-92-8
Perfluorooctanesulfonic acid	PFOS	$C_8F_{17}SO_3H$	1763-23-1
PFOS branched isomer	Br-PFOS	$C_8F_{17}SO_3H$	
Perfluorononanesulfonic acid	PFNS	$C_9F_{19}SO_3H$	68259-12-1
Perfluorodecanesulfonic acid	PFDS	$C_{10}F_{21}SO_3H$	335-77-3
PFDS branched isomer	Br-PFDS	$C_{10}F_{21}SO_3H$	
Perfluoroundecanesulfonic acid	PFUnDS	$C_{11}F_{23}SO_3H$	749786-16-1
Perfluorooctane sulfonamide (FOSA) and Perfluoroalkyl sulfonamido derivatives (FASAAs)			
Perfluorooctane sulfonamide	PFOSA/FOSA	$C_8H_2F_{17}NO_2S$	754-91-6
PFOSA branched isomer	Br-PFOSA		
Perfluorooctane sulfonamidoacetic acid	FOSAA	$C_{10}H_4F_{17}NO_4S$	2806-24-8
FOSAA branched isomer	Br-FOSAA		
N-methyl perfluorooctane sulfonamidoacetic acid	MeFOSAA	$C_{11}H_6F_{17}NO_4S$	2355-31-9

Table 3. Continued

Compound	Acronym / trade name	Structure	CAS-number
Me-FOSAA branched isomer	Br-MeFOSAA		
N-ethyl perfluorooctane sulfonamidoacetate	EtFOSAA	$C_{12}H_8F_{17}NO_4S$	2991-50-6
EtFOSAA branched isomer	Br-EtFOSAA		
Perfluoroether acids			
9-chlorohexadecafluoro-3-oxanonane-1-sulfonic acid	9Cl-PF3ONS	$C_8HClF_{16}O_4S$	756426-58-1
11-chloroeicosafluoro-3-oxaundecane-1-sulfonic acid	11Cl-PF3OUdS	$C_{10}HClF_{20}O_4S$	763051-92-9
Dodecafluoro-3H-4,8-dioxanonanoate, ammonium salt	ADONA	$C_7H_5F_{12}NO_4$	958445-44-8
Fluorotelomer carboxylic acids (FTCA)			
2H,2H,3H,3H-perfluorooctanoic acid	5:3 FTCA	$C_8H_5F_{11}O_2$	914637-49-3
2H,2H,3H,3H-perfluorodecanoic acid	7:3 FTCA	$C_{10}H_5F_{15}O_2$	812-70-4
Fluorotelomer sulfonic acids (FTSs)			
4:2 Fluorotelomer sulfonic acid	4:2FTS	$C_6H_5F_9O_3S$	757124-72-4
6:2 Fluorotelomer sulfonic acid	6:2FTS	$C_8H_5F_{13}O_3S$	27619-97-2
8:2 Fluorotelomer sulfonic acid	8:2FTS	$C_{10}H_5F_{17}O_3S$	39108-34-4
Polyfluoroalkyl phosphoric acid diesters (diPAPs)			
4:2 Fluorotelomer phosphate diester	4:2 diPAP	$C_{12}H_9F_{18}O_4P$	135098-69-0
4:2/6:2 Fluorotelomer phosphate diester	4:2/6:2 diPAP	$C_{14}H_9F_{22}O_4P$	1158182-59-2
6:2 Fluorotelomer phosphate diester	6:2 diPAP	$C_{16}H_9F_{26}O_4P$	57677-95-9
6:2/8:2 Fluorotelomer phosphate diester	6:2/8:2 diPAP	$C_{18}H_9F_{30}O_4P$	943913-15-3
6:2/10:2 Fluorotelomer phosphate diester	6:2/10:2 diPAP	$C_{20}H_9F_{34}O_4P$	
6:2/12:2 Fluorotelomer phosphate diester	6:2/12:2 diPAP	$C_{22}H_9F_{38}O_4P$	

Table 3. Continued

Compound	Acronym / trade name	Structure	CAS-number
6:2/14:2 Fluorotelomer phosphate diester	6:2/14:2 diPAP	$C_{24}H_9F_{42}O_4P$	
8:2 Fluorotelomer phosphate diester	8:2 diPAP	$C_{20}H_9F_{34}O_4P$	678-41-1
8:2/10:2 Fluorotelomer phosphate diester	8:2/10:2 diPAP	$C_{22}H_9F_{38}O_4P$	1158182-60-5
8:2/12:2 Fluorotelomer phosphate diester	8:2/12:2 diPAP	$C_{24}H_9F_{42}O_4P$	1578186-42-1
10:2 Fluorotelomer phosphate diester	10:2 diPAP	$C_{24}H_9F_{42}O_4P$	1895-26-7

Extraction and quantification of organic contaminants in the biogas digestates

All the contaminants were analysed by liquid chromatography tandem mass spectrometry (LS-MS/MS). The extraction procedures for PPCPs are described in detail in Papers I and II along with details on the analysis. The method used for analysis of PFAS follows the procedure described by Kärrman et al. (2021), with minor adaptions in the extraction procedure. Briefly, freeze dried samples were fortified with internal standards, extracted with methanol, and cleaned up with ENVI-carb and glacial acetic acid. The extracts were mixed with recovery standard solution prior to analysis. For analysis of PFAAs, the extracts were also mixed with 4 mM NH_4OAc in water.

Several of the diPAPs were only semi-quantitatively determined, due to a lack of authentic standards. These were 4:2/6:2 diPAP, 6:2/10:2 diPAP, 8:2/10:2 diPAP, 6:2/12:2 diPAP, 10:2 diPAP, 8:2/12:2 diPAP, and 6:2/14:2 diPAP.

Data analysis

Results from the screenings were calculated on a dry weight basis. In Paper I, the results were presented on wet weight basis. Therefore, the results presented here will differ from those presented in Paper I.

Only the full-scale municipal biogas plants were included for presentation in the synopsis. These stations treat large amounts of organic wastes from a large geographic area and therefore best represent the levels of organic contaminants in Norwegian biogas digestates. Therefore two experimental units, which were included in Papers I and II, are excluded. Information on the levels of contaminants in these two digestates can be found in the papers.

Of the municipal biogas plants, all but two (stations A and C) uses either food waste, a mix of food waste and sewage sludge, or sewage sludge as feedstock (Table 1). This gives an opportunity to investigate the relationship between the digestate contaminant profile (i.e., which compounds are present in which concentrations) and these two feedstocks. The Kruskal Wallis H test was used to test for differences between these three categorical groups. Kruskal-Wallis was chosen rather than ANOVA due to the frequent occurrence of outliers and non-detects (observations below the limit of detection (LOD) or the limit of quantification (LOQ)). Significant differences were followed by the Dunn test on the contrasts. Since multiple comparisons were performed, the p-values were adjusted with the Benjamini – Hochberg correction to reduce the false discovery rate.

Linear regression was used to test if: a) the sum of pharmaceuticals in the digestates (based on Papers I and II), b) the number of compounds which were detected in at least one digestate (including compounds in both Table 2 and 3), was a function of the percentage of sewage sludge content in the feedstock. Stations A and C were excluded, so that the amount of food waste in the feedstock was the inverse of the amount of sewage sludge. Only compounds which were screened for in all digestates were included, i.e., pharmaceuticals screened for in only solid or liquid digestates (Table 2) were excluded. In addition, for analysis b), digestate LL was excluded as it was not included in the PFAS screening, and PFBS was excluded because it was not screened for in all digestates. The assumptions of the linear regression were tested by evaluation of diagnostics plot.

The relationship between all compounds detected in the digestates were further explored using a principal component analysis (PCA). PCA requires a complete dataset with no non-detects. Therefore, the same dataset which was used for linear regression part b (see above paragraph) was used. However,

unlike the regression, digestates A and C was included in the PCA. Observations below the LOQ or LOD were substituted by zero. Substitution is generally not recommended (Helsel, 2012), but was necessary to perform the PCA. Note however that the choice of substitution value can significantly affect the result (Helsel, 2012). The dataset was centred and scaled before analysis, as concentrations of the individual compounds varied from the ng kg^{-1} range to the low mg kg^{-1} range. The PCA was performed in R version 4.3.1 using the function prcomp from the stats package.

The levels of the individual compounds in the digestates were included in the PCA. Operating conditions, including feedstock, were not. The biplot presented in the synopsis differs from the biplot presented in Paper I in three respects: (1) Dry weight concentrations are used in the synopsis. (2) More compounds are included in the synopsis. (3) In the synopsis, the feedstock composition of the digestates was not included in the PCA analysis.

Soil concentration of organic contaminants after application of biogas digestate as fertiliser

For pharmaceuticals included in Paper II (see Table 2 for overview), the predicted environmental concentration (PEC) of the pharmaceuticals in the soil after a single fertilising event with biogas digestate was calculated. It was assumed a fertilisation rate of 8 t solid or 45 t liquid (fresh weight) digestate per hectare, and an incorporation to a soil depth of 20 cm. The maximum concentration quantified in any digestate was used, as worst-case scenario. Predicted no effect concentrations (PNEC) for harm towards soil organisms were estimated from ecotoxicity data. For each compound, a risk quotient was calculated as PEC divided by PNEC. Generally, a risk quotient below 0.1 indicates low risk, 0.1 – 1 moderate risk, and a risk quotient > 1 high risk (Menz et al., 2019). Risk quotients for selection of antibiotic resistant bacteria were also calculated for those antibiotics where a PNEC value was available from Menz et al. (2019). Details on the estimation of PEC and PNEC values can be found in Paper II.

For PFOS, PFOA, and the sum of PFASs, PECs were calculated following the procedure described for pharmaceuticals. However, unlike the pharmaceuticals, the PECs were calculated for each individual digestate and the range and mean of PECs are presented in the results.

4.2 Mushroom uptake and degradation of contaminants (Papers III and IV)

Experimental setup

The experimental setup was based on the mushroom cultivation system developed at Lindum AS. The system was originally developed by Ketil Stoknes (Stoknes, 2020) and was further modified by Agnieszka Jasinska (Jasinska et al., 2022). Figures 4 and 5 shows pictures from the preparation of the mushroom growth substrate and from the mushroom cultivation, respectively.

The mushroom growth substrate was fortified with both PFAS (Paper III) and pharmaceuticals (Paper IV). The PFAS spiking solution was mixed into the liquid digestate before preparation of the mushroom substrate (Fig 4B) to ensure a homogenous distribution of the compounds. The pharmaceuticals were added to the ready mushroom substrate (Fig 5A) as these were expected to partly degrade during the preparation of the mushroom substrate.

Two batches of mushroom substrate were prepared, one control and one spiked. Briefly, digestate based on a mix of food waste and manure (4.3% DM, Figure 4A) was mixed with wheat straw and amended with gypsum. Garden compost was used as inoculation. The final dry matter of the mix was 25%. The mix was first composted in two kinds of isolated composting drums for three (Figure 4C and 4D) plus six days (Figure 4E). The compost was very densely packed in the second compost drum, and forced aeration was used to keep the compost aerobe. At last, the compost was pasteurized for two days (Figure 4F). The two batches were prepared in separate equipment, except during pasteurization, when the two batches were treated in two separate chambers of the same pasteurization box (Figure 4F).

Figure 4. Preparation of mushroom substrate. Pictures by Agnieszka Jasinska and Astrid S. Nesse

After pasteurization, each of the two batches were split in 11 units. Four were inoculated with *Agaricus bisporus*, four with *Agaricus subrufescens*, and three were not inoculated (Fig 5A). All 11 units prepared from the PFAS spiked substrate, were now spiked with pharmaceuticals (Fig 5A). Each unit were kept in a closed bag with ventilation filters, until the spawn had overgrown the substrate (Fig 5C). Then, a casing layer of amended peat were added to initiate the development of the mushroom fruiting bodies. Figures 5D and 5F shows the small mushroom pins emerging from the casing layer, and the mushroom hats ready for harvest.

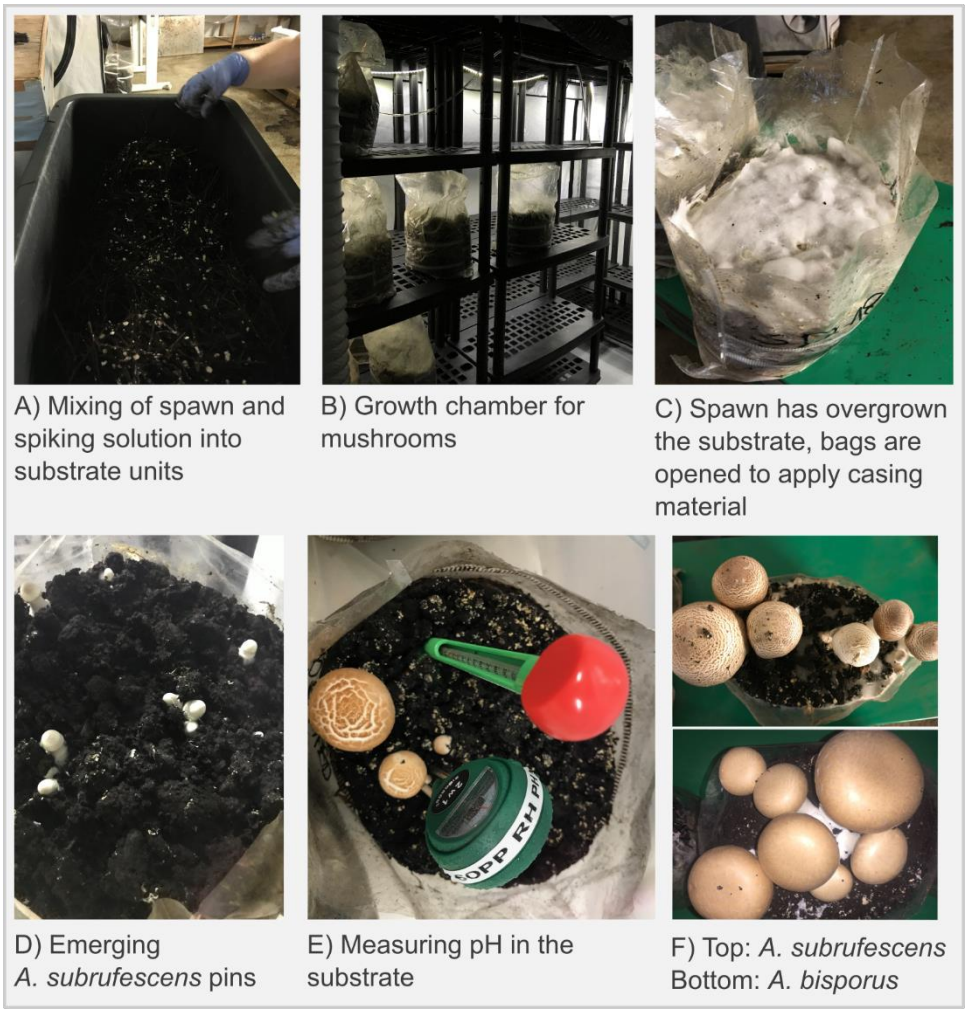

Figure 5. Cultivation of mushrooms. Pictures by Agnieszka Jasinska and Astrid S. Nesse.

Analysis of contaminants

The analysis of contaminants in the mushroom growth substrate and in the mushroom hats are described in Paper III for PFAS and in Paper IV for PPCPs.

Dietary intake of contaminants

The intake of contaminants was estimated by equation 1. The average global mushroom consumption was 5 kg (fresh weight) per capita per year (Royse et al., 2017), and the average dry matter content of the mushrooms in Papers III and IV were 13%. A lower and upper bound was estimated for intake of PFAS. For the lower bound, the average concentration of a contaminant in the control mushrooms were used. For the upper bound, the maximum concentration quantified in mushrooms cultivated in spiked growth substrate was used. For the PPCPs, only the upper bound was estimated because the compounds were not detected in the control mushrooms, except for one instance of DEET uptake. However, the highest concentration quantified in the spiked mushrooms was identical the concentration quantified in the control replicate (i.e., 0.15 µg kg^{-1} DW).

$$Intake\ (mg\ /\ year) = Mushroom\ conc.\left(\frac{mg}{kg\ dm}\right) * \frac{0.13\ kg\ dm}{kg\ fw} * 5\ kg\ fw/\ year \qquad (1)$$

Estimation of bioaccumulation factors

Bioaccumulation factors were determined for each contaminant at both harvests, for each replicate. The bioaccumulation factors were estimated by dividing the concentration of the contaminant in the mushroom hats by the concentration of the contaminant in the growth substrate at the harvest time. The concentrations were given on dry weight basis for the calculation.

5. Results and discussion

5.1 Organic contaminants in Norwegian biogas digestates

In the biogas digestates, 101 compounds were validated for analysis. Of these, 60 were quantified in at least one of the municipal biogas digestates. Nineteen PPCPs were quantified in at least 75% of the digestates and/or with a maximum concentration above 100 µg kg^{-1} DW (Table 4).

Table 4. Overview of PPCPs quantified in at least 75% of the digestates and/or with a maximum concentration above 100 µg kg^{-1} DW.

Compound	Quantification frequency (%)	Median (µg kg^{-1} DW)	Max (µg kg^{-1} DW)
Metoprolol	100	182	343
Carbamazepine	100	100	272
TCPP	94	499	1908
Losartan	94	160	394
Acridine	88	7.3	46
Acetaminophen[1]	86	137	3663
Octocrylene	71	1032	7467
2-hydroxy atorvastatin	71	41	567
Caffeine	53	13	599
DEET	53	2.8	637
Amitriptyline	47	<LOQ	503
Diclofenac	47	<LOQ	307
Ibuprofen[1]	29	<LOQ	1200
Atorvastatin[2]	20	<LOQ	2358
5-hyroxy diclofenac	18	<LOQ	350
Amoxicillin	12	<LOQ	960
Prednisolone*	12	<LOQ	650
Ciprofloxacin	6	<LOQ	430
Sulfadiazine	6	<LOQ	140

[1] Screened for only in liquid digestates. [2] Screened for only in solid digestates. [3] Prednisolone was screened for in both Paper I and II, and were found in one digestate in each screening, i.e., a total of two digestates.

Many of the PPCPs included in the screening are generally used in high quantities (Sommerschild et al., 2020) and were thus quantified frequently, often in concentrations of several hundred µg kg^{-1} DW or even a few mg kg^{-1} DW (Table 4). Out of the 20 antibiotics screened for (Paper I and II) eight were

not detected, four were detected below the LOQ, while eight were quantified in a few digestates (1 – 3 of the municipal digestates). Although the quantification frequency of antibiotics was very low, three compounds were quantified in a few hundred μg kg^{-1} DW in digestates from one biogas plant each, i.e., amoxicillin, ciprofloxacin, and sulfadiazine (Table 4). The presence of easily degradable pharmaceuticals such as amoxicillin and sulfadiazine suggest that other more recalcitrant pharmaceuticals, which were not included in the analysis, may also be present in the digestates (Daughton & Ternes, 1999).

Table 5. PFASs quantified in at least 75% of the digestates and/or with a maximum concentration above 1 μg kg^{-1} DW.

Compound	Quantification frequency (%)	Median (μg kg^{-1} DW)	Max (μg kg^{-1} DW)
PFOA	100	0.66	1.7
6:2 diPAP	94	1.0	94
PFOS	94	1.7	8
PFUnDA	94	0.19	0.69
Br-PFOS	88	0.30	1.3
PFNA	88	0.19	0.5
PFDA	81	0.38	1.7
6:2 FTS	81	0.7	12
PFDoDA	75	0.21	0.66
PFOSA	75	0.12	0.23
5:3 FTCA	69	0.52	10.3
8:2 FTS	63	0.3	1.9
PFHxA	63	0.31	1.4
6:2/8:2 diPAP	56	0.09	2.9
EtFOSAA	50	0.03	1.3
7:3 FTCA	38	<LOQ	3.5
8:2 diPAP	38	<LOQ	1.09
10:2 diPAP	19	<LOQ	42

PFASs were generally quantified in low concentrations compared to the PPCPs, and most of the quantified compounds were found in the concentration range 0.1 – 1 μg kg^{-1} DW. PFASs which were quantified with a maximum concentration above 1 μg kg^{-1} DW and/or in at least 75% of the digestates are listed in Table 5 (eighteen compounds). The levels of PFAS in

the digestates are briefly presented here, as these results are not shown in any of the papers.

Of the 53 PFASs screened for, 33 were quantified in at least one digestate. Several long-chained PFCAs including PFOA, perfluorononaoic acid (PFNA), PFDA and perfluoroundecanoic acid (PFUnDA) were frequently detected, but in relatively low concentrations (< 2 μg kg^{-1} DW). PFOA was the only PFAS which was detected in all digestates. Linear and branched PFOS was also frequently detected, in all but one and two digestates, respectively. Linear and branched PFOS were the dominating PFSAs and made up 74 – 100% of $\sum_{11}$PFSA in the digestates. Also 6:2 FTS and 6:2 diPAP were quantified in more than 80% of the digestates.

The PFASs quantified in the highest concentrations were 6:2 diPAP, 10:2 diPAP, 6:2 FTS, 5:3 FTCA, and linear PFOS (Table 5). Often, diPAPs are amongst the PFASs which are found in the highest concentrations in biosolids (D'Eon et al., 2009; Kärrman et al., 2019), and can be expected to occur in food waste as well due to the use of diPAPs as coatings on food contact materials (FCMs, Taxvig et al., 2014). 10:2 diPAP were quantified in high concentrations in three digestates (FL, FS, and AL, Figure 7) and were not detected in any of the other digestates. However, 10:2 diPAP were only semi-quantitatively determined and these results are somewhat uncertain.

Many short-chained PFCAs and PFSAs were infrequently quantified or not detected, despite that these compounds ought to replace the longer-chained PFAAs (Z. Wang et al., 2013). None of the perfluoroether acids used as replacement for PFAAs were quantified in any of the digestates either.

5.2 Relationship between feedstock and contaminant levels

The feedstock is the source of contaminants in the digestates. As sewage sludge is known to contain several of the compounds included in the screenings, the expectation was to find a higher number of compounds, and in higher concentrations, in digestates based on sewage sludge compared to those based on food waste. However, for most of the individual PPCPs, there was no apparent relationship between feedstock and occurrence, or concentration of contaminants (Figure 6).

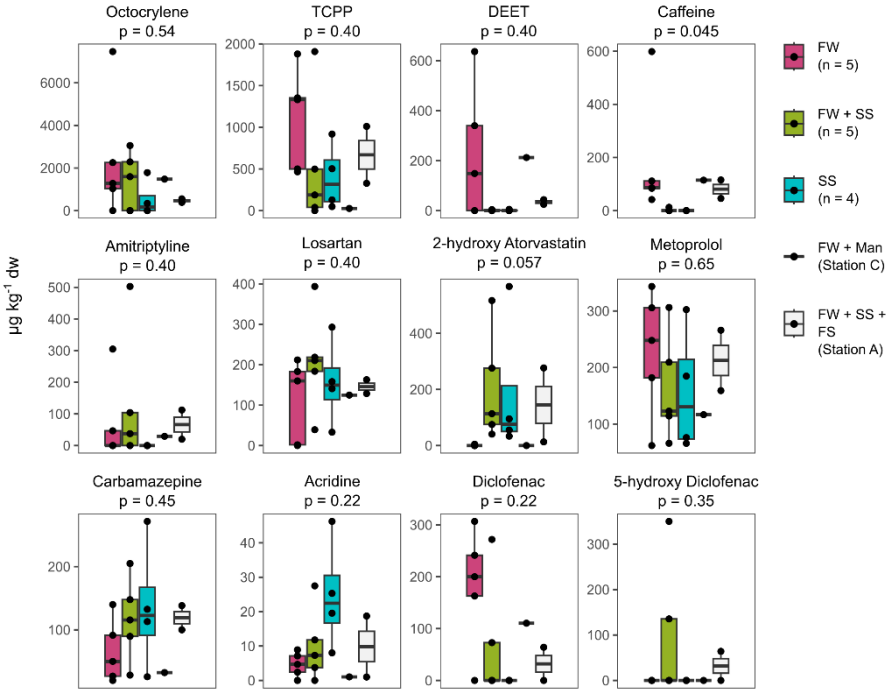

Figure 6. Levels of PPCPs in digestates from Norwegian biogas plants (µg kg^{-1} DW). Values below the LOQ are plotted as zero. The p-values refer to differences in compound concentration between the feedstocks food waste (FW), combined food waste and sewage sludge (FW + SS) and sewage sludge (SS). The test used were Kruskal Wallis H test, and the p-values were adjusted with the Benjamini-Hochberg method. Stations A and C are included only for visual comparison.

In Figure 6, the concentration of compounds validated for both solid and liquid digestates (Paper I) is shown, except carboxy-ibuprofen and prednisolone. Carboxy-ibuprofen was not detected in any digestates, and prednisolone was found only in digestate C (342 μg kg^{-1} DW).

Only for two compounds there was moderate statistical evidence that the compound concentration was different between digestates of different feedstocks. Caffeine was found in higher concentrations in food waste digestates compared to sewage sludge digestates ($p = 0.01$, Dunn's test) and 2-hydroxy atorvastatin was found in lower concentrations in food waste digestate compared to sewage sludge digestate ($p = 0.04$, Dunn's test). For all other compounds there was little to no evidence that the concentration differed depending on feedstock (Kruskal Wallis H-test, Figure 6). Such a comparison was not performed for the antibiotics and steroids determined (Paper II), due to the low detection frequency of all compounds. However, both amoxicillin and sulfadiazine were found exclusively in food waste-based digestates. Ciprofloxacin, sulfamethazine, and prednisolone on the other hand were only quantified in digestates based on sewage sludge alone or in combination with food waste.

For PFAS however, there were differences in contaminant profile depending on the feedstock. In all four digestates based solely on food waste, 6:2 FTS was the dominating compound, with PFOA, PFOS and 6:2/8:2 diPAP as other important contributors to $\sum_{53}$PFAS (Figure 7).

The concentration of 6:2 FTS was considerably higher in food waste digestates (5.5±4.4 μg kg^{-1} DW) than in digestates based partly or fully on sewage sludge (1.1±1.4 μg kg^{-1} DW). 6:2 FTS are used in a range of consumer products, including paints, coatings, adhesives, waxes, and can be applied to e.g. paper and textiles (Hamid et al., 2020), including paper and cardboard FCMs (Dueñas-Mas et al., 2023). However, although 6:2 FTS has been quantified in all investigated FCMs from fast food restaurants in France, the average concentration was only 0.03 μg kg^{-1} FCM (Dueñas-Mas et al., 2023). Thus, it is unlikely that FCMs were the main source of the average concentration of 5.6 μg kg^{-1} DW (average concentration) of 6:2 FTS found in the food waste digestates.

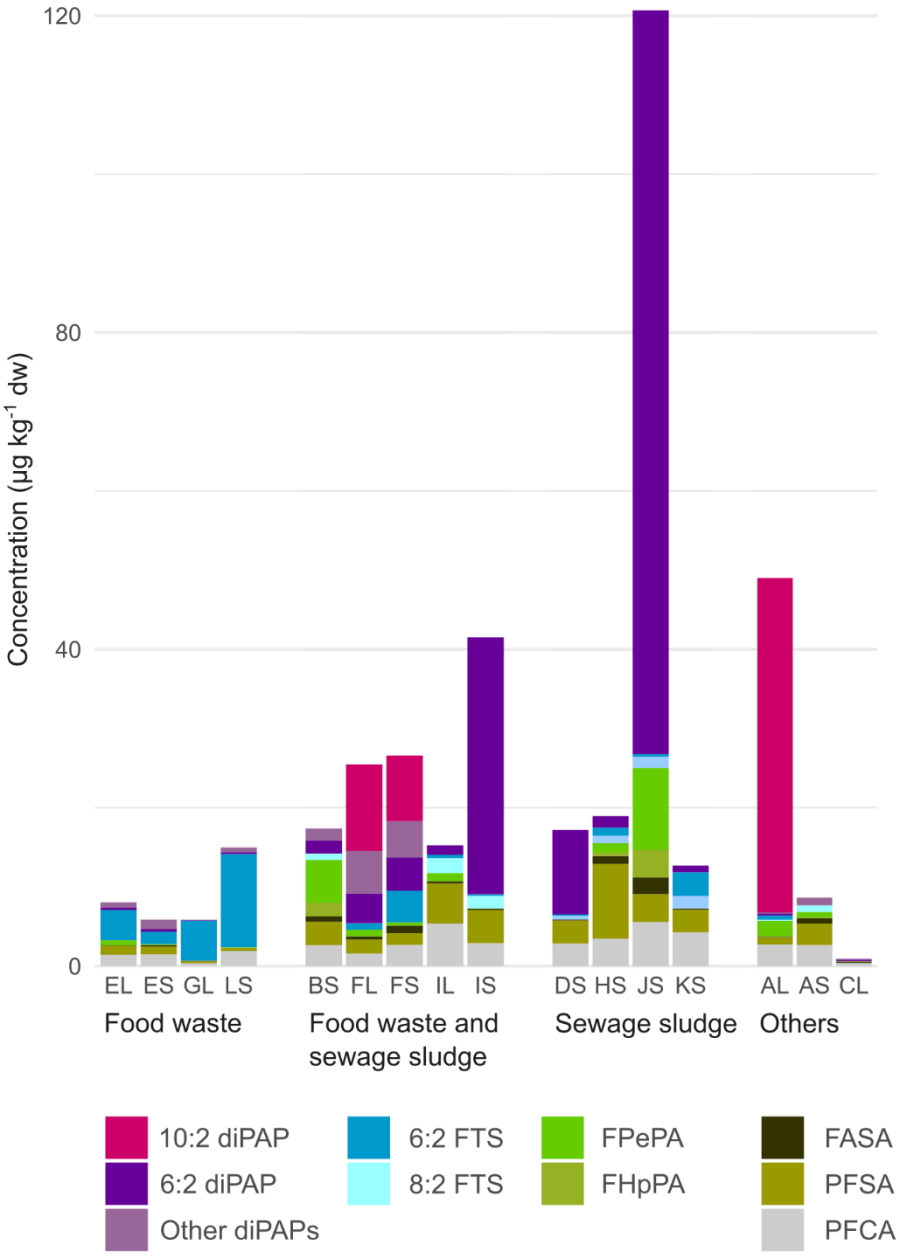

Figure 7. PFASs detected in the digestates. Most of the compounds are grouped together after class, for enhanced readability. Groups: PFCA, PFSA, FASA, green – mono-PAPs

The number of quantified diPAP homologues was larger in food waste digestates (6 out of 11 homologues) than in sewage sludge digestates, where only 6:2 diPAP were quantified. It is reasonable to find a diversity of diPAPs in the food waste digestates. Paper and cardboard FCMs can be coated with diPAPs to repel grease and water, and the diPAPs can migrate from the FCMs into food (Dueñas-Mas et al., 2023; Lerch et al., 2023). Especially 6:2 and 8:2 diPAP are frequently detected in FCMs (Dueñas-Mas et al., 2023; Lerch et al., 2023). The lack of diPAPs other than 6:2 diPAP in the sewage sludge digestates did however contrast with other studies. D'eon et al. (2009) quantified six diPAPs in sewage sludge samples, in concentrations ranging from 47 to 200 μg kg^{-1}, and Kärrman et al. (2019) found 5 different diPAPs in all sewage sludge samples in a Nordic screening.

The within-group variability for digestates based (partly) on sewage sludge was considerable, as has also been found in other studies (Kärrman et al., 2019; Thompson et al., 2023). The variation is reasonable as the influents to the WWTPs can be highly variable. Some WWPTs receive mainly domestic wastewater whereas others also treat industrial effluents or hospital wastewater.

Except for the digestates JS, AL and IS, the $\sum_{53}$PFASs were considerably lower than the levels found in biosolids in a Nordic screening ($\sum_{85}$PFAS 40 – 180 μg kg^{-1} DW, Kärrman et al., 2019) and in the U.S. ($\sum_{92}$PFAS 182 – 1650 μg kg^{-1} DW, Thompson et al., 2023). The numbers are not directly comparable, as a larger number of compounds was included in the study by Kärrman et al. (2019) and Thompson et al. (2023), and the concentration of individual PFASs can both increase and decrease during anaerobic digestion (Thompson et al., 2023).

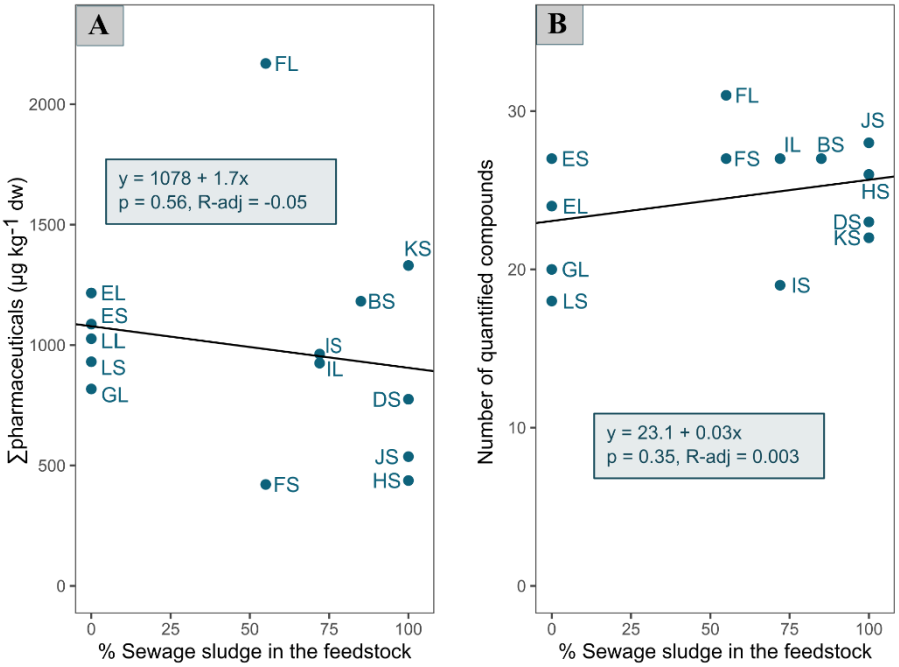

Figure 8. Sum of pharmaceuticals **(A)** and number of all detected compounds **(B)** in the digestates as a function of the amount of sewage sludge in the feedstock. Only digestates using either sewage sludge and/or food waste was included. Digestate LL was not included in Figure 8B because PFASs was not quantified in that digestate.

For digestates based on sewage sludge and/or food waste as feedstock (i.e., all municipal stations but A and C), the total sum of pharmaceuticals (Figure 8A) and the number of detected compounds (incl. PFAS and PCPs, Figure 8B) were plotted vs. the amount of sewage sludge in the feedstock. Note that only the compounds which were screened for in all digestates were included. Hence, e.g., acetaminophen and ibuprofen, which were screened for only in the liquid digestates, are not included. In Figure 8B, digestate LL is not included as PFAS was not screened for in that digestate.

There was no relationship between the sum of pharmaceuticals in the digestates and the amount of sewage sludge in the feedstock (Figure 8A), nor between the number of detected compounds (incl. both PPCPs and PFAS) and the amount of sewage sludge in the feedstock (Figure 8B). Both figures show a large variability between digestates from similar feedstocks. The most pronounced example was found in station F, where the liquid fraction (FL)

had the highest concentration of pharmaceuticals and the solid (FS) one of the lowest (Figure 8A). Prednisolone, diclofenac, 5-hydroxy-diclofenac, and amitriptyline were quantified in FL in concentrations ranging from 37.5 to 650 μg kg^{-1} DW digestate, but were not detected in FS. Other pharmaceuticals were quantified in both, but with the highest concentration in FL. In contrast, digestates FL and FS had similar contaminant profiles for PFAS (Figure 7).

To further investigate the contaminant profiles of the digestates, a principal component analysis was performed. The two first principal components (PC1 and PC2) explained only about 40% of the variation in the dataset. Hence, a large amount of the variability in the contaminant profiles are not captured by the two first principal components and the following information is only indicative.

The first principal component separates the food waste digestates from the sewage sludge digestates. The food waste digestates clustered relatively close to each other, together with digestate CL, which was based on a mix of food waste and manure. This is consistent with the similar PFAS content of the food waste digestates. Further, the three personal care products octocrylene, DEET, and TCPP seem to be associated with food waste digestates. These three compounds had higher average concentration in the food waste digestates than in the sewage sludge digestates (Figure 6). However, the concentrations were not significantly different among the feedstocks (Kruskal Wallis H test).

While the food waste digestates clustered relatively close to each other, the variability within sewage sludge digestates and combined sewage sludge and food waste digestates was considerable. This is consistent with the high variability in PFAS concentration seen for these digestates (Figure 7).

The second principal component separated digestate FL, and to some extent FS and JS, from the other digestates. Digestate JS had the highest concentration of 6:2 diPAP, 5:3 FTCA and 7:3 FTCA, and was the only digestate where perfluorooctadecanoic acid PFOcDA and *N*-methyl perfluorooctane sulfonamidoacetic acid (MeFOSAA) were quantified. Digestates FS and FL differed from the other digestates by having the highest concentration of all diPAPs except 6:2 diPAP. As noted earlier, FS and FL also differed from each other in their content of pharmaceuticals.

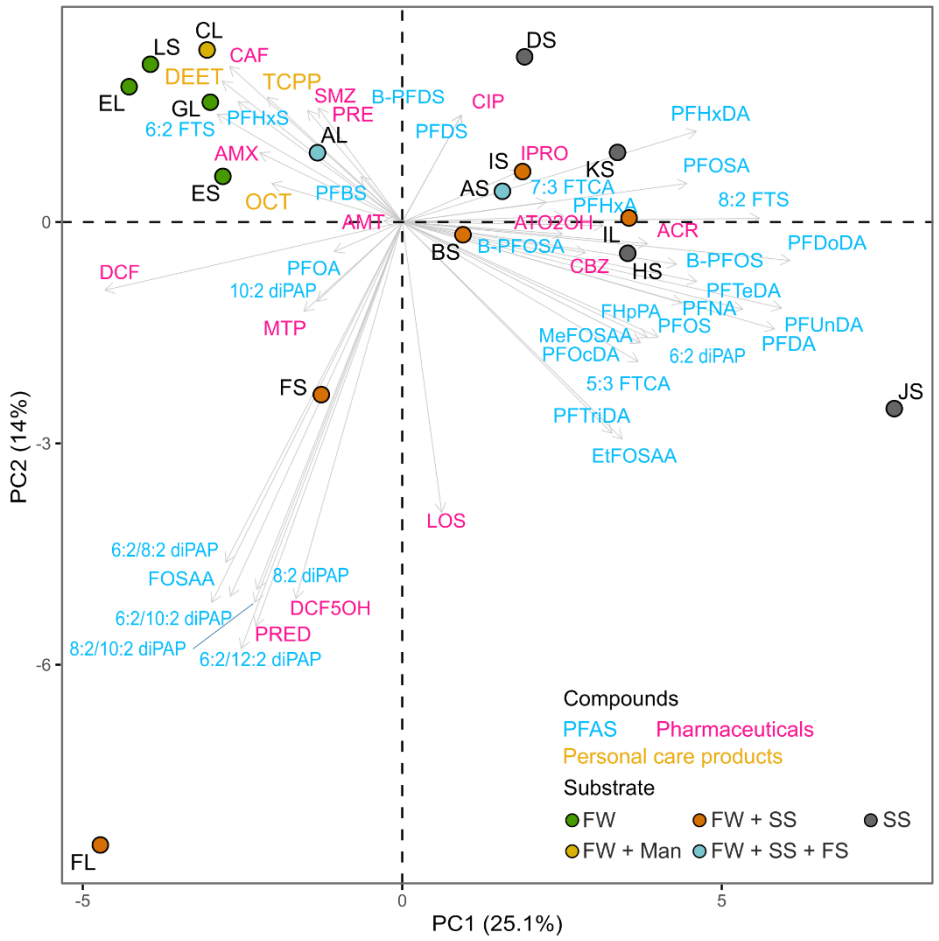

Figure 9: Biplot showing the first two principal components for a PCA performed on the combined dataset of all three screenings (dry weight, scaled and centred). Compound abbreviations are explained in Tables 2 and 3.

The combined results presented indicate that knowledge of the feedstock only gives limited information on the digestate contaminant profiles. Hypothesis 1 and 2, connected to the screening studies, are thus not supported. The number of individual compounds present in digestates were not higher in digestates based on sewage sludge than in digestates based on food waste (Hypothesis 1). The concentration of pharmaceuticals was not higher in digestates based on sewage sludge than in digestates based on food waste (Hypothesis 2).

Regarding hypothesis 3, support in the data set was found. The concentration of PFASs was higher in the digestates based on sewage sludge. Only a few individual PFASs were found in higher concentrations in food waste digestates.

A similar screening of biogas digestates was conducted in Finland almost 10 years ago. Suominen et al. (2014) screened for 10 compounds or compound groups in digestates from 10 Finish biogas plants. The contaminants included PFAS, PCBs, PAHs, polybrominated diphenyl ethers, phthalates, and others. The feedstocks included sewage sludge, municipal biowaste, fat, food industry by-products, and others. The authors found no clear relationship between the type of feedstock and the contaminant profiles of the digestates. The lack of relationship was not discussed. The finding of comparable levels of contaminants in the digestates independent of feedstock also in the Finish study supports that food waste, similar to sewage sludge, should be considered a source of contaminants.

While sewage sludge often contains a range of contaminants (Clarke & Smith, 2011; Daughton & Ternes, 1999) and thus usually is considered as a major source, food waste is less acknowledged as a contaminant source. However, it should not be a surprise that also food waste contains contaminants. Food and food waste can be contaminated during all steps of production, transport, processing, packaging, and during waste management (Nerín et al., 2016). Food and feed plants may take up contaminants from the soil (Wu et al., 2015), antibiotics may be used for growth promotion in livestock production in some parts of the world (J. Chen et al., 2019), and both PFAS as well as antibiotics can be used for plant protection (Glüge et al., 2020a). Many PFASs have shown to be bioaccumulative in fish, including long-chained PFAAs, FOSA, FASAAs, diPAPs, and FTSs (Burkhard, 2021), and can be found in concentrations up to the low – medium µg kg^{-1} range (Pasecnaja et al., 2022). Also antibiotics have been found in the µg kg^{-1} – mg kg^{-1} range in foodstuffs, both in vegetables and animal products (J. Chen et al., 2019).

Migration of chemicals from FCMs is a well-known food contamination source (Nerín et al., 2016). For example, Su et al. (2021) quantified 475 migrants after extraction of polyolefins. One of these was octocrylene, which was detected in 60% of recycled FCMs in China and Spain, at a maximum concentration of 166 µg kg^{-1} "food stimulant". Other substances, e.g.,

organophosphate esters such as TCPP, can be used as plasticizers in FCMs. Hence, TCPP was found at a median concentration of 1.55 µg kg^{-1} ww in Chinese foodstuffs (X. Chen et al., 2023). As mentioned earlier, paper and cardboard FCMs may also be coated with PFAS (Dueñas-Mas et al., 2023).

In addition, source separated food waste does not only contain food waste. Two municipal companies handling food waste report that the municipal food waste contained on average 3.8 – 6% "other wastes", including plastic packaging, diapers, sanitary pads, paper, cardboard, glass, and metal packaging (Bjørnerud & Hultin, 2019; Oslo municipality, 2021).

There are also differences in the management of food waste and wastewater and prior to anaerobic digestion. Food waste is usually transported to the biogas plants in the bags used for source separation, but is pretreated before anaerobic digestion, which can degrade some organic contaminants (Taboada-Santos et al., 2019). Many WWTPs apply treatments intended to biodegrade the organic material of the wastewater (Rout et al., 2021). Although these WWTPs are not specifically designed for the removal of organic contaminants, many compounds are nevertheless degraded during wastewater treatment (Jelic et al., 2011; Rout et al., 2021). Additional treatments such as electrochemical oxidation can further degrade organic contaminants, even PFAS, but may be very energy intensive (Gomez-Ruiz et al., 2017). In general, a part of the remaining contaminants is discharged with the treated wastewater. For pharmaceuticals, Jelic et al. (2011) found that most residues at three different WWTPs were discharged with the treated wastewater. Only 3 – 9% of the residues were retained in the sewage sludge.

5.3 Risks associated with the use of digestates as fertilisers

A simplified risk evaluation showed that fertilisation with digestates implied a high risk of harm towards soil organisms, if the digestate with the highest amoxicillin concentration was used (risk quotient > 1, Table 6). The calculated risk quotient for prednisolone was also above 1, but as this value was extrapolated from aquatic toxicity data it was considered uncertain. No other investigated pharmaceuticals (Paper II) were present in the digestates in concentrations which poses a high risk towards soil organisms, and for all antibiotics the risk of promotion of antibiotic resistance was low (risk quotient < 0.1, Table 6). Only compounds included in Paper II which were quantified in at least one municipal biogas digestate were included in the risk assessment (see Table 2 for overview of compounds).

Table 6. Risk quotients for harm towards soil organism and for selection of antibiotic resistant bacteria after application of biogas digestates as fertiliser. The risk quotients were calculated based on the highest concentrations quantified in the digestates.

	Risk quotient, soil organisms	**Risk quotient, ARB selection**
Amoxicillin	1.4	0.099
Sulfadiazine	0.01	0.002
Sulfamethazine	0.001	
Ciprofloxacin	0.9	0.014
Ipronidazole	NA	
Prednisolone	2	

Amoxicillin is however not expected to persist in soil (Braschi et al., 2013). When Menz et al. (2019) calculated risk quotients for amoxicillin in soil pore water after application of manure, the initial risk quotients were 596 for toxicity towards soil bacteria and 170 for selection of antibiotic resistant bacteria. However, when the PEC were refined and, among others, degradation during manure storage and after soil application were accounted for the risk quotients decreased to below 0.02. Hence, the long-term risk associated with amoxicillin is probably low.

Instead, the presence of ciprofloxacin and ipronidazole can be of higher concern, although the risk quotient for ciprofloxacin was comparatively lower and the risk quotient for ipronidazole was unknown (there was no ecotoxicity data). Ciprofloxacin is of concern because it is a persistent compound which is strongly sorbed to soil particles and has thus been reported to accumulate in soils when repeatedly added (Dalkmann et al., 2012). Both ciprofloxacin and norfloxacin are classified as critically important antimicrobials by the World Health Organization (WHO, 2018). Ipronidazole was found only in low concentrations in a few digestates (up to 2.7 μg kg^{-1} DW). Nevertheless, the presence of ipronidazole was surprising, as there are no registered human or veterinary pharmaceuticals in Norway which contain ipronidazole (H. Østensen, personal communication, July 7, 2021). The risk quotient for prednisolone was also above 1, but this value was rather uncertain as it was based on ecotoxicity data for aquatic organisms.

In Norwegian regulations, soils are classified as polluted if the concentration of selected contaminants is above their respective normative values (Ministry of Climate and Environment, 2004). Currently, the only contaminant in this PhD project included in the regulations is PFOS. The normative value for PFOS was recently proposed to be reduced from 100 μg kg^{-1} dry soil to 2 μg kg^{-1} dry soil (Norwegian Environment Agency, 2020), based on the recommendation of the Norwegian Geotechnical Institute (NGI, Breedveld et al., 2020). NGI recommends normative values of 3 μg kg^{-1} for both PFOA and PFOS. Both compounds were therefore included in Table 7 along with the sum of the 53 PFASs included in the screening for comparison. Note that the concentrations shown in Table 7 are in ng kg^{-1}, not μg kg^{-1} soil, due to the low predicted concentrations. The concentrations listed in Table 7 are also not comparable with the digestate concentrations in Figure 7, as the calculation of predicted soil concentration of PFAS is done on a wet weight basis (see Paper II for details) whereas the digestate concentrations shown in Figure 7 are on a dry weight basis.

Table 7. Calculated soil concentrations of PFOS, PFOA and $\sum PFAS_{53}$ (ng kg^{-1} dry soil, top 20 cm) after a single fertilising event with biogas digestate. The minimum and maximum values are shown, with the average in parenthesis.

Feedstock	PFOS	PFOA	$\sum_{53}$PFAS
FW	0.17 - 0.68 (0.47)	0.19 - 2.0 (0.85)	3.7 - 17 (8.03)
FW_SS	0.78 - 4.1(2.35)	0.42 - 1.3 (0.77)	15 - 51 (23.2)
SS	2.0 - 6.8 (3.72)	0.44 - 0.93 (0.61)	13 - 122 (44.2)

With a proposed limit of 2 µg PFOS kg^{-1} soil (DW), it should be no problem to fertilise soils with Norwegian produced digestates. The highest predicted soil concentration of PFOS was 4.1 ng kg^{-1} dry soil. It will then take 294 years to reach a soil concentration of 2 µg PFOS kg^{-1} soil if the digestate with the highest PFOS concentration is added each year, and assuming no disappearance of PFOS from the soil. For PFOA, it will take 1000 years to reach 3 µg kg^{-1} soil.

On the other hand, it can also be argued that a safe lower limit for PFAS cannot be defined. Due to their persistence, or transformation into persistent compounds, PFASs released to the environment will contribute to the total PFAS load in a cumulative fashion (Cousins et al., 2020). Cousins et al. (2020) argue that increased concentration result in increased exposure, thus, the likelihood of new or already known health effects will increase.

When more research on the fate and toxicity of PFAS is conducted, and/or new methods with lower limits of detection and quantification are developed, the concentrations of PFASs in the digestates may be regarded as too high. Legislative limits on contaminants are not just related to their environmental fate and toxicity, but also to analytical capabilities for the compounds (Breedveld et al., 2020).

Besides harm towards soil organisms, organic contaminants added to the soil can also be taken up by agricultural crops (Lesmeister et al., 2021; Wu et al., 2015). Most of the investigated PPCPs are not accumulated in agricultural crops, due to sorption or degradation in the soil, or low bioaccumulation factors (Wu et al., 2015). Carbamazepine, however, is an example of a

persistent and bioavailable contaminant in soils, and is frequently detected in plants irrigated with treated wastewater or spiked water (Wu et al., 2015).

Furthermore, application of manure or biosolids to farmland can be a source of contaminants in groundwater, where degradation often is slow due to a low abundance of microorganisms and anaerobic conditions (Lapworth et al., 2012). This contributes to the global occurrence of pharmaceuticals in groundwater (Silori et al., 2022). Groundwater contamination is a challenge e.g., in the U.S.A., where 23 million households are estimated to rely on drinking water from private groundwater wells (Murray et al., 2021).

Contaminants, for example carbamazepine, acetaminophen, and ibuprofen, can also be found in the subsurface drainage of soil fertilised with biosolids (Gottschall et al., 2012) and thereby contaminate e.g., surface waters. Carbamazepine has also been detected frequently in leachate or runoff from soils irrigated with treated wastewater (Wu et al., 2015). However, leaching and runoff of contaminants is more pronounced for soil irrigated with treated wastewater than for soils amended with e.g., biosolids, as the organic matter of the biosolids increases the retardation of the contaminants in soil (Wu et al., 2015).

5.4 Mushroom uptake of organic contaminants

The mushrooms generally took up very small amounts of organic contaminants into their fruiting bodies, both PFAS (Figure 10) and PPCPs (Table 8). The detection frequency in the mushroom hats was low for most compounds. For the assimilated compounds, bioaccumulation factors (BAF) were below, and usually far below, 1. The low uptake was expected because fungal cell walls generally only let small molecules pass (Griffin, 1994), and since other studies also have demonstrated a very low potential for mushroom uptake of organic contaminants (Table 8).

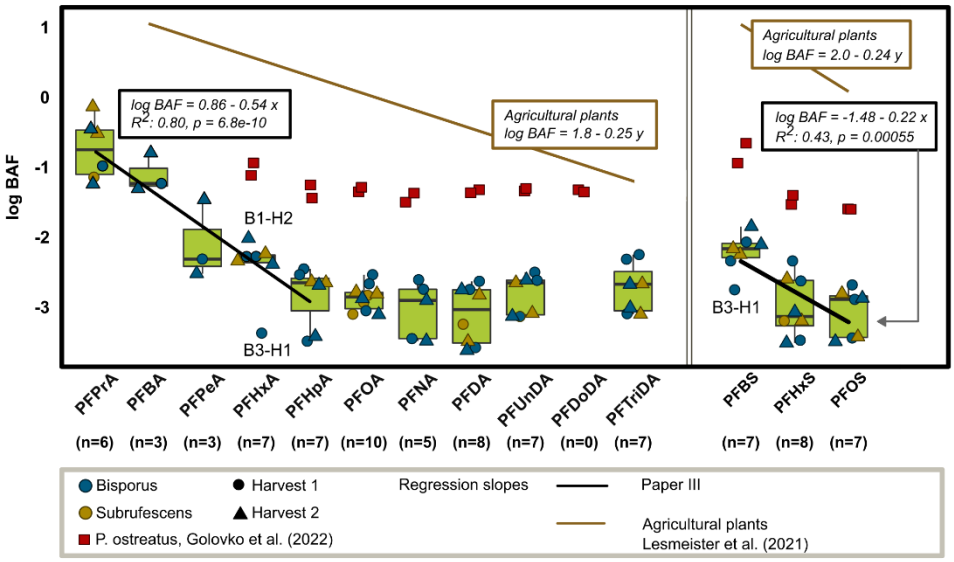

Figure 10. Bioaccumulation factors (log BAF) for PFAS. The figure shows experimental data from Paper III (*A. bisporus* and *A. subrufescens*) with individual observations and boxplots. Included are also log BAF for uptake of PFAS in oyster mushrooms (*P. ostreatus*) from Golovko et al. (2021), and the regression slope for PFAS uptake in agricultural plants calculated in a review by Lesmeister et al. (2021). The figure is adapted from Figure 3, Paper III (Reproduced according to a CC-BY 4.0 licence).

Table 8. Bioconcentration factors (BAF) determined for organic contaminants in mushrooms. BAFs for PFASs are included in Figure 10 and are thus not included here. Note that the BAFs presented for References 2, 3, 4 and 5 are calculated based on information presented in the articles and are approximate. References: PIV: Paper IV. 1: Gbylik-Sikorska (2020). 2: Golovko et al. (2021). 3: Schildt et al. (2021). 4: Carrasco-Cabrera et al. (2019). 5: Moeder et al. (2005). *Fresh weight basis. ** BAF calculated based on nominal contaminant concentration.

Compound	Nominal substrate concentration (µg kg^{-1} DW)	BAF	Species	Ref.
Carbamazepine	1243 – 1382	0.0009 – 0.0021	*A. bisporus*	PIV
		nd	*A. subrufescens*	
Carbamazepine-10,11-epoxide	nd	0.013 – 0.016	*A. bisporus*	
		nd – 0.085	*A. subrufescens*	
Ciprofloxacin	1213 – 1349	*nd*	*A. spp*	
Norfloxacin	1166 – 1297	*nd*	*A. spp*	
Sulfadiazine	1189 – 1323	*nd*	*A. spp*	
Sulfamethazine	1229 – 1367	*nd*	*A. spp*	
DEET	7.5	*nd* – 0.63	*A. bisporus*	
	5.1	*nd*	*A. subrufescens*	
Caffeine	15	*nd*	*A. spp*	
Doxycycline	10 – 250*	*nd*	*A. bisporus*	1
	500 – 5000*	0.003 – 0.012		
Propylparaben	286	*nd*	*P. ostreatus*	2
Clindamycin	286	*nd*		
Sertraline	286	*nd*		
Fenbendazole	286	*nd*		
Clarithromycin	286	0.011 – 0.018		
Ciprofloxacin	1333	*nd*	*A. bisporus*	3
Chloramphenicol	1333	*nd*		
Praziquantel	1333	0.028 – 0.11		
Caffeine	4 670 000	1.1**	*P. ostreatus*	4
	1 070 000	0.083**		
	780 000	0.055**		
$\sum_{30}$PCB	100 000	<0.001**	*P. ostreatus*	5

For PFCAs, the BAFs were strongly dependent on the chain-length and decreased with 0.54 log-units for every additional perfluoroalkyl moiety (Figure 10). Also for PFSAs, the bioaccumulation factors were lower for longer-chained homologues. Unlike plants (Lesmeister et al., 2021), however, the chain-length dependency for PFCAs was evident only for the homologues perfluoropropanoic acid (PFPrA) to perfluoroheptanoic acid (PFHpA). For long-chained homologues there was a consistently low uptake regardless of chain length.

One other study has investigated PFAS uptake in mushrooms. Golovko et al. (2021) assessed PFAS uptake by Oyster mushrooms (*P. ostreatus*) cultivated in two different substrates, one of which was containing biogas digestate. Based on the data presented by Golovko et al. (2021), BAFs were calculated and inserted in Figure 10 (Red squares) along with the BAFs calculated in the present study. The BAFs calculated from Golovko et al. (2021) were similar for PFHpA to perfluorododecanoic acid (PFDoDA), whereas the BAF for PFHxA was slightly higher. There is no apparent chain-length relationship for mushroom uptake of long-chained PFAAs, and the reason is difficult to understand. As the long-chained PFAAs were quantified in the mushroom hats, they must be able to pass the cell wall of the fungi.

Perfluoroether carboxylic (i.e., ADONA) and sulfonic (i.e., F-53B) acids were not quantified in the mushrooms. These compounds were however applied at a lower concentration than the PFAAs; ADONA was spiked at a concentration 11 times lower than each PFAA, and F-53B at a concentration 5.5 times lower. The maximum PFAA concentrations quantified in the mushrooms ranged from 2.1 to 15.5 times the LOQ. Hence, it is plausible that the spiking concentration of the perfluoroether acids was too low to be able to detect any mushroom uptake. Similarly, PFDoDA and perfluorotetradecanoic acid (PFTeDA), which were not quantified in the mushrooms, had LOQs which were higher than the average uptake of PFAAs in the mushrooms.

Among the PPCPs, only carbamazepine, its transformation product carbamazepine-10,11-epoxide, and DEET were detected in the mushroom fruiting bodies (Table 7). With a few exceptions, the compounds were quantified only in *A. bisporus* mushroom hats.

Mushrooms acquire many nutrients as cations, for example potassium and ammonium (Griffin, 1994). Similarly, cadmium can be taken up in large amounts. Using a very similar experimental setup as described in Paper III and IV, Stoknes et al. (2019) showed that about 80% of the cadmium present in the substrate were accumulated in the fruiting bodies of *A. subrufescens* at the first harvest. Subsequent harvests had a much lower cadmium content and met the food quality standards. Also mercury can accumulate in the stem and fruiting body of several mushroom species, whereas bioaccumulation factors of most other metals in mushrooms are below 1 (Ali et al., 2017).

Since the levels of metal ions in biogas digestate may be high (Dragicevic et al., 2018), it is important to be aware of this risk. Especially if sewage sludge is used in the substrate, as iron and aluminium concentrations may be high due to their use as an agent to precipitate phosphorus (Monea et al., 2020). However, sewage sludge is prohibited to be used in vegetable production and in nurseries (Norwegian Ministry of Agriculture and Food, 2003). Thus, it is unlikely that sewage sludge will be accepted for use in mushroom cultivation.

The estimated yearly intake of personal care products through mushroom consumption was below 0.01 mg for all PPCPs which were quantified in the mushroom hats (Table 9). Single doses of exposure through medication (for carbamazepine) or by insect repellents (for DEET) are several hundred mg (Table 9). Hence, the intake of PPCPs through mushroom consumption seems to be of minor importance. For the metabolite carbamazepine epoxide there was no reference dose.

Table 9. Estimated maximum yearly intake of PPCPs through mushroom consumption, for compounds quantified in the mushroom hats.

	Carbamazepine	**Carbamazepine epoxide**	**DEET**
Max yearly intake (mg)	0.0049	0.0012	0.0001
Reference (mg)	200 – 1200[1]		499[2]

[1] Administered dose for adults per day (Norwegian pharmaceutical handbook, 2023),
[2] Amount applied to one arm when using DEET-containing insect repellents (Apotek1, 2023).

For PFAS, estimated dietary intake is often compared with the tolerable weekly intake (TWI) of 4.4 ng kg^{-1} body weight for the sum of PFOS, PFOA, PFHxA and PFNA set by the EU (EFSA, 2020). A lower boundary of PFAS intake through mushroom consumption was calculated from the average concentration of $\sum_4$PFAS in the control mushrooms. For estimation of a maximum, the highest concentrations quantified in the spiked mushrooms were used.

A weekly intake of 1.2 – 10.2 ng $\sum_4$PFAS was estimated, corresponding to 0.4 – 3.3% of the TWI for a person of 70 kg. As mushrooms constitute only a minor portion of a normal diet, this number must be regarded as high. Hence, the

suggestion of Paper III that "it is possible to use PFAS-containing waste material to produce mushrooms that are safe for human consumption", should be reevaluated.

As the BAFs for PFASs were very low, the relatively high contribution of mushroom consumption to the TWI is due to a mismatch between the levels of PFAS in waste materials and the TWI. The TWI is based on available information on health effects of PFASs. Hence, it is the PFAS concentrations in the waste materials which must be lowered for dietary PFAS exposure to stay below the TWI.

Hypothesis no 1. of the mushroom cultivation study was supported. Neither PFAAs nor the selected pharmaceuticals were bioaccumulative in the mushrooms, in the sense that their bioaccumulation factors were below 1.

Hypothesis no 2. was partly supported. The mushroom uptake of PFAAs was seen to be chain-length dependent with a higher uptake of shorter-chain homologues, but only for the homologues PFBA to PFHpA. For long-chained PFCAs, BAFs were not dependent on the chain length. For the PFSAs, there were observed a significant relationship between chain-length and BAFs. The relationship did however explain a rather low portion of the variability in the BAFs (R^2 was 0.43).

5.5 Can mushroom production be used as a bioremediation tool?

The mushroom growth substrate was spiked with the fluoroquinolone antibiotics norfloxacin and ciprofloxacin, the sulfonamide antibiotics sulfadiazine and sulfamethazine, as well as the anti-epileptic drug carbamazepine. The residual concentrations of these compounds at the end of the experiment are shown in Figure 11. The substrate was screened for 37 additional contaminants using the method presented in Paper I, including three transformation products of carbamazepine. Of these, caffeine and the transformation product carbamazepine-10,11-epoxide were detected throughout the experiment, whereas metoprolol and DEET were detected more occasionally.

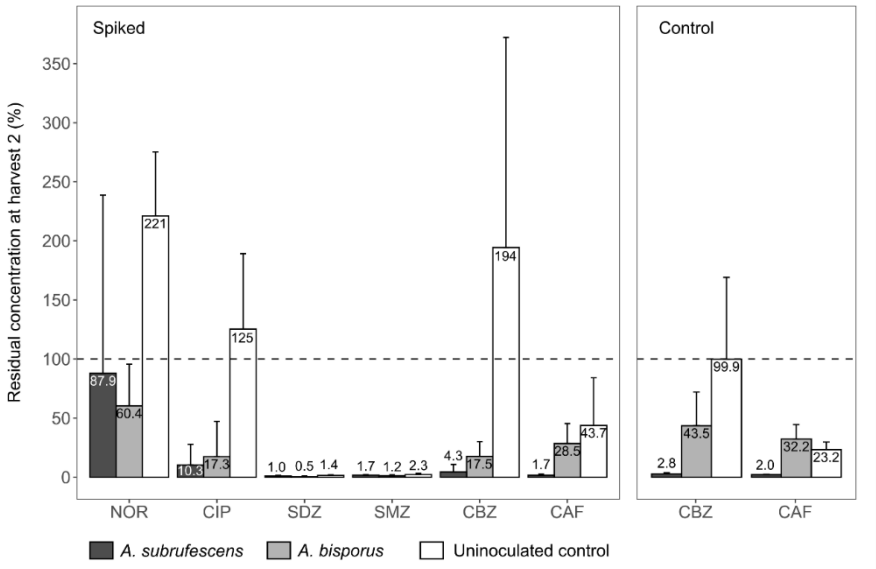

Figure 11. The residual concentration of PPCPs at harvest 2 in the mushroom substrate, calculated on ash basis as the percentage of the initial concentration (i.e., at inoculation).

The compounds displayed great variability in removal efficiency. Both sulfonamides were almost completely removed from the mushroom substrate, regardless of treatment. For fluoroquinolones, the difference in removal between the two representatives was high. While over 80% of ciprofloxacin was removed in both mushroom treatments, only a small

amount of norfloxacin was removed. Carbamazepine and caffeine were removed at similar efficiency as ciprofloxacin. The study design did not allow for mechanistic interpretations, and removal is therefore used as a collective term for all removal mechanisms, including biodegradation by fungi, biodegradation by other organisms, abiotic degradation, and sorption. The target compounds are not volatile, and the mushroom substrate was kept in closed bags. Volatilisation and leaching were therefore deemed to be of minor importance as a removal mechanism.

The general trend was a higher removal of compounds in the substrate of *A. subrufescens* than *A. bisporus*, which in turn showed a higher removal efficiency than the uninoculated control. Carbamazepine and caffeine were present also in the control substrate, as the biogas digestate used for the mushroom growth substrate was taken from a municipal biogas plant. The removal efficiency was similar in the control and spiked substrate, despite the large difference in initial concentration. When data for these two compounds from both the control and spiked treatment were compared, the percentage removal of both compounds were significantly higher in *A. subrufescens* substrate than in *A. bisporus* substrate, and in *A. subrufescens* substrate than in the uninoculated substrate ($p<0.035$ for all comparisons, Dunn's test).

Results from other studies on removal of organic contaminants from the substrate during mushroom cultivation show that many of the investigated compounds were readily removed from the substrate. However, some compounds, i.e., clarithromycin (Golovko et al., 2021), praziquantel (Schildt et al., 2021), and caffeine (Carrasco-Cabrera et al., 2019), did persist.

Caffeine has been shown to be readily removed from WWTPs (Gao et al., 2012; Y. Wang et al., 2018) and was also removed to a high degree in the present experiment (68 – 98% in the mushroom treatments). However, when Carrasco-Cabrera et al. (2019) cultivated oyster mushrooms in a 1:3 mix of spent coffee grounds and sawdust, only 2 – 11% of the caffeine was removed. When the substrate consisted only of spent coffee grounds, 87% of the caffeine was removed despite poor growth of the oyster mushrooms (Carrasco-Cabrera et al., 2019). Hence, the substrate composition can greatly affect the removal efficiency of organic compounds by fungi.

Table 10. Removal efficiency of organic contaminants during cultivation of mushrooms in experiments by other authors. 1: Golovko et al. (2021). 2: Schildt et al. (2021). 3: Carrasco-Cabrera et al. (2019). 4: Moeder et al. (2005). * Highly variable removal rates between individual congeners.

Compound	Nominal substrate concentration (µg kg^{-1} DW)	Removal (%)	Species	Ref.
Propylparaben	286	100	*P. ostreatus*	1
Clindamycin	286	100		
Sertraline	286	96 – 97		
Fenbendazole	286	100		
Clarithromycin	286	35 – 59		
Ciprofloxacin	1333	100	*A. bisporus*	2
Chloramphenicol	1333	100		
Praziquantel	1333	30		
Caffeine	4 670 000	87	*P. ostreatus*	3
	1 070 000	2		
	780 000	11		
$\sum_{30}$PCB	100 000	0 – 100*	*P. ostreatus*	4

Moeder et al. (Moeder et al., 2005) investigated removal and uptake of 30 PCB congeners, as well as relationships between physiochemical properties of the congeners and their fate in the mushroom production system. Moeder et al. (2005) found that congeners with a lower octanol – water partitioning coefficient (log K_{OW}) and higher water solubility were more easily removed from the substrate. Further, the chlorination grade of the congeners affected the removal efficiency.

The combined results shown in Figure 11 (Paper IV) and listed in Table 10, show that although some compounds were recalcitrant towards removal, mushroom cultivation can be an efficient tool to remediate wastes for several organic contaminants. Also, an unexplainable variability in degradation efficiency was observed for structurally related compounds (e.g., norfloxacin and ciprofloxacin, Paper IV), or for the same compound but in different experimental setups (e.g., caffeine) (Carrasco-Cabrera et al., 2019). Most of the compounds shown in Figures 10 and 11 and listed in Tables 8 and 10 have only been examined in one or two studies each, including Papers III and IV. It is thus uncertain how large variability which can be expected under different experimental conditions.

However, PFAS, metals, and other essentially non-degradable contaminants will remain in the substrate unless taken up by the mushrooms. For these persistent compounds, a combination of mycoremediation and safe food production appears not possible. Waste materials which contain a variety of non-degradable contaminants may therefore not be suited for mushroom production. For example, if a digestate with high concentration of both cadmium and PFASs are used, the mushrooms will most likely not meet the food quality standards due to the accumulation of cadmium, and the substrate will at best only be partially remediated as the PFASs remain in the substrate.

Analysis of organic contaminants in the waste materials are costly and cannot be expected to be performed by the individual mushroom producer. Hence, waste materials used in large quantities for commercial mushroom production should be materials with low concentrations of non-degradable contaminants such as PFAS and metals.

Thus, hypothesis no 3 of the mushroom cultivation study was partly supported. The presence of mushrooms increased the removal of carbamazepine and caffeine from the mushroom substrate. For the other pharmaceuticals there was no statistical difference in removal efficiency between the treatments.

5.6 Choice of methods

Screening of digestates

The biogas digestates were screened for 101 compounds in the present work. In the EU under REACH, 26 866 compounds are registered (ECHA, 2023). For PFAS, at least 1400 individual compounds are in use (Glüge et al., 2020b), of which at least 531 are registered under REACH (Rudin et al., 2023). The answer to the question – "does the digestate contaminant profile differ based on which feedstock is utilised?" – is then highly dependent on the compound selection. A non-target screening would perhaps be more appropriate to answer this question, and is also in process at the Norwegian Institute of Marine Research.

The number of samples in the screening was relatively low. There were only 5, 5, and 4 samples for each of the feedstocks food waste, mix of food waste and sewage sludge, and sewage sludge, respectively. It would not have been possible to expand on the number of digestates (if restricted to Norwegian plants), but it would have been possible to take samples better representing the variation, and time average, e.g., weekly composite samples (Ort et al., 2010).

The concentration of contaminants in the digestates also vary with season. Antibiotic consumption is season dependent in many countries, including Norway (Van Boeckel et al., 2014), and antibiotic loads in digested sewage sludge can be considerably higher during winter than during summer (Aydın et al., 2022). In China, it was found that UV-filters such as octocrylene were present in elevated concentrations in WWTPs during the warm season (Li et al., 2007). In a Norwegian biogas plant utilizing food waste as feedstock, it was found that the concentration of certain pesticides in food waste digestate was highest during the winter months, due to a high consumption of citrus fruits (Govasmark et al., 2011). Consequently, samples could have been taken during e.g., a whole year to catch seasonal variations.

In hindsight it would also have been useful to collect information on the sources of the feedstock. Especially for the sewage sludge digestates, since some WWTPs receive mainly domestic wastewater and other receive hospital

effluents or industrial wastewaters. However, also food waste input can vary. For example, certain areas can get a lower population during holidays when many people travel simultaneously. Because the reactors need a steady input, alternative feedstocks must be gathered during these periods.

Mushroom experiments

It was generally challenging to find a good balance between the growth requirements of the mushrooms and analytical chemistry. Mushrooms require certain conditions to thrive, which is not necessarily compatible with the spiking concentrations required to be able to quantify the contaminants at later stages in the experiments or in the mushroom hats, or with the best practices to achieve a homogenous distribution of target compounds in the compost and to take representative samples.

Only one of the above cited reports on mushroom uptake of organic contaminants were available in 2018, when the experiment was started (Moeder et al., 2005). We did not know which concentrations of contaminants the mushrooms could tolerate, especially the antibiotics which could affect the fungi and the microflora of the substrate (which also is an important part of the cultivation system).

During the composting phase (i.e. after spiking with PFAS but before pharmaceuticals), the spiked and control batches behaved differently. Two litres of spiking or control solution were added to each batch of digestate before mixing with the straw. The high volume was chosen to ensure a homogenous distribution of PFASs in the digestate, but may have been too high for the mushrooms to thrive. Towards the end of the composting phase, about one litre of compost liquid was drained from the control batch, which made the control substrate somewhat drier than the spiked substrate. After inoculation with the fungi, three replicates from the spiked treatment went rotten and had to be discarded. The mushroom yield was also lower in the spiked treatment than in the control treatment. The lower yield and the rotting of the substrate could have been due to the higher moisture content and/or due to the spiked compounds.

How to take representative samples to quantify something which is present in minute quantities in a heterogeneous matrix where rigid wheat straws is a

central ingredient? It takes time before the straw softens and mixes with the digestate, and even at the end of the experiment a large part of the straw structure were intact. The ratio of straw to digestate in a sample could affect the contaminant concentration. Whenever possible, subsamples from thoroughly mixed substrate were taken. But the fungal mycelia are very sensitive to disturbances, and during growth (i.e., samples taken at casing application and at harvest 2), it was only possible to take subsamples from the outer edges of the substrate. Looking at the standard deviations for the concentration of contaminants in the substrate it seems likely that the samples were not completely representative (although variation could also be due to different degradation pattern between replicates).

The standard deviations could also have been lowered by having more replicates. At Lindum, two sets of equipment for preparation of mushroom substrate were available – one for control and one for the spiked batch. Each set could prepare a maximum of 33 kg mushroom substrate. The mushrooms require at least 3 kg of substrate to thrive, hence we had 11 replicates to distribute amongst the treatments. In hindsight it would have been better to include only one mushroom species and rather have more replicates for both the mushroom treatment and the uninoculated control.

What to use as control treatment? Harms et al. (2011) describes that "the total contribution of fungi to remediation success is difficult to quantify, as true controls deviating only in the absence of fungi are highly artificial and, in many cases, impossible to establish." That is true for the present experiment. The control did not deviate only in the absence of fungi, because the addition of fungi alters the microbial community of the substrate (Carrasco et al., 2020), and hence, it is highly likely that the microflora in the uninoculated control deviated from the microflora in the inoculated treatments, apart from the absence of fungi. The uninoculated control can also be characterised as artificial in the sense that a digestate/straw compost would not be kept in 3 kg units for any practical purposes. It is not uncommon to compost digestate with a bulking material with high carbon/nitrogen ratio (as the straw), but the scale is much larger. The temperature cannot rise in a 3 kg unit. The non-spiked control on the other hand was necessary to keep track on the concentration of contaminants in the substrate. Both PFASs and pharmaceuticals can be formed from precursors which could have been present in the digestate.

6. Conclusion and outlook

The screening studies showed that Norwegian municipal biogas digestate generally had low concentrations of antibiotics. However, the few instances of elevated antibiotics-concentrations with the accompanying risk towards soil organisms show that even the few instances of antibiotics in waste materials can be a problem. With respect to contamination with PPCPs, the digestates based on food waste had similar concentrations as the digestates based on sewage sludge. Thus, food waste cannot be regarded as safe but must be regarded as a potential source of contaminants to the environment in line with sewage sludge. For PFAS, the number of compounds detected was similar across feedstocks, but the concentrations were generally lower when the feedstock was based on food waste.

Mushroom cultivation shows to be a feasible remediation technology for several PPCPs, with a very low accompanying mushroom uptake. In contrast, mushroom production cannot be recommended for PFAS remediation, PFASs were neither removed by degradation nor mushroom uptake. The mushrooms seem to be safely produced on low-PFAS-containing materials despite the very low tolerable weekly intake of PFASs. However, for higher PFAS concentrations, the mushroom consumption can contribute to exceedance of the TWI despite very low bioaccumulation factors. For waste materials heavily contaminated with PFASs, other technologies must be considered.

Environmental release and recycling of contaminants is a bottleneck to a safe implementation of circular economy. To decrease and control the environmental release of contaminants, restrictions on manufacture and use of chemicals are essential. Even with strict regulations, some chemicals must be used. For example are the use of antibiotics essential. However, consumption should indeed be restricted to the bare minimum to prevent promotion of antibiotic resistance. For PFAS, the concept of essential use has been developed, where it is acknowledged that specific applications of certain PFASs serve a critical role until replacement chemicals are in place (Cousins et al., 2019).

Thus, even with strict regulations on manufacture and use of potential contaminants, it is likely to have some contamination of organic waste streams. It is therefore important to continue to monitor the environmental release of contaminants and to investigate their fate in e.g., food production systems.

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Paper I

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Organic contaminants of emerging concern in Norwegian digestates from biogas production†

Aasim M. Ali, *[a] Astrid S. Nesse,[b] Susanne Eich-Greatorex,[b] Trine A. Sogn,[b] Stine G. Aanrud,[c] John A. Aasen Bunæs,[c] Jan L. Lyche[c] and Roland Kallenborn [acd]

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The aim of this study was to analyze a variety of environmental organic contaminants of emerging concern (CEC) and their metabolites in representative digestate samples from Norwegian biogas production plants. Biogas digestates can be a valuable source for soil amendments and/or fertilizers in commercial agriculture. It is important to assess whether the digestates contain harmful contaminants in order to avoid unintended exposure of human consumers. In total 19 biogas digestates from 12 biogas production plants in Norway were collected and analyzed. Furthermore, process related parameters such as pretreatment of substrates, additives, flocculation and temperature conditions were considered for interpretation of the results. The CEC levels found in the digestates were shown to be dependent on the original composition of the substrate, dry-matter content, and conditioning of the substrate. The sunscreen octocrylene (147 μg L^{-1}) and acetaminophen (paracetamol; 58.6 μg L^{-1}) were found at the highest concentrations in liquid digestates, whereas octocrylene (>600 ng g^{-1}, on a wet weight basis = ww) and the flame retardant TCPP (tris(1-chloro-2-propyl) phosphate, >500 ng g^{-1} ww) were found at the highest levels in solid digestates, exceeding even the upper limit of quantification (uLOQ) threshold. The highest levels of total CECs were measured in solid digestates (1411 ng g^{-1} ww) compared to liquid digestates (354 μg L^{-1} equals 354 ng g^{-1}). The occurrence of CECs in digestate samples, even after extensive and optimized anaerobic digestion, indicates that the operational conditions of the treatment process should be adjusted in order to minimize CEC contamination.

Environmental significance

Biogas digestates are considered valuable fertilizers and soil amendments with agricultural applications. The study reported herein describes the quantification of certain organic contaminants of emerging concern (CECs) in liquid and solid anaerobic digestates from twelve biogas production facilities in Norway. The concentration of CECs was found to depend on the composition of the initial substrate (dry matter content) and how it was conditioned or pretreated prior to anaerobic digestion. The anaerobic digestion process employed at the biogas production facilities did not eliminate the analytes investigated.

Introduction

In order to reduce global anthropogenic CO_2 production and emission, and to further the utilization of suitable renewable resources, the development of sustainable bioeconomic strategies has been given a high priority on the political agenda of today's world leaders. An important prerequisite strategy, in terms of industrial processes based on biological materials in a circular economy, is sustainable management of the residues, and hence recycling of nutrients from the source material will be imperative.[1–5] There is huge diversity in organic residues depending on their origin and/or the type of process involved in their production.[6,7] Application of organic residues as a soil amendment and fertilizer is expected to recycle most of the nutrients contained within. However, it may also imply a risk for the dispersal of contaminants on agricultural soils.[8,9] From soils, contaminants may be absorbed into food and fodder plants, from where it may ultimately find its way into animal and human food chains.

The use of biological (waste) material in anaerobic digestion (AD), both in decentralized biogas plants on farms and in municipal plants for handling, among others, organic household waste, has increased significantly in Europe and North America.[10–14] AD is a microbiological process where organic

[a]Faculty of Chemistry, Biotechnology and Food Science (KBM), Norwegian University of Life Sciences (NMBU), NO-1432 Aas, Norway. E-mail: aasimali@nmbu.no
[b]Faculty of Environmental Sciences and Natural Resource Management (MiNa), Norwegian University of Life Sciences (NMBU), NO-1432 Aas, Norway
[c]Faculty of Veterinary Medicine (VetFak), Norwegian University of Life Sciences (NMBU), NO-0454Oslo, Norway
[d]University Centre in Svalbard, Arctic Technology, NO-9171 Longyearbyen, Svalbard, Norway

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material (hereafter named the substrate) is degraded in an oxygen free environment (digesters), producing biogas and biogas digestate. This biogas is an energy rich gas consisting of methane, carbon dioxide and other trace gases (including H_2). This development has not only led to an increasing amount of bioenergy being produced, but has also contributed to a considerable amount of organic residue being properly handled (*i.e.*, biogas digestate). Consequently, digestates are currently applied as organic fertilizer to agricultural land, allowing the recovery of nutrients, primarily nitrogen and phosphorus, and, in addition, potentially improving soil quality by adding organic matter.[15–18] Unfortunately, these biogas digestates may also contain harmful chemical pollutants and/or pathogenic bacteria, which may represent both environmental and human health risks.[19–22] Biogas digestate is today considered an excellent bio-fertilizer and a soil amendment for agricultural applications.[23–28] The physicochemical properties of digestate depend mainly on the nature of the substrate and operating conditions of the digester. Compared to raw animal manures and slurries, digestates generally contain low total solids and have a low organic carbon content, a low carbon to nitrogen ratio (C : N), and low viscosity.[29–31] The typical pH value of fresh digestate ranges from 7.5 to 8.0, comparable to that of raw animal manures and slurries.[32] Anthropogenic contaminants and hazardous pollutants have previously been reported in digestates.[19,22,33] Such contaminants may influence the function of microorganisms during the AD process if present in the substrate or generated in the digester.[25,34]

However, only a few scientific studies have reported on the presence and fate of organic pollutants, such as dioxin-like compounds,[35–37] polycyclic aromatic hydrocarbons (PAH),[38,39] polychlorinated biphenyls (PCBs) and pesticides,[39,40] in digestates (in a ng kg^{-1} to μg kg^{-1} range). Some organic contaminants such as mycotoxins may be effectively removed during the anaerobic digestion process.[41] However, contaminants of emerging concern (CECs) have not yet been comprehensively investigated in biogas digestates.

Ultimately, nutrient rich and unpolluted digestate is expected and required for safe recycling and application in agricultural production. Hence, an important premise for producing high-quality digestate as a fertilizer in agricultural production is the use of high-quality substrates (rich in plant nutrients and without pollution and impurities) for the digestion process.[42]

Our study focuses on the occurrence of organic CECs including a phosphate containing flame retardant tris-(1-chloro-2-propyl) phosphate (TCPP), an insect repellant (DEET), a sunscreen ingredient, 25 pharmaceuticals and personal care products (PPCPs) and 11 selected metabolites in digestate samples collected from several representative biogas plants in Norway. The effect of the substrate composition and plant specific pretreatment procedures on the concentration of these contaminants in digestate samples was elucidated. For this purpose, a new optimized quantitative trace-analytical method was developed. The optimized quantification method was applied for the characterization of CEC patterns in 19 representative Norwegian biogas digestate samples (including one substrate sample). Based upon these results, a first assessment of levels and consequences of CEC residues in soils treated with digestates as soil amendments is reported.

Materials and methods

Contaminants and reagents

The sample preparation and quantification method are fully described in the ESI material section.† 41 reference standards and 10 isotope-labelled internal standards (ISTD) were purchased in trace analytical quality (≥97%) from Sigma-Aldrich (St. Louis, MI, USA) and Toronto Research Chemicals (Toronto, Ontario, Canada). These target compounds were selected based on their high prescription rate in Norway and their reported presence in related environmental samples. Metabolites for some compounds were selected based on their availability as purified standard materials. Complete information on the contaminants studied and the standards applied for the present reported study can be found in Table S1 (ESI material section†).

Biogas process conditions

Representative digestate samples from 12 major commercial biogas plants in Norway were collected during late autumn 2017. The general process conditions are listed in Table 1. Since most biogas plants use sewage sludge as a substrate, most of digestates are dewatered and decomposed. However, some food waste biogas plants supply liquid digestate as fertilizer for agriculture. In order to separate the digestate into a liquid and a solid digestate, a flocculant (*e.g.* cationic polymer) is added to destabilize colloidal materials and hence improve their sedimentation. Digestate is subsequently dewatered by centrifugation. Both liquid and solid digestates were analyzed in this study.

For dewatering of the digestate at 9 biogas plants, the cationic polymers listed in Table S11† were added to the digestates. The list of contributing Norwegian biogas plants was anonymized and coded as plants A–L in the following sections (Table 1). For this first survey, 19 samples were investigated (all samples were prepared and analyzed in replicates). The sample set also included one liquid substrate sample (I_{sub}) and a liquid digestate sample (I_{dig}) from an experimental biogas reactor associated with plant I. Biogas plant I uses 20% sludge from young fish and 80% manure as a raw substrate (Table 1). As preparation for effective anaerobic digestion and for optimization of the biogas yield (mainly CH_4), the biogas plants use different conditioning steps, pretreatments and process adaptations, which may influence the contaminant levels in the digestates. Hence, in addition to the levels of organic contaminant residues, the influence of the biogas production parameters including pretreatment, additives, and temperature conditions on the resulting levels of these organic pollutants was considered in the statistical interpretation of the obtained CEC levels. The 12 stations and the production processes investigated in our study are considered representative of today's biogas production strategies both in Norway and

internationally. Modifications of reactor conditions (*i.e.*, reactor temperature), chemical composition of the substrate (*i.e.* by thermal hydrolysis or chemical reactions) and physical state (*i.e.* polymer addition or flocculants) are necessary for process optimization purposes (see Table 1). Differences in the substrate composition (various mixtures of food waste, fish silage, biosolids, manure, sewage sludge, *etc.*) and the resulting physical consistency of the starting material require specific adjustments for optimal processing conditions. An inoculum (*e.g.*, manure) is added to initiate biogas production. Thermal pretreatment (including thermal hydrolysis = THP) is often used as a hygienization step but can also make the substrate more biodegradable for the subsequent biogas production process. Furthermore, additives like organic polymers and flocculants/precipitants (inorganic salts) are added to the digestates in order to bind nutrients like phosphorous. The chosen pretreatment procedures are also dependent on the dimensions and operational conditions of the respective biogas production plants (Table 1).

Analytical methods

For this study, a method for simultaneous quantification of 41 contaminants of emerging concern (CECs), including some transformation products of some compounds, was developed and applied (for details see the ESI†). The target CECs were extracted by solvent extraction followed by cleanup with solid phase extraction (SPE). For the quantification of the target compounds, liquid chromatography-electrospray ionization/tandem mass spectrometry (LC-ESI-MS/MS) was used.

The quality control protocol for the quantitative analysis, including linearity testing and documenting of non-linear matrix effects on the quantification of the target substances, was performed according to a method described in earlier related studies.[43,44]

Statistical methods

Principal component analysis (PCA) was performed and Pearson's correlation coefficients were calculated with R-software (R-Studio Version 1.1.143 based on R version 3.5.2.) under the GNU public license (Boston, MA, USA) and Matlab (Version 8, Mathworks, Natick, MA, USA), respectively. The significance threshold for Pearson's correlation coefficient calculation was considered to be $p < 0.05$.

Results and discussion

Detection of CECs in digestate samples

Concentration levels of the target CECs which met the quality control criteria of the method validation are listed in Tables S9 and S10.† Representative chromatograms and mass transitions used for their quantitative analysis are presented in Fig. S3–S17 in the ESI.† For the liquid digestates, 28 target compounds were quantified, whereas for solid digestates only 24 target contaminants met the quality control criteria for quantification and were reported in ng g^{-1} wet weight (ww). Ranitidine, metronidazole, trimethoprim, norfluoxetine HCl, warfarin, and carbamazepine-10,11-epoxide were not found in liquid samples (Table S10†). Of the 24 contaminants quantified in the solid digestate, 16 compounds were detected. For two solid digestate samples ($E_{(S)}$ and $B_{(s)}$), TCPP and octocrylene concentrations were found outside the confirmed linear range of the quantification method. The levels were reported to be above the upper limit of quantification (>uLOQ, see Table S10†).

Substrate composition

In general, the concentrations in solid digestates were considerably higher compared to those in liquid sample materials. For solid digestates (dry matter content of 22–47%), sum-CEC concentrations exceeding 1000 ng g^{-1} were determined (Table S10:† $E_{(S)}$, $B_{(s)}$). The highest sum-CEC levels in liquid digestates (dry matter content of 2–5%) were found in the concentration range 22.3–353.6 μg L^{-1} (Table S9†).

The CECs found in the various digestates, and their levels, seem directly related to the substrate used. The highest residue levels for pharmaceuticals exclusively used in human therapy (*i.e.* carbamazepine, metoprolol, losartan) were found in liquid and solid digestates originating mostly from sludge and food waste substrates. Monesin, an antibacterial agent only used in livestock breeding, was found in one digestate sample from a 100% food waste-based substrate. High levels of octocrylene (a sun-screen ingredient), in some cases even exceeding the uLOQ method limit, were found almost exclusively in digestates when sewage sludge was used for biogas production. The highest sum-CEC concentration was confirmed in a solid digestate sample after processing a mixed food waste/sewage sludge substrate ($B_{(S)}$).

Biogas production and processing

For the optimisation of the biogas process with respect to quality and yield, plant-specific adjustments and treatment procedures are implemented in the production process (Table 1). These treatment steps may also have effects on the levels and distribution patterns of the quantified target CECs.

The calculation of Pearson correlation coefficients for the various preparation steps listed in Table 1 revealed the complex picture of the influence of substrate conditioning on the CEC levels in the resulting digestates (Fig. 1). The correlation calculations revealed a significant positive correlation between the CEC level and the use of thermal hydrolysis (PTTHP) in the pre-treatment of the substrate prior to AD as well as the dry-matter content (% DM). This is not surprising since earlier investigations have confirmed that pollutants, immobilised during biological sewage treatment through phase II metabolising enzymes such as glutathione S-transferases or UDP-glucoronosyltransferases, may be reactivated again by cleaving the conjugates with subsequent hydrolysis.[45–48] In fact, such cleavage methods are frequently used for quantification of conjugated anthropogenic pollutants in biological samples.[49–53] In this context combined thermal treatment, including thermal hydrolysis, where high temperature is applied in combination with catalytic or direct chemical reactions (oxidation, reduction, elimination, photochemistry, *etc.*) is often performed for

Table 1 Plant specific procedure strategies for optimized biogas production in representative Norwegian biogas production plants[a]

Location	Solid (S)/liquid (L) sample available	Substrate	Reactor temperature [°C]	Retention time [days]	Inoculum	Pre treatment	Precipitant added	Dry matter [%]	Polymer
A	L + S	45% food waste, 53% sewage sludge, 2% fish silage	40	16	No	THP	$FeCl_3$	L = 5; S = 47	Yes
B	S	15% food waste, 85% sewage sludge	40	40	Yes	THP	No	S = 26	Yes
C	L	72% food waste, 18% manure	35	35	Yes	Temp.	$FeCl_3$	L = 5; S = 47	Yes
D	S	100% sewage sludge	37	20	No	No	$FeCl_3$ & $AlCl_3$	S = 47	Yes
E	S + L	100% sewage sludge	41	35	Yes	THP	No	L = 3; S = 27	Yes
F	S + L	45% food waste, 65% sewage sludge	62	20	Yes	Temp.	$FeCl_3$	S = 22	Yes
G	L	100% food waste	37	20	No	No	No	L = 5	No
H	S	100% sewage sludge	37	15	No	No	No	S = 26	Yes
I	S + L	20% sludge from young fish, 80% manure	40	40	No	Temp.	No	L = 5; S = 33	Yes
J	S	100% sewage sludge	55	14	No	No	$FeSO_4 \times 7H_2O$	S = 32	Yes
K	S	100% sewage sludge	40	25	No	THP	EcoFloc	S = 33	Yes
L	S + L	100% food waste	53	20	No	No	FeS	L = 2; S = 35	No

[a] THP = thermal hydrolysis processing; temp. = pre-treatment at high temperature (70–165 °C for 20–60 min); L = liquid samples; S = solid samples; Act. Sl. = activated sludge; EcoFloc = liquid, commercially available flocculant (Ecolab, Naperville, Il, USA) mainly composed of $FeCl_3$ in water solution. For details on polymers added to the digestates, see Table S11.

cleaving of conjugates, initiating the breakdown of cell walls and solubilization of organic matter.[54] Thermal hydrolysis is, hence, expected to change the availability and the partitioning of the target contaminants.

Adding a reactive inoculum as well as providing high dry matter content (% DM) may probably contribute to elevated CEC levels in the final digestates (Fig. 1). Adding precipitants (flocculants) to the digestate is also not expected to contribute to reduced CEC levels according to our preliminary findings.

The results presented in Fig. 1 indicate that the careful selection of substrate composition (including dry matter content) and optimised conditioning strategies for biogas production may be considered a first important step to reduce the occurrence of potential CECs in the digestate.

CEC distribution profiles

Levels and distribution profiles of contaminants in digestate samples depend on a variety of ambient factors. These include the original contaminant profile in the substrate sample, physicochemical properties of the substance, interactive processes with the substrate material during the anaerobic digestion (AD) process, and microbiological transformation processes during the digestion process. Hence, in the literature, selected contaminants are found with high removal efficiency by AD. Among these, caffeine,[55,56] trimethoprim[55,57] and sulfamethoxazole[58] have been reported to be readily degraded and removed in such processes. However, other substances like carbamazepine and fluoxetine were found to be stable and, thus, are not expected to degrade during AD.[55,57–59]

To our knowledge, no previous studies have been reported in the literature yet, where indications for the partition of CECs (like those selected here) and their transformation products between liquid and solid biogas digestates were found. Based on the results available here, a first attempt is made to evaluate the physicochemical properties of the target contaminants and their influence on the final distribution profiles found in the respective digestates (Tables S9 and S10†). For this purpose, calculated partitioning coefficients (directly derived from the ACD/Labs Percepta Platform – PhysChem Module, Toronto, CA) were compared and discussed in relation to the distribution profiles identified in the digestates of the twelve plants (Table S1†). Hence, soil organic carbon–water partitioning coefficients ($\log K_{oc}$) along with the octanol–water partitioning coefficient ($\log P$) and the pH dependent octanol–water distribution constant ($\log D$) for all target CECs are listed in Table S1† for comparison. Since these coefficients are quotients of concentrations, the calculated values are dimensionless.

Log D is considered the most reliable descriptor for the ionisable CECs in our study. Substances with low $\log D$, $\log P$ and $\log K_{oc}$ coefficients are expected to be enriched in solution whereas high values indicate sorption to particulate material and thus a tendency to reach higher levels in the solid phase (Table S1†). Carboxy-ibuprofen has the lowest $\log D$ ($\log D = -2.65$) and should thus mainly be found in liquid digestates. However, carboxy-ibuprofen, a major transformation product of ibuprofen was only found in one liquid and one solid sample.

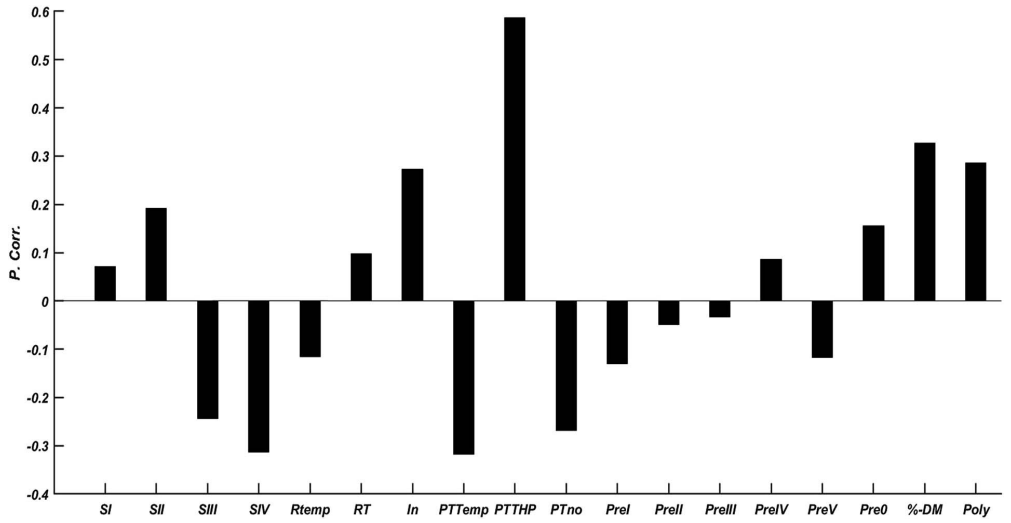

Fig. 1 Relative Pearson correlation coefficients (P. corr. maximum range: −1 to +1) for the conditioning processes related to the obtained CEC concentrations. Statistical data related to available information on biogas processing and CEC concentrations measured in the respective digestate samples. *% DM and PTTHP showed positive and significant correlations (P.corr. = 0.57 and 0.59 respectively, p < 0.05).*

Octocrylene belongs to the CECs with the highest concentrations in both liquid and solid digestates. The high log *D* (=6.34) and log *P* (=7.53) indicate that octocrylene is found mainly adsorbed to particle surfaces. TCPP, on the other hand, is also found in high concentrations in both solid and liquid digestates (see Table S1†), even exceeding the uLOQ threshold in solid samples. The log *D* (=1.53) and log *P* (=0.48) indicate that TCPP is more likely to be detected in aqueous environments. It is also important to note that solid digestate contains a considerable amount of water (53–78%). Thus, it is not surprising to also detect water-soluble compounds in solid digestate samples (and *vice versa*).

Hence, the theoretical partitioning coefficients are to be considered indicators based upon inherent physicochemical properties of the investigated target contaminants.

The relative distribution of the CECs, calculated from the concentrations reported in Tables S9 and S10,† and presented in Fig. 2, indicates a matrix dependent distribution. While atorvastatin, TCPP, and octocrylene are predominant in solid digestate samples (sum = 54%), acetaminophen, prednisolone and octocrylene are observed to be the predominant CECs in liquid digestates (sum = 65%).

These differences are, however, not caused only by the water content of the samples (Table 1: liquid digestates 95–98% water content, solid digestates: 53–78% water content). As earlier outlined, the final distribution patterns are rather a consequence of the variability and interaction between substrate composition, water content, substrate preparation and physicochemical properties of the target contaminants. This is illustrated in Fig. 3, where principal component analysis (PCA) describes the influence of the identified variables (see Table 1) and the physicochemical properties (Table S1:† partitioning coefficients) on the CEC profiles in the investigated digestates.

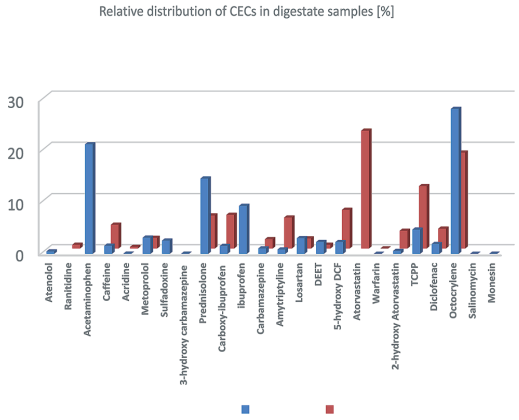

Fig. 2 Average relative distributions of selected CECs (100% = sum CECs) detected and quantified in solid (red) and liquid (blue) digestate samples.

The two major principal components (PC1 & PC2) account for only approximately 40% of the overall variability in the dataset. Thus, the complexity of the influencing variables and their interactions are not completely explained by the parameters identified here.

The liquid samples (red circle) are separated in a well-defined group, where the water soluble CECs dominate the distribution profile (*i.e.*, ACE, DCF, and CAR-3OH). The substrate composition (SI–SIV) seems to have a stronger influence on the CEC profile of liquid samples compared to the solid digestates. In particular, the composition and content of SI (food waste), SIII (fish silage) and SIV (bio waste) may influence the CEC patterns in the liquid digestates investigated here.

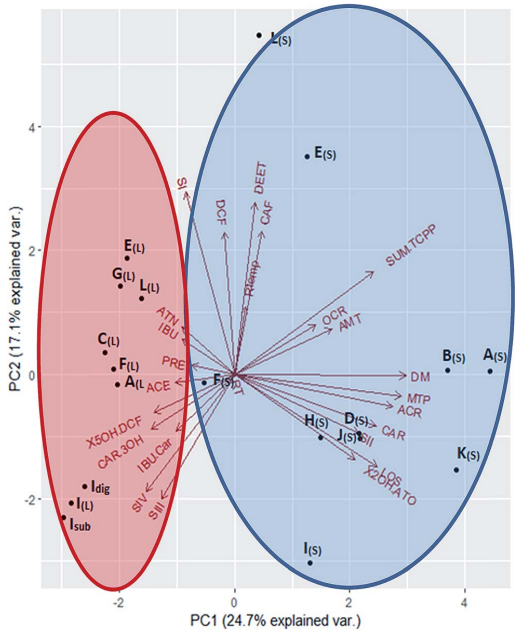

Fig. 3 Bi-plot for Principal Component Analysis (PCA) conducted for variables potentially influencing the CEC distribution profile in the analysed digestate samples. Red circle (liquid digestates); blue circle (solid digestates). All abbreviations are explained in Table S1† and Fig. 1.

For the solid digestates (dry matter: 22–47%), microbial transformation products, such as acridine (carbamazepine metabolite), and 3-hydroxy-atorvastatin seem to have a large influence on the overall CEC patterns.

Levels of CECs and their major transformation products in biogas digestates

For identification of potential CEC transformation during the anaerobic microbial digestion processes (resulting in the production of biogas), the CEC patterns in a substrate sample (I_{sub}) and the corresponding digestate (I_{dig}) were compared (marked grey in Table S9†). The comparison of the sum-CEC concentrations indicates that the digestion process will result in an overall but minor reduction of the target CECs (substrate I_{sub}: 25.8 μg L^{-1} to digestate 5*I*: 22.3 μg L^{-1}) as summarised in Fig. 4. Fig. 4 shows the level comparison between the target CECs in a substrate and a digestate sample collected from an experimental reactor at station I. It is worth mentioning that the substrate sample I_{sub} was not thermally treated, and no additives were added to the digestate sample I_{dig}. However, some compounds were detected in the digestate but not found in the substrate, namely, 3-hydroxy carbamazepine (CAR-3OH), diclofenac (DCF) and metoprolol (MEP; see Fig. 4). Hence, the occurrence of these contaminants, which were not found in the substrate, indicates that cleavage of phase-II conjugates, present in the raw substrate (20% sludge from young fish, 80% manure), may occur during the digestion process. They also contribute to the final composition and concentrations of CECs and the transformation products in the resulting digestate. However, this could also reflect the significant temporal variation in the composition of the raw substrate loaded, even in the same biogas reactor.

The role of conjugate cleavage for the remobilisation of previously immobilised contaminants in biologically active matrices has been reported previously for similar matrices to those studied here.[60–65] There is apparent degradation for some compounds (*e.g.*, ACE and OCR) but not for the majority of target substances (Fig. 4). This observed degradation may be attributed to the degradation of ACE and OCR in the resulting digestate after the anaerobic digestion as earlier reported.[66,67]

The levels of CAR are higher in solid digestates compared to the liquid samples, but the presence of the transformation product CAR-3OH was not confirmed in solid samples (Fig. 5). This different profile between liquid and solid digestates indicates partitioning of the compounds, for instance, the partitioning of CAR-3OH (more polar) into the liquid phase before separating the digestate into a liquid and a solid fraction.

Contaminants of emerging concern in liquid digestates

The compounds with the highest concentrations in liquid digestates (Table S9†) were acetaminophen, caffeine, metoprolol, prednisolone, losartan, DEET, TCPP, ibuprofen, and octocrylene, which were found at levels from tens to hundreds of μg L^{-1}. Acetaminophen, ibuprofen, prednisolone, losartan, and metoprolol are currently among the most widely used pharmaceuticals available without a prescription but also prescribed in Norway for medical treatment.[68] Octocrylene is a commonly used UV-blocker and sunscreen ingredient. Therefore, the occurrence of these compounds (pharmaceuticals and sunscreen) in substrate and digestate samples is directly attributed to their wide application in cosmetics and medical treatment in Norwegian society. The stimulant caffeine, commonly used in beverages and refined food products, was found in most digestate samples at a maximum concentration of 10 μg L^{-1}. Similar levels for some of these compounds have been reported in primary sludge samples during anaerobic digestion.[69]

Carbamazepine (CAR), used as an anticonvulsant in medical therapy, was detected in all liquid digestate samples with concentrations varying from 0.07 to 5.0 μg L^{-1}. It has earlier been reported that CAR is unaffected by an aerobic digestion.[57] However, as shown in Fig. 5, the CAR metabolites acridine and 3-hydroxy carbamazepine were found in the digestate samples, indicating effective transformation in the substrate or during biogas production (AD). Surprisingly, the CAR transformation product carbamazepine-10,11-epoxide, usually identified as the main CAR transformation product in waste water and other biologically active matrices,[70–72] was not detected in the digestate samples investigated here.

Ibuprofen was detected in two liquid biogas digestates mainly derived from sewage sludge based substrates ($E_{(L)}$ and $A_{(L)}$) at concentrations of 36 μg L^{-1} and 26.7 μg L^{-1}, respectively. Carboxy-ibuprofen (ibuprofen transformation product) was

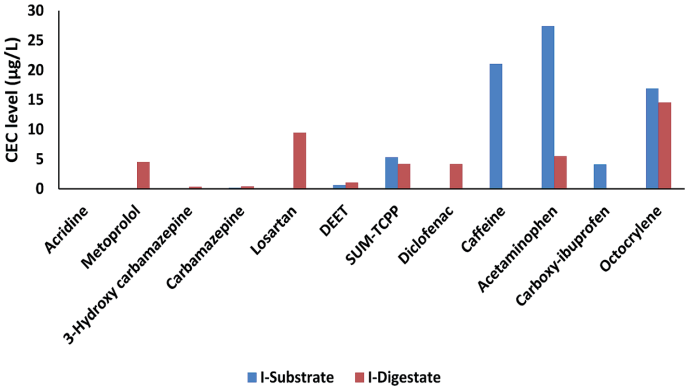

Fig. 4 Concentration comparison between the target CECs in a substrate (blue – I_{sub}) and a digestate sample (red – I_{dig}) collected from an experimental reactor at station I. *Please note these samples originate from the same production plant but are not produced during the same biogas process.*

detected in one liquid digestate at a <LOQ concentration level ($F_{(L)}$). Formation of carboxylated and oxidized transformation products during the AD process is not considered a common process. However, their detection in the digestate samples may be attributed to their formation during substrate conditioning and treatment.

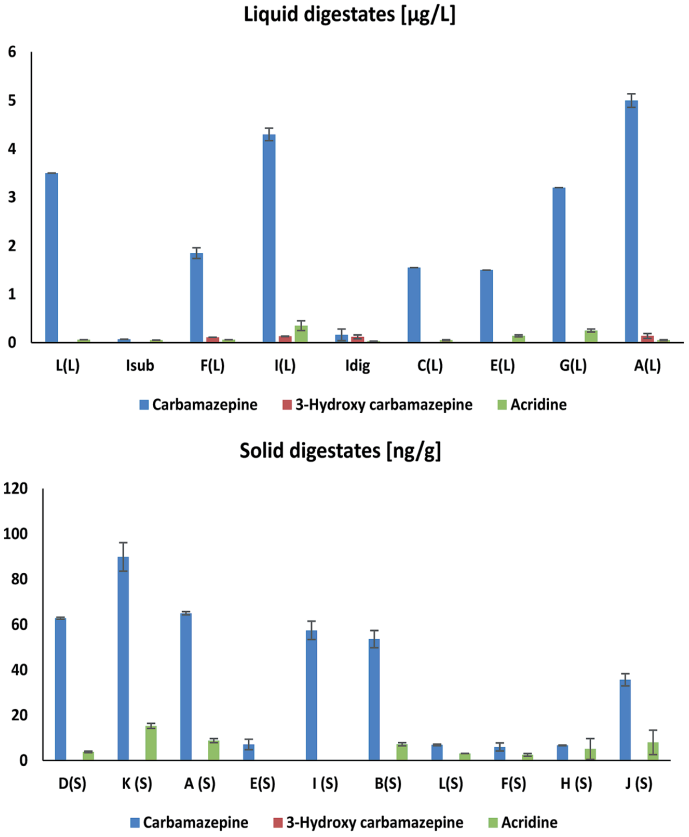

Fig. 5 Levels of carbamazepine (CAR) and its transformation products acridine [ACR] and 3-hydroxy-carbamazepine [CAR-3OH] in liquid and solid digestates.

All other target PPCPs were found at average concentrations below and around 1–5 μg L^{-1}. The levels and patterns of the CECs in liquid digestate reported here are found to be in agreement with earlier findings; for instance, carbamazepine, diclofenac, and metoprolol are reported to mainly partition into the aqueous phase.[73,74]

Contaminants of emerging concern in solid digestates

In solid digestates, higher CEC levels were found compared to those in liquid digestate samples. However, only 17 out of 24 target CECs were detected and quantified in solid digestates. The novel flame-retardant tris(chloroisopropyl) phosphate (TCPP), octocrylene (sunscreen ingredient), the angiotensin II-receptor antagonist losartan, and the β-blocker metoprolol are the predominant CECs found in solid samples. Atorvastatin was found in two cases at concentration levels above the uLOQ (samples $I_{(S)}$ & $F_{(S)}$; Fig. 2). Caffeine (frequently detected in liquid digestates) was found in concentrations of 30–210 ng g^{-1} wet weight (ww) in the solid digestates (3 out of 10 samples). DEET, acridine, carbamazepine, and metoprolol were quantified in both liquid and solid samples although the levels found in solid digestates were higher.

Perspectives

Biogas production is acknowledged as an important tool in terms of today's international efforts aimed at establishing sustainable circular bioeconomic structures in global economies. Many nations support this development in order to reduce the still increasing CO_2 emissions on Earth. Circular strategies and improved sustainability imply that waste from production processes like biogas production is used as the basis for other production lines. To succeed, it is mandatory to assess the entire life cycle of these product waste chains in order to avoid any uncontrolled negative effects on society and the environment.

Our study shows that the many CEC residues that are present during biogas production are not retained or degraded during anaerobic digestion. In fact, most of the CECs in our study are found in potential substrate materials (Table S12†). As demonstrated earlier for similar recycling-based production processes, in order to reduce and minimize potential hazards and provide a safe platform for reuse of the resulting residues, a thorough monitoring and understanding of the production, with respect to potential anthropogenic pollutants, is required.[75–80] We, therefore, recommend optimising biogas production processes also with respect to a minimal output of pollutants. This should be done, at least, when the digestate is further applied as a soil amendment or fertilizer.

The results of the current study revealed that the AD process in biogas plants does not efficiently eliminate organic micropollutants associated with substrates. Furthermore, the potential cleavage of undetected phase-II conjugates during substrate pretreatment and subsequent digestion may in some cases lead to increased CEC levels. Hence, in addition to appropriate selection and treatment of the biogas substrate, we recommend appropriate treatment of biogas digestates before their subsequent agricultural application.

According to the results obtained here, separation of digestates into liquid and solid fractions significantly enhances the partitioning of organic contaminants. Polar and ionized organic pollutants such as pharmaceuticals tend to partition into the liquid digestate. Thus, we recommend enhancing the separation process and even performing successive water extraction of the separated solid digestate.

The polar and ionized organic contaminants in the liquid fraction (digestate supernatants or diluted liquid digestate) can further be eliminated by physicochemical treatment such as UV photolysis and ultrasonication. Several pharmaceuticals undergo fast photodegradation even under solar irradiation.[81] It is also recommended to enhance the recovery of phosphorus by precipitation using iron(II)-sulfate prior to digestate separation.[82]

As anaerobic processes are not very efficient in removing CECs from solid digestates, other technologies such as composting could be used for the solid digestate fractions.[66]

Conflicts of interest

There are no conflicts to declare.

Abbreviations

SI	Substrate food waste
SII	Substrate sewage sludge
SIII	Substrate fish silage
SIV	Substrate manure
RTemp	Reactor temperature
RT	Retention time
In	Inoculum added
PTTemp	Pre-treatment high temperature
PTTHP	Pre-treatment thermal hydrolysis processing
PTno	No pre-treatment
PreI	Precipitant $FeCl_3$
PreII	Precipitant $AlCl_3$
PreIII	Precipitant FeS
PreIV	Precipitant EcoFloc90
PreV	Precipitant $FeSO_4 \times 6H_2O$
Pre0	No precipitant
% DM	% dry matter
Poly	Polymer added

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SUPPLEMENTARY INFORMATION

Organic contaminants of emerging concern in Norwegian digestates from biogas production

Aasim M. Ali [*,a], *Astrid S. Nesse*[b], *Susanne Eich-Greatorex*[b], *Trine A. Sogn*[b], *Stine G. Aanrud*[c], *John A. Bunæs*[c], *Jan L. Lyche*[c], *Roland Kallenborn* [a,c,d]

[a] Faculty of Chemistry, Biotechnology and food Sciences (KBM); Norwegian University of Life Sciences (NMBU), NO-1432 Aas, Norway.

[b] Faculty of Environmental Sciences and Natural Resource Management (MiNa), Norwegian University of Life Sciences (NMBU), NO-1432 Aas, Norway.

[c] Faculty of Veterinary Medicine (VetFak), Norwegian University of Life Sciences (NMBU), NO-0454 Oslo, Norway

[d] University Centre in Svalbard, Arctic Technology, NO-9171 Longyearbyen, Svalbard, Norway

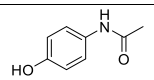
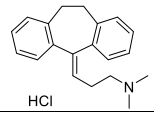
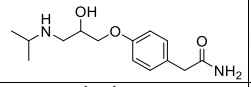
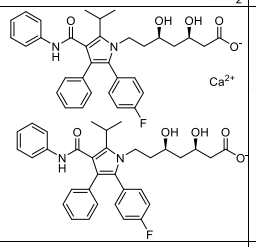
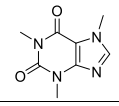
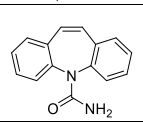
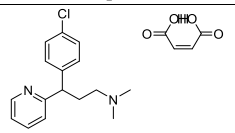
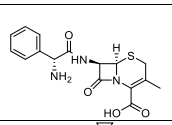
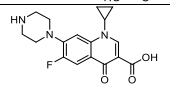
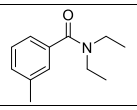
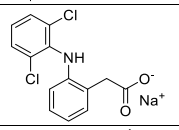
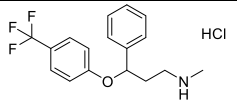
Method description

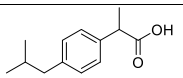
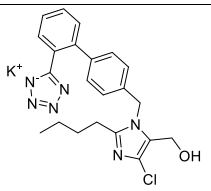
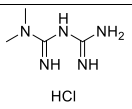
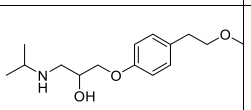
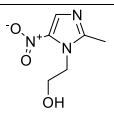
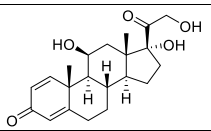
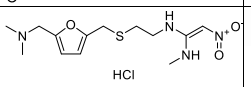
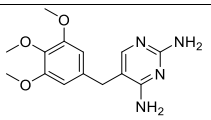
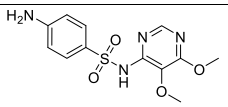
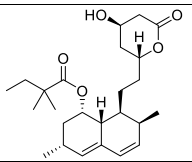
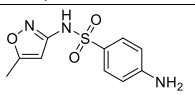
The methods applied for the quantification of targeted analytes for the here reported study was validated and optimised as follows**.**

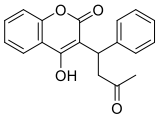
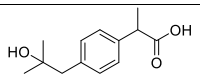
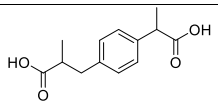
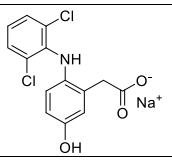
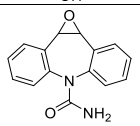
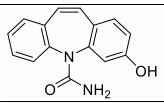
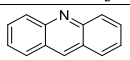
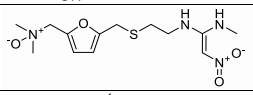
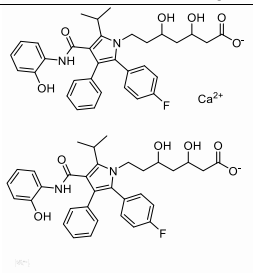
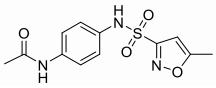
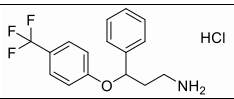
S1. Target substance characterisation

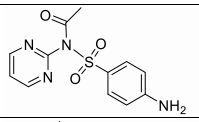
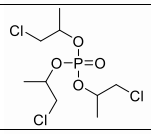
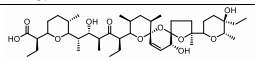
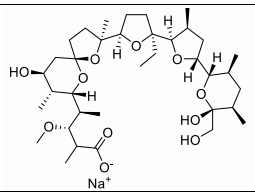
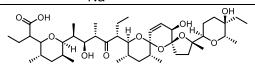
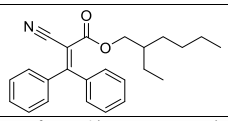
The selection of the compounds for the here performed study was based on their relatively high consumption rate and their previous detection in some environmental samples collected from Norway and the European environment. Table S1 lists the starting list of the selected CECs.

Table S1 : Target chemicals of emerging concern (CECs)

No.	Compound (Abbreviation)	Mol. formula	Structure	CAS Number	LogP*	LogD (pH 7.4)*	$LogK_{OC}$ (pH 7.4)*	Description	Supplier
1	Acetaminophen (ACE)	$C_8H_9NO_2$		103-90-2	0.34	0.40	1.59	nonsteroidal anti-inflammatory	Sigma Aldrich, Oslo, Norway
2	Amitriptyline hydrochloride (AMT)	$C_{20}H_{23}N \cdot HCl$		549-18-8	4.92	2.96	2.18	antidepressant	Sigma Aldrich, Oslo, Norway
3	Atenolol (ATN)	$C_{14}H_{22}N_2O_3$		29122-68-7	0.10	-1.85	0	beta-blocker	Sigma Aldrich, Oslo, Norway
4	Atorvastatin calcium salt trihydrate (ATO)	$C_{66}H_{68}CaF_2N_4O_{10}$		134523-03-8	4.13	1.25	0.64	antilipidemic	Toronto Research Chemicals, Toronto, Canada
5	Caffeine (CAF)	$C_8H_{10}N_4O_2$		58-08-2	-0.13	0.28	1.53	Psychostimulants	Sigma Aldrich, Oslo, Norway
6	Carbamazepine (CAR)	$C_{15}H_{12}N_2O$		298-46-4	2.67	2.28	2.61	anticonvulsant	Sigma Aldrich, Oslo, Norway
7	(±)-Chlorpheniramine maleate salt (CPA)	$C_{16}H_{19}ClN_2 \cdot C_4H_4O_4$		113-92-8	3.39	1.16	1.12	antihistaminic	Sigma Aldrich, Oslo, Norway
8	Cephalexin (CPX)	$C_{16}H_{17}N_3O_4S$		15686-71-2	0.65	-2.83	0	antibiotic	Sigma Aldrich, Oslo, Norway
9	Ciprofloxacin (CIP)	$C_{17}H_{18}FN_3O_3$		85721-33-1	0.65	-2.23	0	antibiotic	Sigma Aldrich, Oslo, Norway
10	*N,N*-Diethyl-3-methylbenzamide (DEET)	$C_{12}H_{17}NO$		134-62-3	1.96	2.24	2.59	insect repellent	Sigma Aldrich, Oslo, Norway
11	Diclofenac sodium salt (DCF)	$C_{14}H_{10}Cl_2NNaO_2$		15307-79-6	4.06	1.37	5.07	nonsteroidal anti-inflammatory	Sigma Aldrich, Oslo, Norway
12	Fluoxetine hydrochloride (FLX)	$C_{17}H_{18}F_3NO \cdot HCl$		56296-78-7	4.09	1.75	1.17	antidepressant	Sigma Aldrich, Oslo, Norway

No.	Compound (Abbreviation)	Mol. formula	Structure	CAS Number	LogP*	LogD (pH 7.4)*	$LogK_{OC}$ (pH 7.4)*	Description	Supplier
13	Ibuprofen (IBP)	$C_{13}H_{18}O_2$		15687-27-1	3.75	0.45	0.29	nonsteroidal anti-inflammatory	Sigma Aldrich, Oslo, Norway
14	Losartan potassium (LOS)	$C_{22}H_{22}ClKN_6O$		124750-99-8	3.56	1.29	1.17	Anti-hypertensive	Sigma Aldrich, Oslo, Norway
15	Metformin hydrochloride (MEF)	$NH_2C(=NH)NHC(=NH)N(CH_3)_2 \cdot HCl$		1115-70-4	-2.31	-3.36	0	Antidiabetic	Sigma Aldrich, Oslo, Norway
16	Metoprolol (MTP)	$C_{15}H_{25}NO_3$		37350-58-6	1.79	-0.25	0.28	β-blocker	Sigma Aldrich, Oslo, Norway
17	Metronidazole (MET)	$C_6H_9N_3O_3$		443-48-1	-0.01	0.05	1.40	antibiotic	Sigma Aldrich, Oslo, Norway
18	Prednisolone (PRE)	$C_{21}H_{28}O_5$		50-24-8	1.50	1.66	2.28	Corticosteroid	Sigma Aldrich, Oslo, Norway
19	Ranitidine hydrochloride (RAN)	$C_{13}H_{22}N_4O_3S \cdot HCl$		66357-59-3	1.23	-0.63	0.57	Histamine H_1 and H_2 receptor antagonist	Sigma Aldrich, Oslo, Norway
20	Trimethoprim (TRI)	$C_{14}H_{18}N_4O_3$		738-70-5	0.79	1	1.86	antibiotic	Sigma Aldrich, Oslo, Norway
21	Sulfadoxine (SUL)	$C_{12}H_{14}N_4O_4S$		2447-57-6	0.34	-1.04	0	antibiotic	Sigma Aldrich, Oslo, Norway
22	Simvastatin (SMV)	$C_{25}H_{38}O_5$		79902-63-9	4.41	4.60	3.88	antilipidemic	Chiron AS, Trondheim, Norway
23	Sulfamethoxazole (SMX)	$C_{10}H_{11}N_3O_3S$		723-46-6	0.89	-0.56	0.52	antibiotic	Sigma Aldrich, Oslo, Norway

No.	Compound (Abbreviation)	Mol. formula	Structure	CAS Number	LogP*	LogD (pH 7.4)*	$LogK_{OC}$ (pH 7.4)*	Description	Supplier
24	Warfarin (WAR)	$C_{19}H_{16}O_4$		81-81-2	3.42	0.30	0.26	Anticoagulant	Sigma Aldrich, Oslo, Norway
25	2-hydroxyibuprofen (IBP-OH)	$C_{13}H_{18}O_3$		51146-55-5	1.69	-0.51	0	transformation product of ibuprofen	Sigma Aldrich, Oslo, Norway
26	Carboxy ibuprofen (IBP-Car)	$C_{13}H_{16}O_4$		15935-54-3	1.82	-2.65	0	transformation product of ibuprofen	Sigma Aldrich, Oslo, Norway
27	5-Hydroxydiclofenac (5OH-DCF)	$C_{14}H_{11}Cl_2NO_3$		69002-84-2	3.91	0.96	0.45	transformation product of diclofenac	Sigma Aldrich, Oslo, Norway
28	Carbamazepine 10, 11-epoxide (CAR-1011)	$C_{15}H_{12}N_2O_2$		36507-30-9	1.26	1.31	2.09	transformation product of carbamazepine	Sigma Aldrich, Oslo, Norway
29	3-Hydroxycarbamazepine (CAR-3OH)	$C_{15}H_{12}N_2O_2$		68011-67-6	2.44	2.27	2.61	transformation product of carbamazepine	Sigma Aldrich, Oslo, Norway
30	Acridine (ACR)	$C_{13}H_9N$		260-94-6	3.40	3.34	3.18	transformation product of carbamazepine	Sigma Aldrich, Oslo, Norway
31	Salicylic acid (SA)	$C_7H_6O_3$		69-72-7	2.06	-0.77	0	anti-inflammatory	Sigma Aldrich, Oslo, Norway
32	Ranitidine N-oxide (RAN-O)	$C_{13}H_{22}N_4O_4S$		73857-20-2	-1.54	-0.76	0.96	transformation product of ranitidine	Sigma Aldrich, Oslo, Norway
33	2-Hydroxy Atorvastatin Calcium Salt (2OH-ATO)	$C_{66}H_{68}CaF_2N_4O_{12}$		265989-46-6	4.13	1.05	0.53	transformation product of atorvastatin	Toronto Research Chemicals, Toronto, Canada
34	N-Acetyl Sulfamethoxazole (ACY-SMX)	$C_{12}H_{13}N_3O_4S$		21312-10-7	1.48	-0.28	0.61	transformation product of sulfameth-oxazole	Toronto Research Chemicals, Toronto, Canada
35	Norfluoxetine hydrochloride (NOR)	$C_{16}H_{16}F_3NO \cdot HCl$		57226-68-3	4.36	2.23	1.84	antidepressant	Sigma Aldrich, Oslo, Norway

No.	Compound (Abbreviation)	Mol. formula	Structure	CAS Number	LogP*	LogD (pH 7.4)*	$LogK_{OC}$ (pH 7.4)*	Description	Supplier
36	N-Acetyl Sulfadiazine (ACY-SAD)	$C_{12}H_{12}N_4O_3S$		127-74-2	0.41	-0.86	0.38	transformation product of Sulfadiazine	Toronto Research Chemicals, Toronto, Canada
37	Tris (1-chloro-2-propyl) phosphate, mixture of isomers (TCPP)	$C_9H_{18}Cl_3O_4P$		13674-84-5	0.48	1.42	2.14	flame retardant	Sigma Aldrich, Oslo, Norway
38	Salinomycin (SLM)	$C_{42}H_{70}O_{11}$		53003-10-4	6.10	2.77	1.53	anticoccidial drug	Sigma Aldrich, Oslo, Norway
39	Monensin sodium salt (MON)	$C_{36}H_{61}NaO_{11}$		22373-78-0	3.72	0.45	0.23	anticoccidial drug	Sigma Aldrich, Oslo, Norway
40	Narasin (NAR)	$C_{43}H_{72}O_{11}$		55134-13-9	6.59	3.20	1.76	anticoccidial drug	Sigma Aldrich, Oslo, Norway
41	Octocrylene (OCR)	$C_{24}H_{27}NO_2$		6197-30-4	7.53	6.34	4.82	Sunscreen agents	Sigma Aldrich, Oslo, Norway

All structures were prepared with ChemDraw Professional (version 15.0.0.106), PerkinElmer Informatics, Inc. (Boston, Massachusetts, USA)

* Predicted data is calculated with ACD/Labs Percepta Platform – PhysChem Module, Toronto, CA. (http://www.chemspider.com/Chemical Structure.18219.html).

S.2. Chemicals

Acetonitrile (CH_3CN, HPLC grade) and methanol (MeOH, HPLC grade) were purchased from Sigma-Aldrich and VWR (West Chester, PA, USA). Reagent grade formic acid (CH_2O_2), hydrochloric acid (HCl), disodium ethylene diamine tetra acetate (Na_2EDTA), and ammonium hydroxide (NH_4OH) were purchased from Sigma-Aldrich. The water used was grade 1 purified with a Milli-Q water purification system (Millipore, Bedford, MA, USA).

S.3. Extraction and sample preparation

S.3.1. Extraction of the target compounds from solid digestate: An aliquot of 1.0 g (wet weight, ww) sample of a solid digestate was weighed into 15 mL polypropylene centrifuge tube. Subsequently, 6.0 mL of extraction solution A (MeOH: CH_3CN: Water with 0.1% Na_2EDTA and 0.2% formic acid; 70:20:10) was added into the sample and the mixture was vortexed for 20 s and then the tube was mechanically shaken for 10 min at 1400 rpm using IKA Vibrax VXR vibrator (Janke & Kunkel, Staufen, Germany). The mixture was further ultrasonically extracted for 10 min and then centrifuged for 5 min at 3000 rpm. Subsequently, the supernatant was transferred to another 15 mL polypropylene centrifuge tube. The sample was further extracted with 6.0 mL of extraction solution B (MeOH: CH_3CN: Water 0.1%NaEDTA, 0.2% NH_4OH; 70:20:10). The supernatants were combined and directly passed through an SPE cartridge PRiME HLB (60 mg, 3 mL) and collected. The collected solution was evaporated to dryness under air stream at 40 °C using a Reacti-Therm III evaporating unit (Thermo Fisher Scientific Inc., Rockford, USA). After addition of recovery standard (10 µg/mL, 2.5 µL), the residue was dissolved with 20 % CH_3CN in water until the final volume reached 0.5 mL, and the sample was then vortexed and subsequently filtered through a 0.2 µm microcentrifuge filter (Spin-X, Costar, Corning Inc., Corning, NY, USA). The resulting sample was finally transferred to polypropylene vials for quantitative LC–MS/MS analysis.

S.3.2. Extraction of the target compounds from liquid digestate: Aliquots*:* An aliquot of 2.0 mL of a liquid digestate sample was pipetted into 15 mL polypropylene centrifuge tube. Subsequently, 4.0 mL of extraction solution A was added, and the mixture was vortexed for 20 s. Subsequently, the tube was mechanically shaken for 10 min at 1400 rpm using an Vibrax VXR vibrator (IKA, Janke & Kunkel, Staufen, Germany). The mixture was further ultrasonically extracted for 10 min and then centrifuged for 5 min at 3000 rpm. Subsequently, the supernatant was transferred to a clean 15 mL polypropylene centrifuge tube. The extraction was repeated again using 4.0 mL of extraction solution B. The supernatants were combined, evaporated until the volume reached approximately 3 mL, 6.0 mL of Milli Q water was added, and the resulting solution was then directly loaded onto a SPE cartridge with PRiME HLB (60 mg, 3 mL) as adsorbnet. SPE was conducted by applying a low vacuum to the manifold (water jet), assuring a flow rate of 1- 3 drops per second. The cartridges were then washed with 2 mL of 5% MeOH in water and dried under low vacuum for 10 min. Elution was performed with 3.0 mL of (CH_3CN:MeOH; 9:1). The resulting eluates were evaporated to dryness under a gentle air stream (O_2, 5.0 quality, AGA, Oslo, Norway) at 40 °C using a Reacti-Therm III evaporating unit (Thermo Fisher Scientific Inc., Rockford, USA). After addition of the recovery standard (10 µg/mL, 2.5 µL), the residue was dissolved with 20 % CH_3CN in water until the final volume reached 0.5 mL, and the sample was then vortexed and subsequently filtered through a 0.2 µm microcentrifuge filter (Spin-X, Costar, Corning Inc., Corning, NY, USA) prior to quantification. The resulting sample was finally transferred to a polypropylene vial for immediate quantitative LC–MS/MS analysis.

S.4. High Performance Liquid chromatography (HPLC)

The quantitative determination all targeted analytes was performed on an Agilent 1200 series HPLC (Agilent Technologies, Waldbronn, Germany). The analytical column used for chromatographic separations was a Zorbax Eclipse plus C_{18} RRHD (2.1 x 100 mm, 1.8 µm) (Agilent, Palo Alto, USA) with a respective Guard Cartridge (4 µm x 3.0 mm ID) (Zorbax, Agilent, Palo Alto, USA). The column temperature was held isothermal at 25 ˚C. The injection volume was 10 µL. Separations were performed using a binary gradient with mobile phase consisting of water with 0.1% formic acid (A) pure CH_3CN (B) with a mobile phase flow rate of 0.35 mL/min (v:v). The initial mobile phase proportion was 100 % (A). B was then linearly increased to 100 % over 8 min and held for 7 min. Initial mobile phase conditions were restored over 1.0 min and the column was allowed to equilibrate for 4 min resulting in a total run time of 20 min. Combined chromatograms of the MRM transition for the product ions for each analyte are shown in Figure S1.

S.5. Electrospray ionization Mass spectrometry

An Agilent 6460 (Agilent Technologies, Santa Clara, CA, USA) triple quadrupole mass spectrometer with an Agilent Jet Stream electrospray ion source was used for the detection and quantitative analysis. The ions were monitored in positive and negative dynamic multiple reaction monitoring (dMRM). The ion source parameters are shown in Table S2. Table S3 contains information on the ion transitions monitored and their individual settings. Agilent MassHunter software (Version B.07.00 /Build 7.0.457.0, 2008) was used for instrument control, method validation and quantification.

Table S2: Ion source Parameters

Table 2 Ion source Parameters

Parameter	Value (+)	Value (-)
Gas Temp (°C)	320	320
Gas Flow (l/min)	10	10
Nebulizer (psi)	35	35
Sheath Gas Heater	390	390
Sheath Gas Flow	12	12
Capillary (V)	4000	3500

Table 3 Monitored ion transitions and their individual instrument settings; Precursor ion (Prec Ion), product ion (PI), Fragmentor voltage (FV), Collision Energy (CE), Retention time (RT), Retention time Window (RTW), and Polarity. For abbreviations and structure information on the target compounds, see table S1.

For abbreviations of CECs please consult table S1. <MLOQ: below method quantification limit, n.d. = not detected, <MLOD = below method detection limit.

Peak No	Compound Name	Prec Ion	PI	RT (min)	ΔRT	Frag (V)	CE	Polarity
1	MEF	130	71	0.76	2	60	20	+
1	MEF	130	60	0.76	2	60	20	+
2	ATN	267.2	190	1.4	2	80	20	+
2	ATN	267.2	145	1.4	2	80	30	+
3	RAN	315.1	170	1.6	2	60	10	+
3	RAN	315.1	130	1.6	2	60	20	+
4	RAN-O	331.1	176	1.8	2	90	20	+
4	RAN-O	331.1	130	1.8	2	90	30	+
5	ACE	152	110	2	3	60	15	+
5	ACE	152	65.1	2	3	60	35	+
6	MET	172	128	2	2	60	10	+
6	MET	172	82	2	2	60	25	+
7	CAF	195	138	4	2	110	20	+
7	CAF	195	110	4	2	110	30	+
8	$^{13}C_3$-CAF	198.2	140.2	4	2	110	20	+
8	$^{13}C_3$-CAF	198.2	112	4	2	110	20	+
9	CPX	348.1	174	4.3	2	70	15	+
9	CPX	348.1	158	4.3	2	70	5	+
9	CPX	348.1	106	4.3	2	70	20	+
10	$^{2}H_9$-TMP	300.3	264	4.3	2	80	30	+
10	$^{2}H_9$-TMP	300.3	243.1	4.3	2	80	30	+
10	$^{2}H_9$-TMP	300.3	122.9	4.3	2	80	30	+
11	TMP	291.5	261.1	4.3	2	100	25	+
11	TMP	291.5	123.2	4.3	2	100	25	+
12	ACY-SAD	293.1	198	4.4	2	100	20	+
12	ACY-SAD	293.1	134.2	4.4	2	100	30	+
12	ACY-SAD	293.1	65.2	4.4	2	100	35	+
13	ACR	180.1	152	4.5	2	70	60	+
14	CIP	332	288	4.6	2	80	20	+
14	CIP	332	245	4.6	2	80	30	+
15	MEP	268.3	116.2	5	2	70	20	+
15	MEP	268.3	98.1	5	2	70	20	+
15	MEP	268.3	74.1	5	2	70	20	+
16	$^{2}H_7$-MEP	275.3	191	5	2	100	20	+
16	$^{2}H_7$-MEP	275.3	121	5	2	100	20	+
16	$^{2}H_7$-MEP	275.3	105.2	5	2	100	20	+
17	CPA	275	230	5.3	2	60	10	+
17	CPA	275	167	5.3	2	60	30	+
18	$^{2}H_5$-Enrofloxacin	365	347	5.5	2	250	20	+

Table S3 continue

Peak No	Compound Name	Prec Ion	PI	RT (min)	ΔRT	Frag (V)	CE	Polarity
18	$^{2}H_{5}$-Enrofloxacin	365	321	5.5	2	250	20	+
18	$^{2}H_{5}$-Enrofloxacin	365	245	5.5	2	250	30	+
19	SUL	311	156.1	5.8	2	90	20	+
19	SUL	311	108	5.8	2	90	30	+
19	SUL	311	92.1	5.8	2	90	30	+
20	$^{2}H_{3}$- SUL	314.1	156	5.8	2	60	20	+
20	$^{2}H_{3}$- SUL	314.1	108	5.8	2	60	30	+
20	$^{2}H_{3}$- SUL	314.1	92.1	5.8	2	60	30	+
21	SMX	254	156	6	2	90	10	+
21	SMX	254	108	6	2	90	20	+
21	SMX	254	92	6	2	90	30	+
22	$^{13}C_{6}$-SMX	260	162	6	2	40	10	+
22	$^{13}C_{6}$-SMX	260	114	6	2	40	20	+
22	$^{13}C_{6}$-SMX	260	98	6	2	40	30	+
23	ACY-SMX	296.1	198	6.1	2	100	20	+
23	ACY-SMX	296.1	134	6.1	2	100	30	+
23	ACY-SMX	296.1	65	6.1	2	100	30	+
24	$^{2}H_{4}$-N-acetyl SMX	300.1	201.8	6.1	2	100	20	+
24	$^{2}H_{4}$-N-acetyl SMX	300.1	138	6.1	2	100	30	+
24	$^{2}H_{4}$-N-acetyl SMX	300.1	69	6.1	2	100	50	+
25	CAR-10,11	253.1	236.1	6.19	2	90	10	+
25	CAR-10,11	253.1	180.1	6.19	2	90	30	+
26	SA	137	93	6.19	2	90	20	-
26	SA	137	65	6.19	2	90	35	-
27	CAR-3OH	253.1	210.1	6.2	2	100	20	+
27	CAR-3OH	253.1	167.1	6.2	2	100	30	+
28	PRE	361.1	325.1	6.4	2	80	10	+
28	PRE	361.1	146.7	6.4	2	80	30	+
29	IBP-OH	221.2	177.1	6.48	2	100	10	-
29	IBP-Car	235.1	191	6.5	2	100	0	-
29	IBP-Car	235.1	73	6.5	2	100	30	-
30	AMT	278.2	105	6.7	3	40	20	+
30	AMT	278.2	91	6.7	3	40	30	+
31	NOR	296.2	134.2	6.7	2	100	10	+
31	NOR	296.2	105	6.7	2	100	10	+
32	CBZ	237	194	6.8	2	70	15	+
32	CBZ	237	179	6.8	2	70	35	+
33	$^{2}H_{10}$-Car	247.1	204.1	6.84	2	125	20	+
33	$^{2}H_{10}$-Car	247.1	187.1	6.84	2	125	40	+
33	$^{2}H_{10}$-Car	247.1	174.1	6.84	2	125	50	+
34	ATO	559.2	440.1	7	2	135	20	+
34	ATO	559.2	292.1	7	2	135	40	+
34	ATO	559.2	250	7	2	135	50	+
35	LOS	423.2	404.9	7.2	2	100	10	+
35	LOS	423.2	377	7.2	2	100	10	+
35	LOS	423.2	207	7.2	2	100	30	+
36	$^{2}H_{10}$-DEET	202.2	119	7.4	2	80	20	+
36	$^{2}H_{10}$-DEET	202.2	91.1	7.4	2	80	30	+
37	DEET	192	119	7.5	2	120	20	+
37	DEET	192	91	7.5	2	120	30	+
38	5OH-DCF	312	294	7.7	3	125	10	+
38	5OH-DCF	312	230.1	7.7	3	125	30	+
39	FLX	310	148	8	2	100	10	+
39	FLX	310	117	8	2	100	20	+
40	WAR	307.2	161	8.4	2	80	15	-
40	WAR	307.2	117.1	8.4	2	80	30	-
41	2OH-ATO	575.2	466.1	8.5	2	125	10	+
41	2OH-ATO	575.2	440	8.5	2	125	30	+

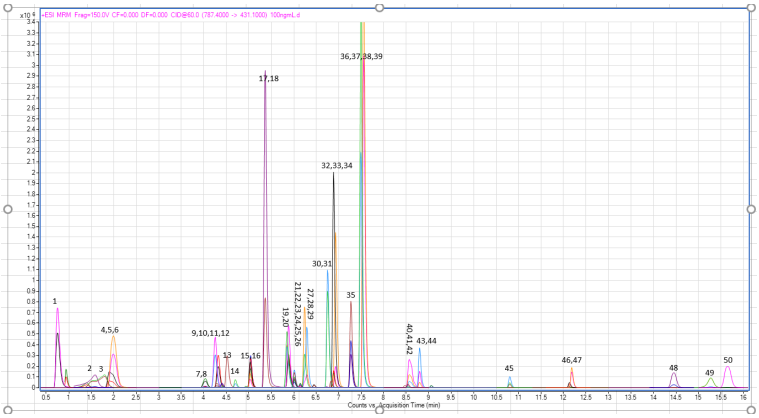


Figure S1.Typical combined MRM chromatograms of the target analytes with their respective internal standards. Individual peak assignments are listed in Table S3.

S.6. Quality control and calibration

S.6.1. Calibration: Matrix match and solvent matched calibration curves for targeted analytes and their calibration criteria of the method are shown in Table S4 in comparison. Calibration curves of five concentration levels (10, 20, 50, 80, and 100 ng/mL) were prepared for all isotope-labeled internal standards;$^{13}C_3$-CAF, $^{2}H_9$-Trimethoprin, $^{2}H_7$-Metoprolol, $^{2}H_3$-Sulfadoxine-d3, $^{13}C_6$-SMX, $^{2}H_4$-N-acetyl SMX, $^{2}H_{10}$-CBZ, $^{2}H_{10}$-DEET, $^{2}H_{15}$-Octocrylene and applying $^{2}H_5$-Enrofloxacin at concentration of 50 ng/mL as a recovery standard. The percentage recovery of internal standards was calculated based on these calibration curves.

S.6.2. Detection limits: The method detection limit (MDL) is defined as the minimum analyte concentration that can be detected and identified with a 99% confidence that its concentration is greater than zero [1]. MDL was calculated by multiplying the standard deviation of 5 spiked digestate samples at concentration of 5 and 10 ng/mL by student-t-test at the appropriate degree of freedom, the spiked samples were prepared and analysed according to the above described methods. The instrumental limit of detection (LOD) and limit of quantification (LOQ) were determined as LOD = 3*S/M and LOQ = 10*S/M, where S is signal standard deviation obtained by injecting solutions with a concentration of 5 ng/mL seven times, and M is the slope of the calibration curve[2].

S.6.3. Matrix effect: Matrix effect (ME %) was estimated using the following equation, where Sm and Ss are the slope of the matrix matched and solvent matched calibration curves respectively.

$$ME\% = \left[\left(\frac{Sm}{Ss}\right) - 1\right] \times 100$$

Positive ME% values indicate signal enhancement and negative values indicate ion suppression by the matrix. In general, ME% values ranging from −20% to +20% indicate acceptable matrix effect, while ME values <−20% or >+20% indicate significant matrix effects. Thus, in liquid digestate all compounds except ranitidine, narasin, and monesin experienced a significant matrix affect as shown in Table S6. Similarly, in solid digestate samples, all compounds experienced a significant matrix affect as shown except warfarin, fluoxetine and cephalexin (see table S5).

Table 4 Matrix match and solvent matched calibration curves. For abbreviations and structure information on the target compounds, see table S1

Compound	ISTD	Retention Time (min)	Conc. range (ng/mL) Solvent	R^{2**}	Conc. range (ng/mL) Solid digestate	R^2	Conc. range (ng/mL) Liquid digestate	R^2
2OH-ATO	$^2H_{10}$-DEET	8.5	0.2-500 (10)	0.993	0.2-500 (8)	0.991	0.5-300(7)	0.993
CAR-3OH	$^2H_{10}$-CBZ	6.2	0.2-200 (7)	0.997	0.5-500 (10)	0.990	0.2-300 (7)	0.994
5OH-DCF	$^2H_{10}$-DEET	7.7	5-600 (8)	0.994	1-500 (6)	0.992	10-500(5)	0.990
ACE	$^2H_{10}$-CBZ	2.0	0.2-200 (9)	0.995	25-100 (5)	0.979	0.5-500 (10)	0.975
ACR	$^2H_9$-TMP	4.5	0.2-500 (9)	0.994	0.2-500 (9)	0.996	0.2-500 (8)	0.982
AMT	$^2H_{10}$-DEET	6.7	0.2-500 (7)	0.995	0.2-200 (7)	0.994	0.2-500(6)	0.999
ATN	$^{13}C_3$-CAF	1.4	0.2-500 (10)	0.996	1-300 (7)	0.989	0.2-300 (8)*	0.991
CAF	$^{13}C_3$-CAF	4.0	0.2-500 (9)	0.994	0.2-500 (9)	0.992	0.2-500 (7)	0.986
IBP-Car	$^2H_{10}$-CBZ	6.5	5-600 (9)*	0.992	0.2-300 (5)	0.998	25-500(5)	0.986
CAR	$^2H_{10}$-CBZ	6.8	0.2-500 (11)	0.994	0.2-300 (7)	0.992	0.2-500 (10)	0.995
CAR-10,11	$^2H_{10}$-CBZ	6.1	0.2-200 (9)	0.990	0.2-500 (9)	0.994	0.2-300 (7)	0.997
CPX	$^2H_9$-TMP	4.3	1-600 (9)	0.994	1-500 (8)	0.993	5-500 (7)	0.984
CPA	$^2H_3$- SUL	5.3	0.2-300 (8)	0.997	25-600 (6)	0.980	0.5- 500 (7)	0.990
CIP	$^2H_9$-TMP	4.6	0.2-500 (11)	0.980	5-500 (6)	0.992	25-500 (5)	0.996
DCF	$^2H_{10}$-DEET	9.0	1-600 (8)	0.993	25-600 (5)	0.983	25-500 (4)*	0.999
DEET	$^2H_{10}$-DEET	7.5	0.2-100 (7)	0.990	0.2-600 (10)	0.995	0.2-500 (8)	0.995
FLX	$^2H_{10}$-DEET	8.0	0.2-500 (10)	0.994	5-500 (8)	0.99	5-500 (5)	0.990
IBP	$^2H_{10}$-DEET	5.0	50-600 (6)	0.992	-	-	25-500 (3)	0.985
LOS	$^2H_{10}$-DEET	7.2	0.2-200 (9)	0.991	0.2-600 (8)	0.993	25-500 (5)	0.993
MEF	$^{13}C_3$-CAF	0.76	0.2-100 (8)	0.996	0.5-200 (6)	0.995	0.2-300 (7)	0.980
MTP	$^2H_7$-MEP	5.0	0.2-500 (11)	0.994	0.2-500 (7)	0.994	5-300 (5)	0.978
MET	$^{13}C_3$-CAF	2.0	0.2-100 (7)	0.993	1-200 (5)	0.999	0.2-300(6)	0.997
MON	$^2H_{10}$-DEET	15.2	0.2-600 (6)	0.995	10-600 (5)*	0.987	0.2-500 (8)	0.999
ACY-SMX	$^2H_4$-N-acetyl SMX	6.1	0.5-500 (9)	0.993	25-500 (5)	0.989	25-500 (4)	0.996
ACY-SAD	$^2H_7$-MEP	4.4	0.2-300 (8)	0.990	5-600 (7)	0.992	5-500 (5)	0.996
NAR	$^2H_{10}$-DEET	15.5	0.2-100 (6)*	0.994	0.2-200 (5)*	0.986	1-500 (6)	0.975
NOR	$^2H_{10}$-DEET	6.7	1-500 (9)	0.997	5-300 (6)	0.991	5-500 (6)*	0.991
OCR	$^2H_{10}$-DEET	12.1	0.5-500 (6)	0.995	0.2-600 (6)	0.989	0.2 -500 (8)	0.997
PRE	$^2H_{10}$-DEET	6.4	0.5-100 (7)	0.987	25-500 (5)	0.996	5-500(6)	0.985
RAN	$^2H_7$-MEP	1.6	0.2-500 (11)	0.994	0.2-500(9)	0.997	0.2-500 (8)	0.993
RAN_O	$^{13}C_3$-CAF	1.9	0.2-200 (9)	0.992	0.5-600 (9)	0.990	0.2-500 (9)	0.990
SUL	$^2H_7$-MEP	5.8	0.2-200 (9)	0.993	5-500 (6)	0.996	5-500 (6)	0.986
SA	$^2H_{10}$-DEET	6.19	5-300 (6)	0.995	10-600 (4)	0.986	0.5-500 (8)*	0.990
SLM	$^2H_{10}$-DEET	14.4	0.2-500(5)	0.991	25-600 (6)	0.980	0.2-500 (8)	0.989
SMV	$^2H_{10}$-DEET	10.8	0.2-200 (6)	0.988	1-200 (5)*	0.993	10-500 (4)	0.978
SMX	$^2H_4$-N-acetyl SMX	6.0	0.2-500 (11)	0.994	5-500 (6)	0.995	5-500 (6)	0.980
TCPP	$^2H_{10}$-DEET	8.5	0.2-500 (11)	0.990	0.5-500 (7)	0.995	0.2-500(6)	0.992
TMP	$^2H_9$-TMP	4.3	0.2-500 (10)	0.991	1-500 (10)	0.991	5-500 (7)	0.987
WAR	$^2H_{10}$-DEET	8.4	0.5-500 (10)	0.982	5-500 (6)	0.991	1-500(6)	0.990

*Quadratic

**R^2 = Regression coefficient

Table 5 Matrix effect (ME %) values are color-coded (red: ion suppression, blue: signal enhancement, blank: no matrix effect). For abbreviations and structure information on the target compounds, see table S1.

Compound	ME % in liquid Digestate	ME% in solid Digestate
MEF	-68	-59
ATN	-55	-88
RAN	0	-59
RAN-O	-33	-76
ACE	-93	-94
MET	29	-72
CAF	-61	-81
CPX	98	224
ACY-SAD	-62	-52
TMP	-78	-87
ACR	-74	-70
CIP	-95	-79
MEP	-66	-69
SUL	-93	-88
SMX	-87	-79
CPA	-60	-80
ACY-SMX	-94	-77
NOR	-94	-80
SA	-71	-28
CAR-10,11	-81	-74
CAR-3OH	-85	-68
PRE	-98	-87
IBP-Car	-80	-55
IBP	-100	-100
CAR	-68	-64
IBP-OH	-85	-60
AMT	-94	-33
FLX	-98	0.6
LOS	-86	-75
DEET	-70	-60
5OH-DCF	-85	-56
ATO	-100	-100
WAR	-23	4
2OH-ATO	-67	-25
TCPP	-79	-85
DCF	-26	-45
SMV	-91	-78
OCR	-99	-98
SLM	79	-75
MON	7	-40
NAR	2	-57

The apparent recoveries for all analytes were also calculated using the following equation:

$$\text{Apparent Recovery}\% = 100 * \frac{\text{Calculated concentration of spiked sample} - \text{Calculated concentration of matrix blank}}{\text{Spiked concentration}}$$

A spiked sample is prepared by addition of a known concentration of native and internal standards before extraction to the matrix blank material before extraction and then the spiked sample was treated as real sample, and the concentration of the analytes in the spiked samples and blank was using matrix matched calibration graphs.

S.7. Method validation and quality control

The complete method for sample preparation procedure and quantitative analysis was subjected for comprehensive validation before the method was applied for quantitative analysis. The linear quantification range of the analytical instrument was confirmed with matrix-matched calibration.

Method validation: Extraction and clean-up

For complex matrices, such as biogas digestates, matrix matched calibration is mandatory in addition to efficient clean-up procedures as basis for high quality quantification of organic contaminants. In our studies a Hydrophilic – Lipophilic Balanced (HLB) solid phase extraction method was applied since this material proved to be well-suitable for sample preparation for multi-compound quantification in such complex matrices [3]. Sodium ethylene diamine tetra acetate-acetic (Na_2EDTA) was added during extraction in order to bind metals that may be present in the sample extract or adsorbed onto the surface of the sorbent [4]. Confirmed by earlier studies, free metal traces may covalently bind to target organic contaminants and, thus, significantly reduce their recoveries for quantitative analysis[5, 6].

As a part of the extraction method optimization, Oasis PRiME HLB was tested in two different modes, namely two- step clean-up (*pass-through*) and conventional three- step clean-up (*Catch and Release*) for solid and liquid digestate respectively. Furthermore, the effect of

sample size and acidification on matrix effect and recovery of target compounds were investigated for the optimum extraction of the target compounds. Based on the low matrix effect and good recovery of the target compounds, the two extraction methods (figure S2) were selected as optimal for the extraction of target compounds from solid and liquid digestate samples. The finally chosen method was based on 1g solid wet weight (ww) and 2 mL liquid samples, respectively, prepared for SPE with OASIS PRiME HLB as stationary phase. Please refer to the detailed description of extraction methods in section S3. The finally applied sample extraction and preparation methods for quantitative analysis are summarised in the flow chart in Figure S2.

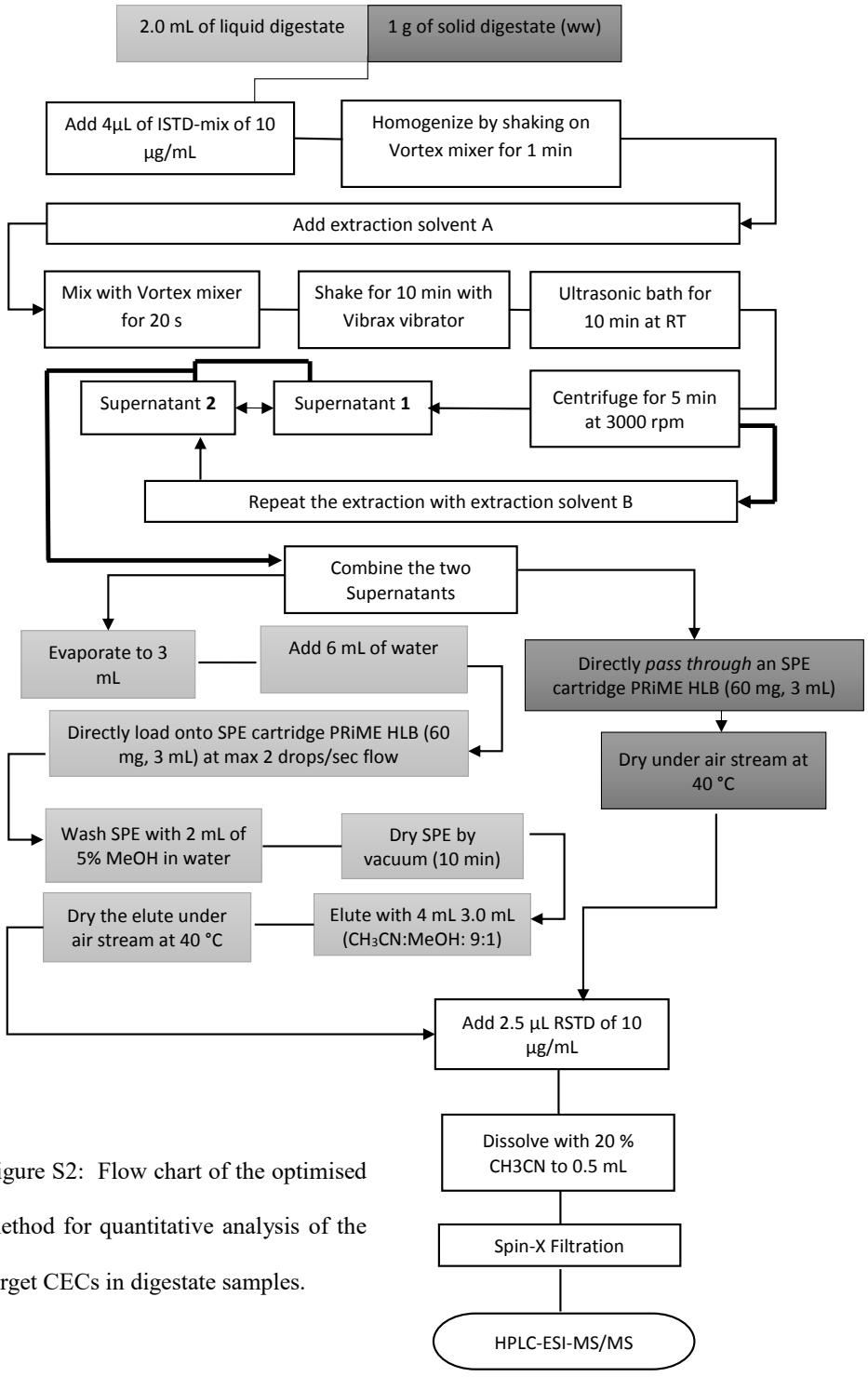


Figure S2: Flow chart of the optimised method for quantitative analysis of the target CECs in digestate samples.

Analytical method validation and quality control

The ultra-trace quantification method is based on high-performance liquid chromatography coupled to a triple-quadrupole mass selective detector with electrospray ionization (HPLC–ESI-MS/MS). This analytical technology is a well-established, validated and considered as a versatile scientific tool for the quantitative trace level detection of polar environmental contaminants. However, for the HPLC-ESI-MS/MS quantification method, non-linear matrix effects (ME) are often reported. MEs are usually attributed to co-eluting residual matrix components that affect the ionization efficiency of the target compounds. Typically, this results in either non-linear suppression or enhancement of target compound signal[7]. MEs are generally not reproducible or repeatable between various sample batches or even replicates of the same sample and, thus, compromise the quantitative analysis if not appropriately assessed [8, 9]. Therefore, the evaluation of MEs was also integrated in the quality control protocol of this study. In fact, for the present applied methods, considerable MEs were found for the quantification of the majority of the CECs. Thus, matrix-matched calibration was conducted using an experimental digestate prepared from a representative mixture of substrates in order to confirm the linear range of the method for reliable quantification (Table S4). For details on the evaluation, please refer to the detailed description in section S6.

All target analytes were separated in single chromatographic runs within a total analysis time of 20 min as depicted in Figure S1 (HPLC/MSxMS quantification). Method detection limit (MDL) in liquid and solid digestate, instrumental limit of detection (LOD), and instrumental lower limit of quantification (LOQ) values are summarised in Table S6. Instrument limits of quantification ranged from 0.2 pg/ mL^{-1} to 3.0 ng/mL^{-1}. For liquid digestate, MDL values for most compounds ranged from 0.25 to 20.55 ng/g while for solid digestate MDL values for most compounds are in the range 0.09 to 49.05 ng/g. The procedure for recovery calculation is summarised in Section S6 in the supplementary information. All individual target CEC recoveries are listed in Table S7. In total, 28 compounds in liquid digestates and 24 compounds in solid digestates showed satisfying recoveries (42 – 120%) in the initial method

validation. In the following spiking experiments, Atorvastatin, Ciprofloxacin (CIP), Sulfamethoxazole, N-acetyl sulfamethoxazole, Salicylic acid, Fluoxetine, Simvastatin, and Narasin were, however, not recovered in liquid samples and were therefore excluded from further quantitative analysis. For solid samples, CIP and Ranitidine *N*-oxide were not recovered from spiked solid samples and were, thus, also excluded from further quantitative analysis. All compounds which met the quality control criteria for final quantification in both liquid and solid digestate samples are marked orange in Table S7. Sample specific recovery rates for all internal standards (ISTD) from liquid and solid digestate samples were calculated applying known concentrations of $^{2}H_5$-enrofloxacin as a recovery standard. The recovery results are summarised in Table S7. The isotope labelled standards $^{2}H_3$-Sulfadoxine, $^{13}C_6$-Sulfamethoxazole and $^{2}H_{10}$-Octocrylene were excluded as internal standards for quantification since they were lost or showed low recovery rates during sample preparation and extraction (table S8). Therefore, Sulfadoxine, Sulfamethoxazole and Octocrylene were quantified in all digestate samples using the alternative internal standards as $^{2}H_7$-Metoprolol, 2H4-N-acetyl SMX and $^{2}H_{10}$-DEET, respectively.

Table 6: Method detection limit (MDL), instrumental limit of detection (LOD), and limit of quantification (LOQ). For abbreviations and structure information on the target compounds, see table S1

Compound	MDL (ng/g) in Solid Digestate	MDL (ng/mL) in Liquid Digestate	LOD (pg/µL)	LOQ (pg/µL)
MEF	4.95	0.45	0.01	0.04
ATN	2.5	2.8	0.01	0.04
RAN	1.35	1.1	0.01	0.03
RAN-O	0.04	0.15	0.02	0.08
ACE	44.75	1.3	0.007	0.02
MET	1.1	1.05	0.05	0.17
CAF	1.2	0.7	0.005	0.02
CPX	0.90	0.20	0.009	0.03
ACY-SAD	0.95	1.25	0.01	0.05
TRM	1.80	0.40	0.92	3.00
ACR	2.15	0.65	0.005	0.01
CIP	0.80	0.70	0.03	0.12
MEP	7.05	1.20	0.01	0.06
SUL	2.2	2.10	0.003	0.01
SMX	1.45	8.15	0.01	0.02
CPA	0.09	7,00	0.01	0.05
ACY-SMX	9.10	2.60	0.01	0.05
NOR	11.3	13.35	0.01	0.05
SAA	70.3	8.55	0.03	0.09
CAR-10,11	1.20	0.32	0.01	0.02
CAR-3OH	1.25	0.70	0.02	0.08
PRE	16.90	20.55	0.01	0.04
IBP-Car	18.75	3.45	0.03	0.12
IBP	66.40	66.40	17.5	58.6
CAR	3,00	0.745	0.03	0.11
IBP-OH	128.55	118.25	0.07	0.24
AMT	5.90	0.62	0.01	0.04
FLX	2.30	5.80	0.04	0.16
LOS	20.45	1.15	0.003	0.01
DEET	1.10	0.25	0.01	0.05
5OH-DCF	49.05	2,00	0.01	0.05
ATO	12.15	221.95	0.005	0.02
WAR	3.60	0.45	0.06	0.21
OH-ATO	57.4	0.60	0.01	0.03
TCPP	97.5	7.45	0.01	0.03
DCF	24.7	3.60	0.07	0.23
SMV	4.90	0.60	0.004	0.01
OCR	62.25	10.10	0.004	0.013
SLM	0.12	0.85	0.01	0.03
MON	0.35	3.40	0.02	0.06
NAR	0.13	7.95	0.25	0.85

Table 7. Recovery rates for all target compounds determined by repeated spiking of liquid and solid digestate sample. RSD = Relative standard deviation. For abbreviations and structure information on the target compounds, see table S1.

Compound/ concentration	**Recovery from liquid ± RSD (%, n = 6)**		**Recovery from solid ± RSD (%, n = 6)**
	25 ng/g	**100 ng/g**	**100 ng/g**
Metformin	5.7±22.0	6.6±19.1	83.6±13.9
Atenolol	83.8±11.8	84.7±15.0	82.4±15.0
Ranitidine	48.3±9.9	77.6±14.5	75.5±9.4
Ranitidine-*N*-oxide	2.7±13.8	5.10±10.0	0.2±109.9
Acetaminophen	66.2±8.8	25.1±21.8	136.8±7.8
Metronidazole	88.2±14.4	96.7±15.0	78.6±14.8
Caffeine	76.5±9.8	97.8±3.7	119.8±13.7
Cephalexin	2.0±33.5	1.7±8.5	42.5±3.6
N-acetyl sulfadiazine	2.5±138	11.7±29.8	96.1±14.8
Trimethoprim	75.6±14.5	88.8±11.4	68.9±10.5
Acridine	68.5±16.5	79.4±22.4	59.9±14.2
Metoprolol	80.5±7.7	98.1±12.9	99.6±13.1
Sulfadoxine	83.0±11.2	89.5±12.5	114.0±13.2
Chlorphenamine	5.9±42.2	14.1±47.6	2.6±110.2
Norfluoxetine HCL	54.2±18.9	3.23±31.1	108.4±15.0
CBZ-*10,11*-epoxide	85.2±7.2	89.3±13.3	96.8±14.0
3-hydroxy carbamazepine	83.2±9.5	87.4±14.1	86.8±14.0
Prednisolone	194.9±7.2	93.2±12.2	65.1±15.6
Carboxy-ibuprofen	42.2±1.3	7.3±55.5	138.4±14.5
carbamazepine	86.8±5.7	93.5±12.7	94.6±12.3
Ibuprofen	116.6±17.3	102.0±29	-

Compound	Recovery from liquid ± RSD (%, n = 6)		Recovery from solid ± RSD (%, n = 6)
	25 ng/g	100 ng/g	100 ng/g
2-hydroxy ibuprofen	156.7±96.2	91.5±38.7	-
Amitriptyline	42.4±16.8	58.3±17.8	44.8±16.8
Losartan	58.3±9.9	89.4±15.0	121.6±14.7
DEET	74.0±14.1	90.3±11.11	82.2±11.9
5-hydroxy diclofenac	28.2±15.0	57.7±12.1	138.8±12.0
WAR	67.1±13.5	68.8±14.4	36.2±51.8
2-hydroxy Atorvastatin	31.3±15.6	47.8±9.5	109.4±17.1
TCPP	67.2±7.3	93.1±14.9	108.6±16.4
Diclofenac	74.5±11.1	87.4±8.7	24.6±33.7
Octocrylene	44.6±29.7	43.4±14.9	50.4±36.9
Salinomycin	39.4±23.4	118.8±17.1	19.2±10.8
Monesin	81.1±11.0	92.3±4.0	10.7±14.9
Narasin	51.7±4.7	83.1±10.9	18.4±13.3
Sulfamethoxazole	-	-	108.8±13.4
N-acetyl sulfamethoxazole	-	-	87.4±13.5
Salicylic acid	-	-	12.4±308
Fluoxetine	-	-	13.8±60.3

Table 8 Sample specific recovery rates of internal standards from liquid and solid digestate samples calculated with solvent matched calibration curves using $^{2}H_5$-Enrofloxacin as a recovery standard. For abbreviations and structure information on the target compounds, see table S1

Compounds	**Average total recovery ±RSTD (%)**	**Average total recovery ±RSTD (%)**
$^{13}C_3$-CAF	63.1±19.6	52.3±9.0
$^{2}H_9$-Trimethoprin	207.8±7.4	77.5±15.4
$^{2}H_7$-Metoprolol	78.0±18.8	61.6±5.8
$^{2}H_3$-Sulfadoxine	-	-
$^{13}C_6$-SMX	9.2±67.5	31.49±5.4
$^{2}H_4$-N-acetyl SMX	109.9±18.6	97.0±25.1
$^{2}H_{10}$-CBZ	60.2±42	15.9±17.7
$^{2}H_{10}$-DEET	108.13±30.4	55.5±16.6
$^{2}H_{15}$-Octocrylene	-	-

- : not detected

Table 9 CEC levels in Liquid digestate [µg/L]

Station	$L_{(L)}$ (avg±std)	I_{sub} (avg±std)	$F_{(L)}$ (avg±std)	$I_{(L)}$ (avg±std)	I_{dig} (avg±std)	$C_{(L)}$ (avg±std)	$E_{(L)}$ (avg±std)	$G_{(L)}$ (avg±std)	$A_{(L)}$ (avg±std)
Atenolol	ND	ND	ND	ND	ND	0.34±0.48	0.60±0.11	2.2±0.01	ND
Acetaminophen	6.4±1.1	27.4±4.8	58.6±6.2	2.5±0.7	5.5±1.2	5.1±0.6	4.1±0.7	ND	8.9±0.9
Caffeine	2.1±0.02	10.0±0.85	ND	0.6±0.85	ND	5.5±0.15	1.25±0.1	3.0±0.90	2.3±0.03
Acridine	0.06±0.0001	0.051±0.001	0.06±0.0003	0.35±0.10	0.03±0.001	0.05±0.01	0.14±0.02	0.25±0.03	0.05±0.01
Metoprolol	6.2±0.13	ND	4.9±0.15	10.05±1.0	2.4±0.22	5.6±0.22	10.3±0.2	10.7±0.1	13.3±1.3
Sulfadoxine	ND	ND	ND	ND	ND	1.5±0.4	ND	ND	ND
3-hydroxy carbamazepine	ND	ND	0.11±0.02	0.13±0.005	0.12±0.04	ND	ND	ND	0.14±0.05
Prednisolone	-<LOQ	ND	<LOQ	ND	ND	16.4±2.5	ND	ND	ND
Carboxy-ibuprofen	ND	4.11±0.14	<LOQ	ND	ND	ND	ND	ND	ND
ibuprofen	ND	ND	ND	ND	ND	ND	36±51	ND	26.7±30.5
Carbamazepine	3.5±0.10	0.07±0.03	1.85±0.01	4.3±0.45	0.16±0.05	1.55±0.05	1.5±0.1	3.2±0.3	5.0±0.06
Amytriptyline	ND	ND	0.60±0.05	5.0±4.5	ND	1.4±0.25	1.4±0.60	ND	1.0±0.0005
Losartan	5.3±0.06	ND	6.3±0.05	10.5±0.5	7.7±0.03	6.0±0.04	5.5±0.1	5.6±0.05	8.15±0.1
DEET	8.50±0.45	0.35±0.06	0.045±0.06	ND	0.60±0.15	10.2±0.5	19.1±0.5	5.2±0.15	2.2±0.15
5-hydroxy DCF	ND	ND	5.6±0.3	6.5±1.7	ND	ND	ND	ND	3.2±0.7
2-hydroxy Atorvastatin	ND	ND	0.65±0.07	3.6±1.6	ND	ND	ND	0.17±0.001	0.65±0.01
TCPP	12.5±0.55	2.0±0.08	3.0±0.15	<LOQ	2.9±1.05	1.25±0.20	40.6±0.15	16.3±0.4	16.4±0.96
Diclofenac	5.0±0.35	ND	4.35±0.04	3.5±0.4	2.2±0.17	5.3±0.3	9.2±0.1	5.7±3.8	3.2±0.05
Octocrylene	25.8±1.3	4.5±5.4	25.5±11	146.6±8.0	6.2±1.45	71.0±5.0	224±113	44.8±6.8	19.3±8.4
Salinomycin	ND	ND	ND	ND	<LOQ	ND	ND	ND	ND
Monesin	0.050±0.005	ND	ND	ND	ND	ND	ND	ND	ND
SUM CEC	**75.1**	**25.8**	**114.4**	**193.6**	**22.3**	**131.1**	**353.6**	**97.1**	**110.3**

Table 10 CEC levels in solid digestate samples [ng/g ww]

Station	$D_{(S)}$ (avg±std)	$K_{(S)}$ (avg±std)	$A_{(S)}$ (avg±std)	$E_{(S)}$ (avg±std)	$I_{(S)}$ (avg±std)	$B_{(S)}$ (avg±std)	$L_{(S)}$ (avg±std)	$F_{(S)}$ (avg±std)	$H_{(S)}$ (avg±std)	$J_{(S)}$ (avg±std)
Ranitidine	15.8±1.4	ND	ND	ND	ND	ND	ND	ND	ND	ND
Caffeine	ND	ND	54.3±5.3	29.7±5.1	ND	ND	208.3±8.2	ND	ND	ND
Acridine	3.8±0.34	15.3±1.1	8.8±0.9	ND	ND	7.2±0.7	3.1±0.02	2.5±0.6	5.1±4.6	8-0±5.4
Metoprolol	36.1±0.5	100.1±2.6	74.8±12.2	48.4±2.1	44.5±8.7	32.2±4.9	21.6±2.0	14.0±0.8	17.2±9.1	58.4±0.5
Prednisolone	ND	ND	ND	<LOQ	ND	ND	ND	ND	ND	ND
Carboxy-IBP	ND	ND	ND	ND	ND	ND	ND	<LOQ	ND	ND
Carbamazepine	62.8±0.4	89.9±6.3	65.0±0.7	7.1±2.3	57.5±4.0	53.7±3.8	6.9±0.33	6.0±1.8	6.7±0.12	35.7±2.7
Amitriptyline	ND	ND	52.8±74.7	81.1±1.4	ND	131.7±15.3	ND	ND	ND	ND
Losartan	15.7±9.5	46.6±22.0	60.48±5.5	0.5±0.7	71.5±2.2	55.12±3.0	ND	8.3±1.2	76.5±65.8	50±33
DEET	<LOQ	<LOQ	11.8±1.7	<LOQ	<LOQ	1.07±0.05	<LOQ	<LOQ	<LOQ	1.6±0.12
5-hydroxy DCF	ND	ND	ND	ND	<LOQ	ND	ND	ND	ND	ND
Atorvastatin	ND	ND	ND	ND	>500	ND	ND	>500	ND	ND
2-hydroxy Atorvastatin	45.1±6.0	187.6±7.1	130±118	<LOQ	201±26	29.7±1.9	<LOQ	58.5±2.5	8.7±11.6	17.5±20.1
TCPP	238.5±88.9	304±102	475 ±135	>500	15.2±4.0	>500	463.2±36.5	105.7±12.5	33.6±4.3	15.3±21.7
Diclofenac	ND	ND	ND	ND	ND	ND	84.0±2.3	ND	ND	ND
Octocrylene	ND	ND	260±368	>600	<LOQ	>600	ND	ND	466±659	107±152
SUM CEC	**417.4**	**743.4**	**1202.9**	**1266.8**	**889.7**	**1410.7**	**849.3**	**691.3**	**613.8**	**614**

Table S11. Polymer used in biogas plants

Biogas plant	Polymer (Nature)	Amount	Added at which point
F	FLOPAM™ FO 4240, (cationic)	2-4 kg / 1000 kg digestate	Before centrifugation
E	long chained (cationic)	7-8 kg / 1000 kg digestate	Before centrifugation
D	Zetag 8185 (cationic)	3.35 kg Dry matter polymer / 1000 kg digestate	Before pressing in a filter press
A	Zetag 7563 (cationic)	17 tonnes / 40.000 tonnes digestate (annual amout)	Before centrifugation
B[1]	CC FLOC D 6144 K, (cationic)	At dewatering: Ca. 8 kg polymer / 1000 kg digestate At thickening: 4 kg polymer / 1000 kg digestate	At centrifugation
H	Zetag 7563 (cationic)	5-6 kg / 100 kg digestate	At centrifugation
K[2]	Zetag 8147 (cationic) – at “pre dewatering” Zetag 8180 (cationic) – at “end dewatering”	10 kg / tonnes of dry matter 10 kg / tonnes of dry matter	
J	Zetag 8125 (cationic)	6.5-7.2 kg / 1000 kg digestate	Before centrifugation
I	Zetag 7563 (cationic)	2-3 kg / 1000 kg digestate	Before centrifugation

[1] In addition, PRAESTOL K 144 L (cationic liquid polymer) is used as a lubricant in pipes to transport dewatered excreted digestate via pumps into digestate silos.

[2] K plant uses polymer at two steps.

Table 12. Literature comparison of concentration levels for selected CECs in related matrices.

Sample type (country of origin)	CAF (ng/g)	CAR (ng/g)	DCF (ng/g)	TCPP (ng/g)	OC (ng/g)	AMT (ng/g)	LOS (ng/g)	Ref.
Feather meal intended as fertilizer (USA)	<6.0 - 201							10
Feather meal intended as fertilizer (China)	59.6 - 153							10
Sewage sludge (Spain)	<MLOQ-74	12-42	<MLOQ-83					11
Fish fillet (USA)		<MDL–0.60						12
Sewage sludge (Spain)				429- 912				13
Sewage sludge (Catalonia, Spain)					1060-9170			14
US Herring gull eggs (*Larus argentatus*)				<MLOQ – 4.1				15
Whole Fish (Spain)	1.6 - 3.3				<MDL – 30.4			16
Soil, irrigated with reclaimed water (Spain)		0.10 - 8.2				<MLOQ – 9.8		17
Sewage sludge (Japan)		n.d. - 12	8.1 – 29				25 – 86	18
Soil (China)			n.d. - 1.16					19
Soil (UK)			0.14 -0.21					20
Sewage sludge (Spain)		73.6 – 89.7	n.d.–424.7					21
Sewage sludge (Ireland)		120						22
Sewage sludge (Switzerland)					32 0– 18740			23
Solid digestate (Norway)	**n.d .- 210**	**6 - 90**	**n.d . -84**	**14 - >500**	**100 - >600**	**n.d. – 132**	**n.d. - 76**	**This study**

For abbreviations of CECs please consult table S1. <MLOQ: below method quantification limit, n.d. = not detected, <MLOD = below method detection limit.

Table 13 List of the used instruments and software

Item	Specification	Producer	Supplier
MS	6460 series triple quadrupole LC/MS	Agilent Technologies, Santa Clara, CA, USA	Matriks AS, Oslo, Norway
HPLC	Agilent 1200 series with auto sampler, binary pump and column oven	Agilent Technologies, Santa Clara, CA, USA	Matriks AS, Oslo, Norway
Software	MassHunter, Quantitative analysis for QQQ, Versjon B.07.00/Build 7.0.457.0	Agilent Technologies, Santa Clara, CA, USA	Matriks AS, Oslo, Norway
Software	MassHunter, Qualititative analysis for QQQ, Versjon B.06.00/Build 6.0.633.10	Agilent Technologies, Santa Clara, CA, USA	Matriks AS, Oslo, Norway
Evaporator	Reacti-Vap III™ Evaporator	Thermo Scientific, Waltham, MA, USA	VWR International AS, Oslo, Norge
Shaker	VXR basic Vibrax	IKA® Werke GmbH & Co, KG. Staufen, Tyskland	Sigma Aldrich, Oslo, Norge
Centrifuge	Rotanta, 50 mL	Andreas Hettih GmbH & Co. KG, Tuttlingen, Tysklan	Dipl.ing. Houm AS, Oslo, Norge
Vortex	MS 3 basic	IKA-Werke GmbH & Co, KG. Wilmington, N.C, USA	

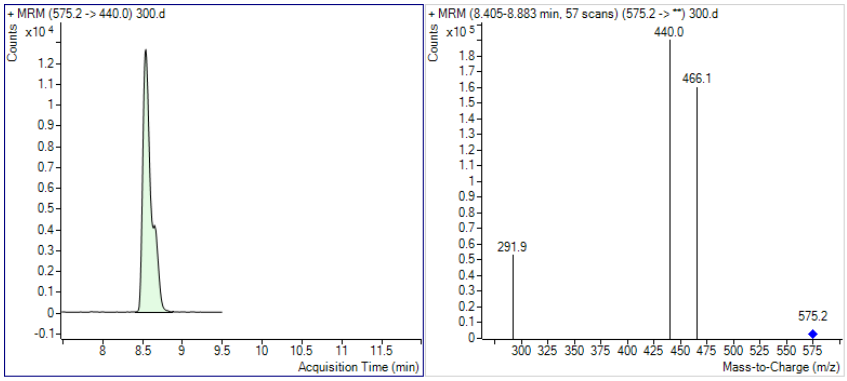


Figure S3a. MRM chromatogram and mass spectrum of 2-Hydroxy Atorvastatin standard

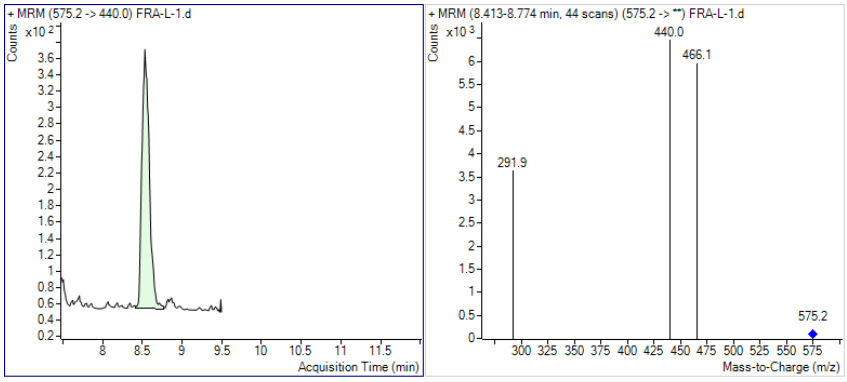


Figure S3b. MRM chromatogram and mass spectrum of 2-Hydroxy Atorvastatin detected in digestate sample ($F_{(S)}$)

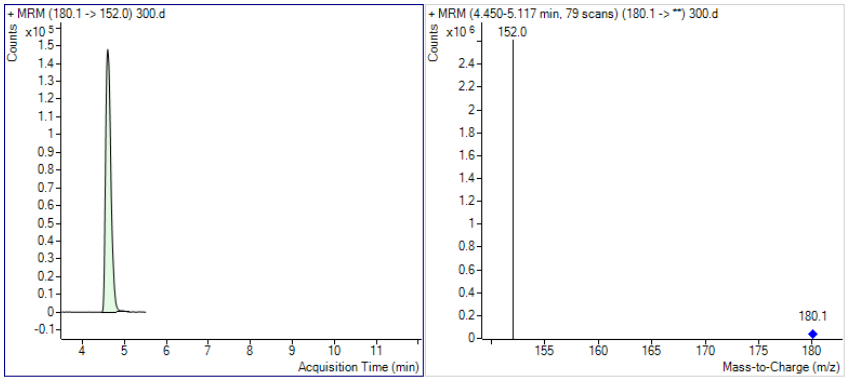


Figure S4a MRM chromatogram and mass spectrum of Acridine standard

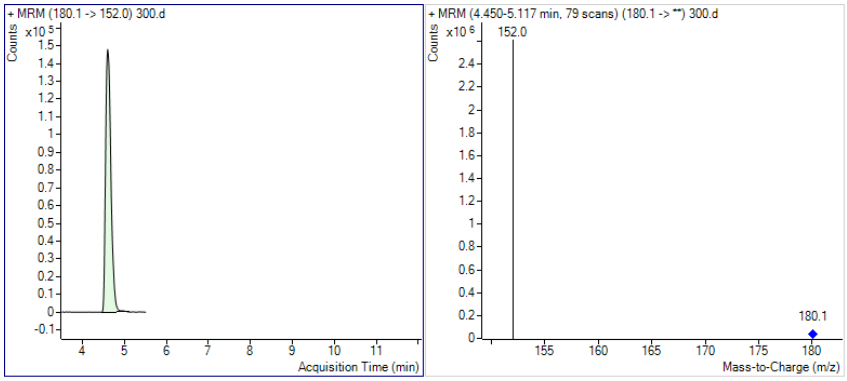


Figure S4b MRM chromatogram and mass spectrum of Acridine detected in digestate sample ($I_{(L)}$)

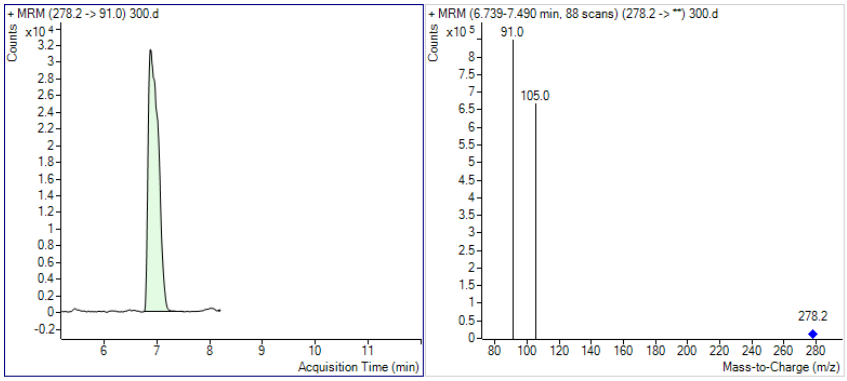


Figure S5aMRM chromatogram and mass spectrum of Amitriptyline standard

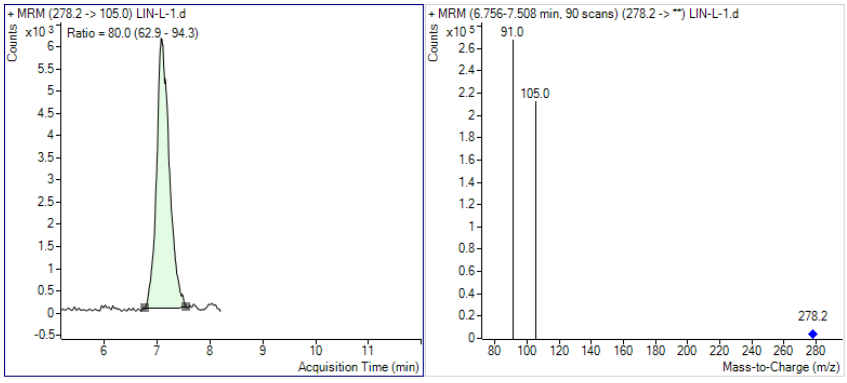


Figure S5b MRM chromatogram and mass spectrum of Amitriptyline detected in digestate sample ($I_{(L)}$)

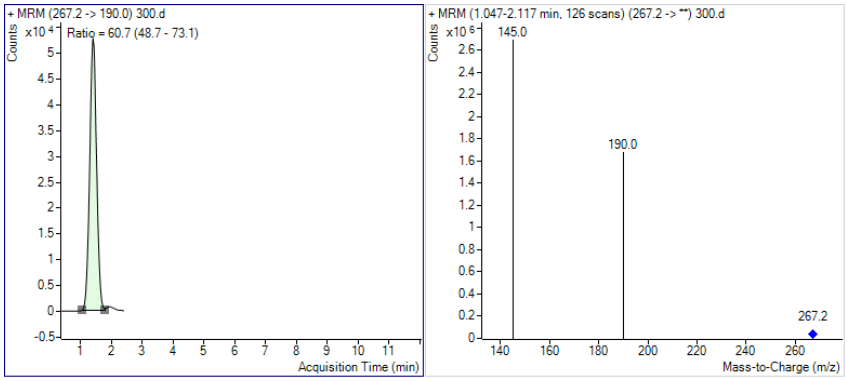


Figure S6a MRM chromatogram and mass spectrum of Atenolol standard

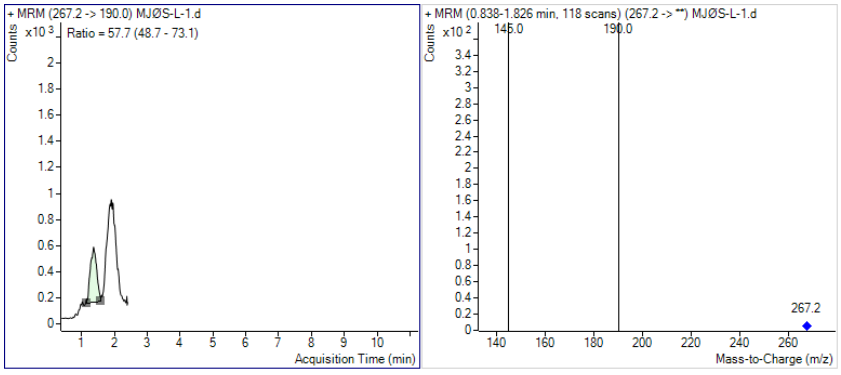


Figure S6b MRM chromatogram and mass spectrum of Atenolol detected in digestate sample ($C_{(L)}$)

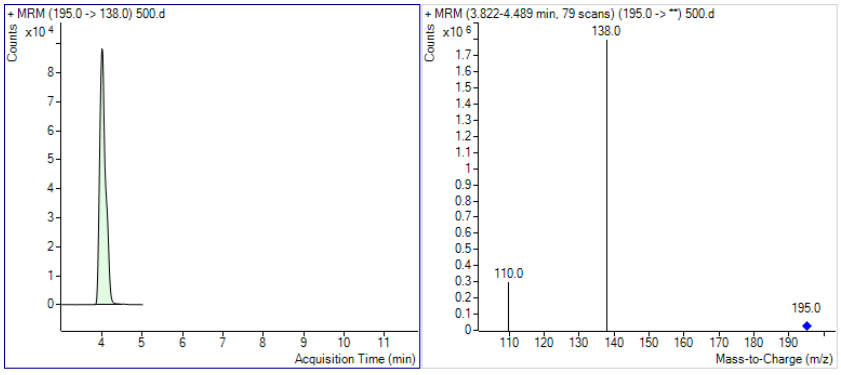


Figure S7a MRM chromatogram and mass spectrum of Caffeine standard

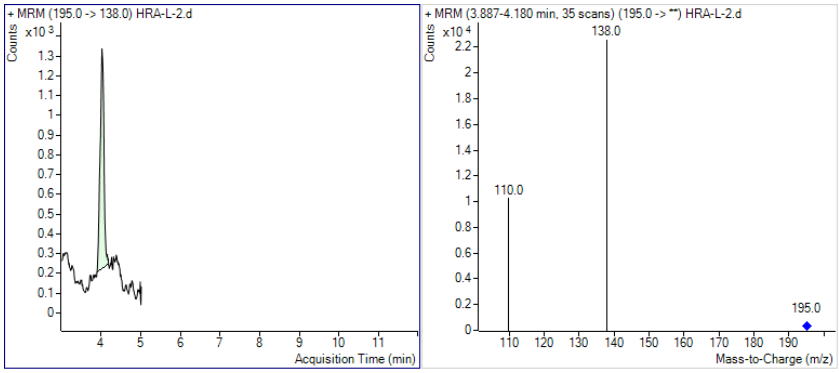


Figure S7b MRM chromatogram and mass spectrum of Caffeine detected in digestate sample ($L_{(S)}$)

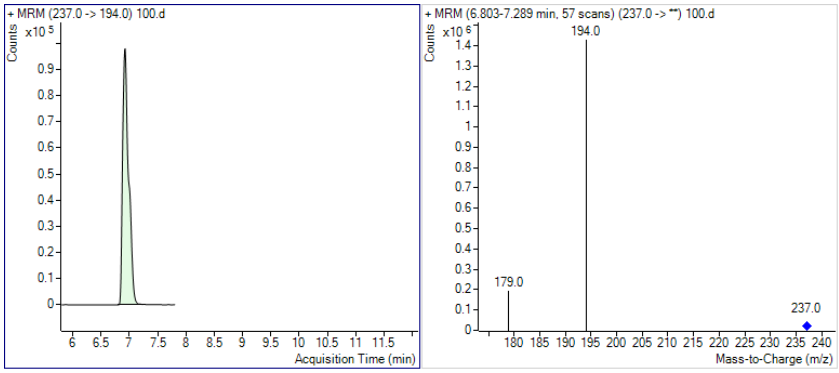


Figure S8a MRM chromatogram and mass spectrum of Carbamazepine standard

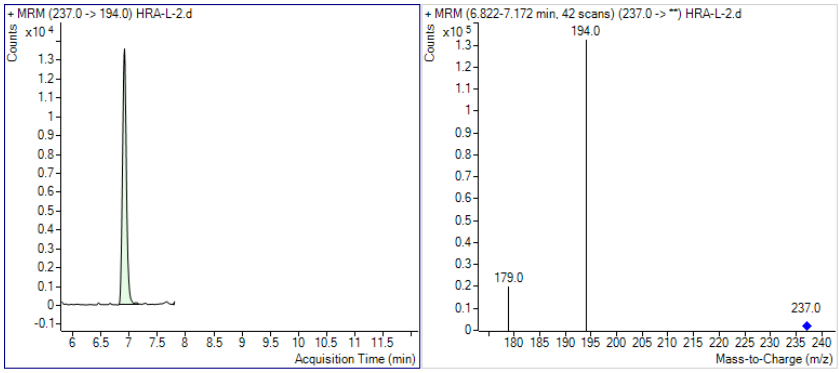


Figure S8b MRM chromatogram and mass spectrum of Carbamazepine detected in digestate sample ($L_{(S)}$)

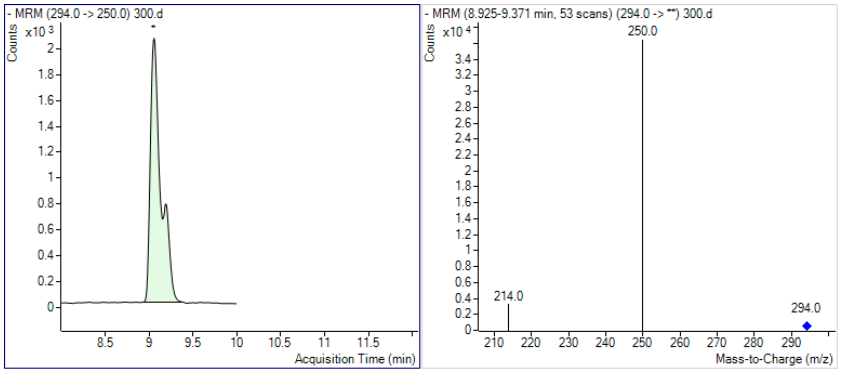


Figure S9a MRM chromatogram and mass spectrum of Diclofenac standard

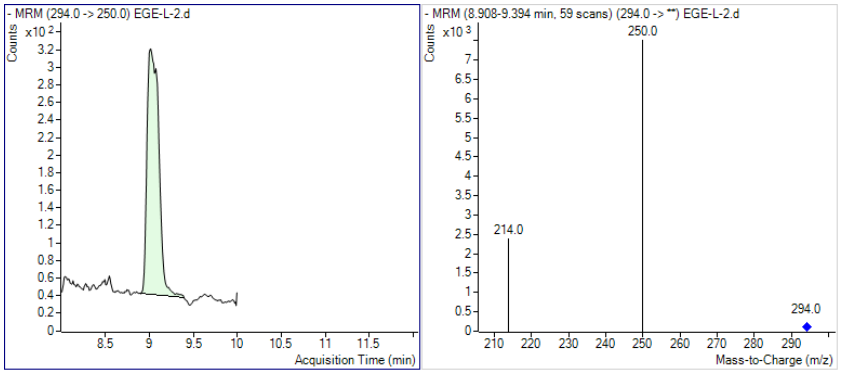


Figure S9b MRM chromatogram and mass spectrum of Diclofenac detected in digestate sample ($E_{(L)}$)

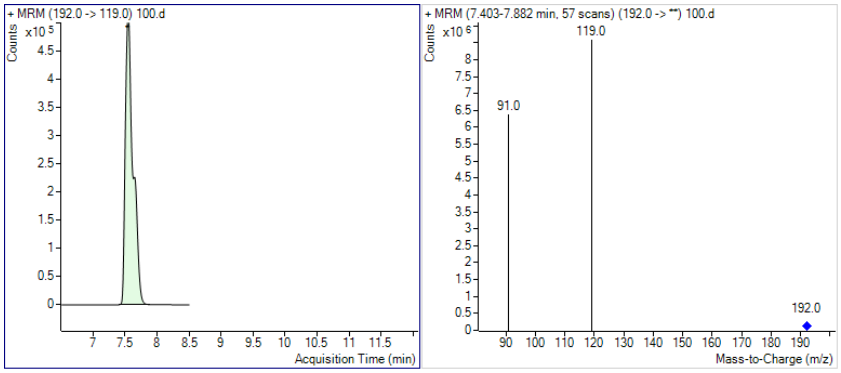


Figure S10a MRM chromatogram and mass spectrum of DEET standard

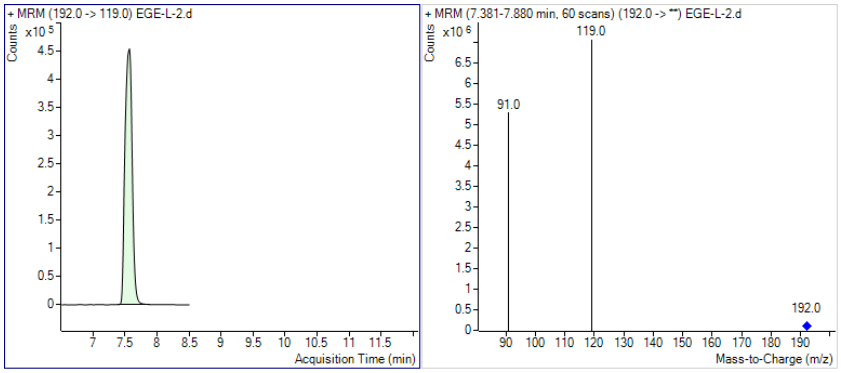


Figure S10b MRM chromatogram and mass spectrum of DEET detected in digestate sample ($E_{(L)}$)

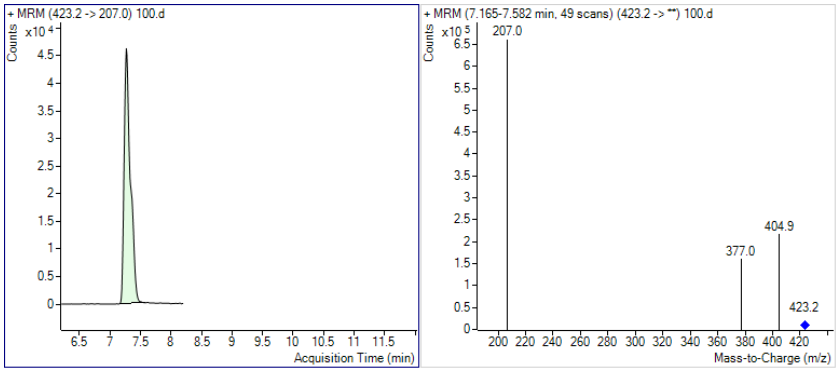


Figure S11a MRM chromatogram and mass spectrum of Losartan standard

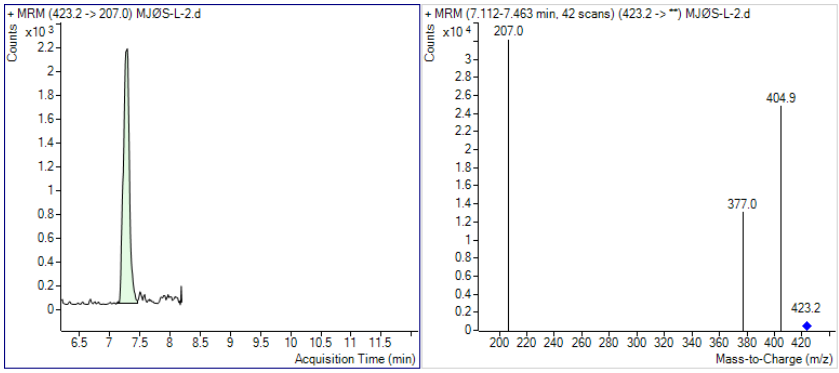


Figure S11b MRM chromatogram and mass spectrum of Losartan detected in digestate sample ($K_{(S)}$)

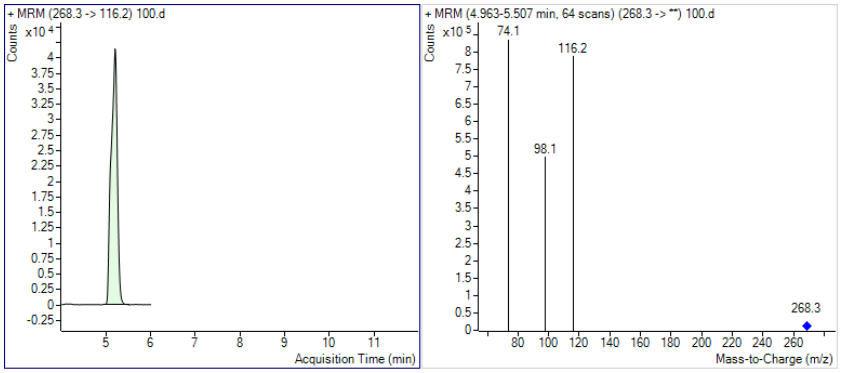


Figure S12a MRM chromatogram and mass spectrum of Metoprolol standard

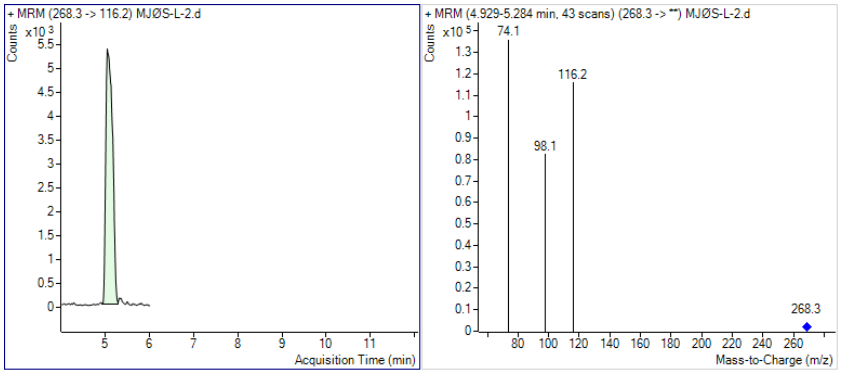


Figure S12b MRM chromatogram and mass spectrum of Metoprolol detected in digestate sample ($K_{(S)}$)

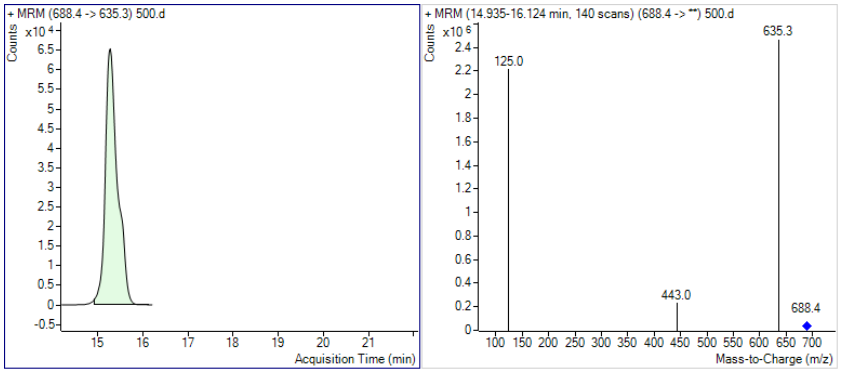


Figure S13a MRM chromatogram Monesin standard

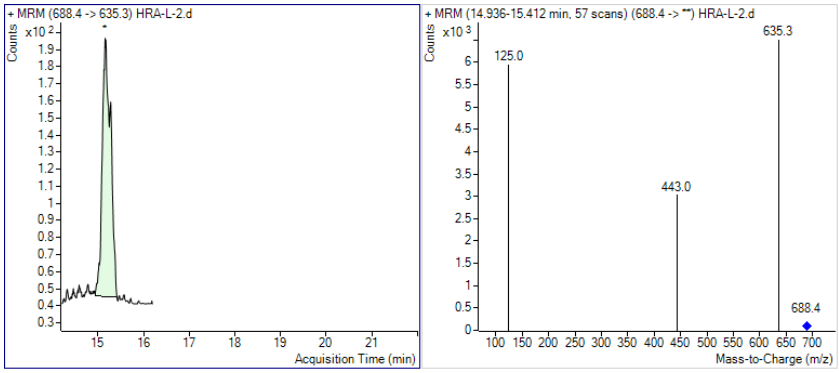


Figure S13b MRM chromatogram Monesin detected in digestate sample ($L_{(L)}$)

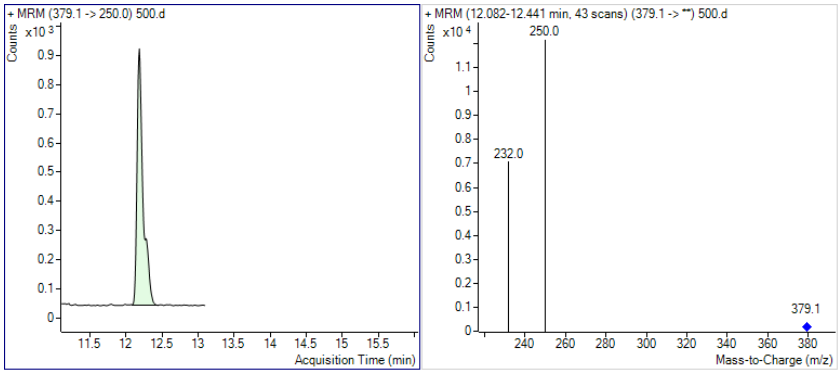


Figure S14a MRM chromatogram and mass spectrum of Octocrylene standard

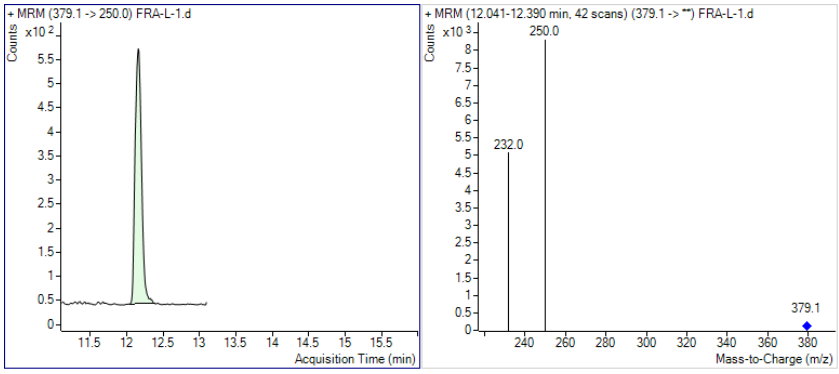


Figure S14b MRM chromatogram and mass spectrum of Octocrylene detected in digestate sample ($B_{(S)}$)

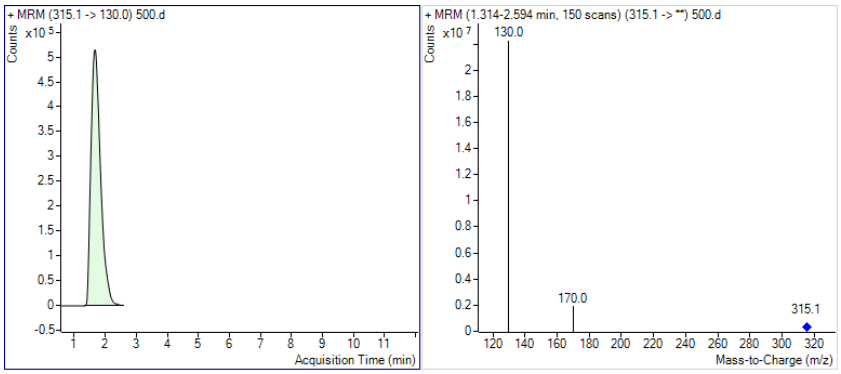


Figure S15a MRM chromatogram and mass spectrum of Ranitidine standard

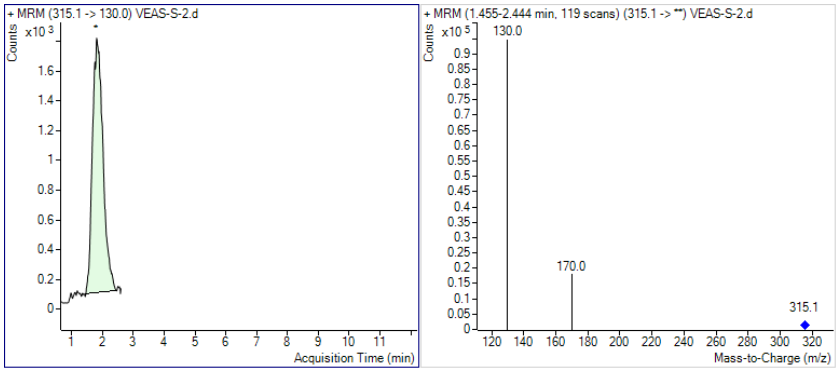


Figure S15b MRM chromatogram and mass spectrum of Ranitidine detected in digestate sample ($B_{(S)}$)

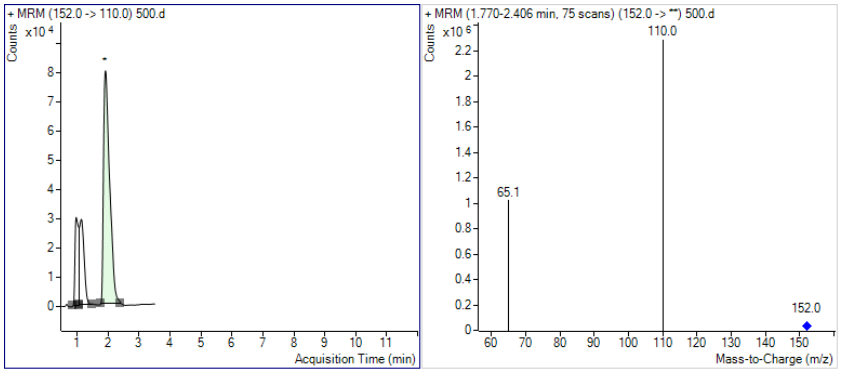


Figure S16a MRM chromatogram and mass spectrum of Acetaminophen standard

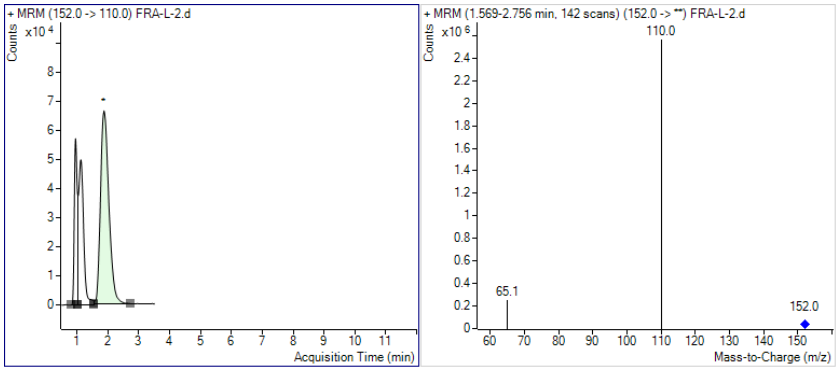


Figure S16b MRM chromatogram and mass spectrum of Acetaminophen detected in digestate sample ($F_{(L)}$)

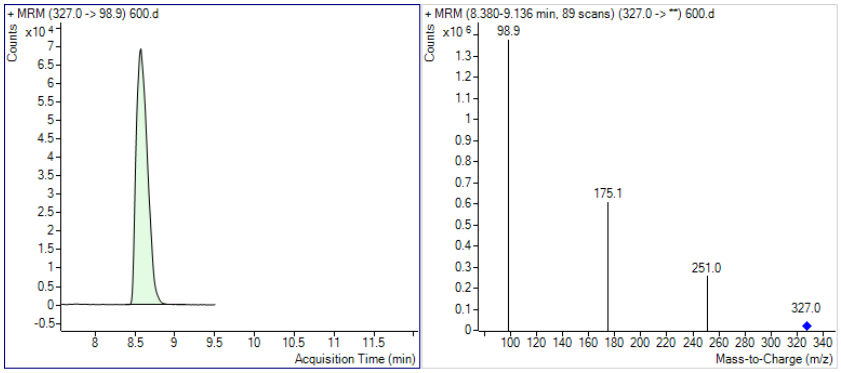


Figure S17a MRM chromatogram and mass spectrum of SUM TCPP standard

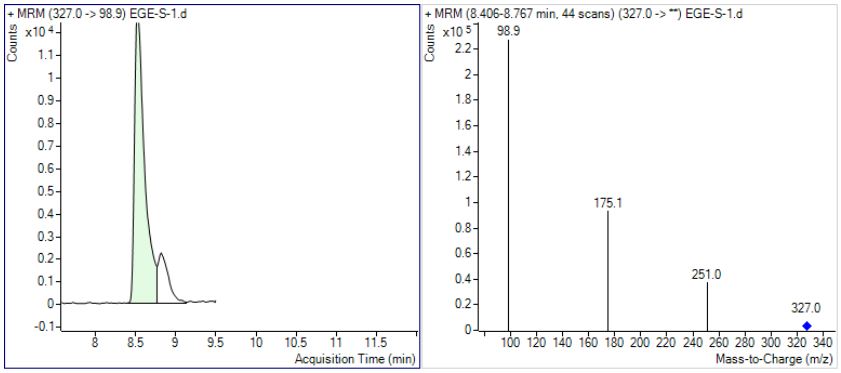


Figure S17b MRM chromatogram and mass spectrum of SUM TCPP detected in digestate sample ($E_{(S)}$)

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CORRECTION

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Correction: Organic contaminants of emerging concern in Norwegian digestates from biogas production

Aasim M. Ali,*[a] Astrid S. Nesse,[b] Susanne Eich-Greatorex,[b] Trine A. Sogn,[b] Stine G. Aanrud,[c] John A. Aasen Bunæs,[c] Jan L. Lyche[c] and Roland Kallenborn[acd]

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Correction for 'Organic contaminants of emerging concern in Norwegian digestates from biogas production' by Aasim M. Ali *et al.*, *Environ. Sci.: Processes Impacts*, 2019, **21**, 1498–1508.

After revisiting and evaluating the data of our recent publication "Organic contaminants of emerging concern in Norwegian digestates from biogas production", we identified several minor erroneous details in Table 1 which need to be corrected in order to allow correct interpretation of the results. In Table 1, the operating conditions of the biogas processes are listed according to information from the respective biogas plants. An updated version of Table 1 is included here, as well as amendments to sections as corrections of the earlier assumptions.

Corrections

p. 1499 **Materials and methods – Biogas process conditions**

Original interpretation: "The sample set also included one liquid sample (I_{sub}) and a liquid digestate sample (I_{dig}) from an experimental biogas reactor associated with plant I. Biogas plant I uses 20% sludge from young fish and 80% manure as a raw substrate (Table 1)."

Correction: Biogas plant I and the experimental biogas reactor are two separate reactors with different operating conditions, but are listed as one reactor in the original paper. The experimental reactor is now added to the modified Table 1 (named I_{EXP} in Table 1). I_{EXP} uses 20% sludge from young fish and 80% manure as a substrate, while biogas plant I uses 72% sewage sludge and 28% food waste as substrate.

Corrected text: "The sample set also included one liquid sample (I_{sub}) and a liquid digestate sample (I_{dig}) from an experimental biogas reactor I_{EXP}, associated with plant I. This experimental biogas plant I_{EXP} uses 20% sludge from young fish and 80% manure as a raw substrate (Table 1)."

p. 1500 **Results and discussion – Substrate composition**

Original interpretation: "High levels of octocrylene (a sun-screen ingredient), in some cases exceeding the uLOQ method limit, were found almost exclusively when sewage sludge was used for biogas production."

Correction: Elevated levels of octocrylene were found across all investigated biogas digestates produced from food waste, in amounts which are comparable to the concentrations found in those produced from sewage sludge. In $E_{(S)}$ (solid digestate from biogas plant E) the concentration was >600 ng g^{-1}. In the liquid digestates, the concentrations in digestates produced from food waste were 25.8 ng g^{-1} (plant L), 224 ng g^{-1} (plant E), and 44.8 ng g^{-1} (plant G).

Corrected text: "High levels of octocrylene (a sunscreen ingredient), in some cases exceeding the uLOQ method limit, were found in food waste and sewage sludge-based biogas digestates."

p. 1500 **Results and discussion – Substrate composition**

Original interpretation: "The correlation calculations revealed a significant positive correlation between the CEC level and the use of thermal hydrolysis (PTTHP) in the pre-treatment of the substrate prior to AD as well as the dry-matter content (% DM)."

Correction: The correlation analysis was repeated with the updated information from Table 1. PTTHP still has a positive correlation with the CEC level, but this corrected correlation is not significant, whereas the correlation between CEC level and dry matter is still significant.

[a]*Faculty of Chemistry, Biotechnology and Food Science (KBM), Norwegian University of Life Sciences (NMBU), NO-1432 Aas, Norway. E-mail: aasimali@nmbu.no*
[b]*Faculty of Environmental Sciences and Natural Resource Management (MiNa), Norwegian University of Life Sciences (NMBU), NO-1432 Aas, Norway*
[c]*Faculty of Veterinary Medicine (VetFak), Norwegian University of Life Sciences (NMBU), NO-0454 Oslo, Norway*
[d]*University Centre in Svalbard, Arctic Technology, NO-9171 Longyearbyen, Svalbard, Norway*

Table 1 Plant specific procedure strategies for optimized biogas production obtained from the representative Norwegian biogas production plants[a]

Location	Solid (S)/liquid (L) sample available	Substrate	Reactor temperature [°C]	Retention time [days]	Inoculum	Pre-treatment	Precipitant added	Dry matter [%]	Polymer
A	L + S	45% food waste, 53% sewage sludge, 2% fish silage	40	16	No	THP	No	L = 1; S = 27	Yes
B	S	15% food waste, 85% sewage sludge	38	40	Yes	THP	No	S = 26	Yes
C	L	27% manure, 72% food waste	39	35	Yes	Temp.	$FeCl_3$	L = 5	No
D	S	100% sewage sludge	37	20	No	No	$FeCl_3$ & $AlCl_3$	S = 47	Yes
E	L + S	100% food waste	41	35	Yes	THP	No	L = 3; S = 27	Yes
F	L + S	45% food waste, 55% sewage sludge	62	20	Yes	Temp.	$FeCl_3$	L = 2; S = 21	Yes
G	L	100% food waste	40	20	No	THP	Micronox	L = 4	No
H	S	100% sewage sludge	54	15	No	Temp.	$FeCl_3$, PAX	S = 26	Yes
I	S + L	72% sewage sludge, 28% food waste	40	20	No	THP	No	L = 5; S = 39	Yes
I_{EXP}	Substrate and digestate	20% sludge from young fish, 80% manure	40	20	No	No	No		No
J	S	100% sewage sludge	55	14	No	No	$FeSO_4 \times 7H_2O$	S = 32	Yes
K	S	100% sewage sludge	40	25	No	THP	Ecofloc	S = 33	Yes
L	L + S	100% food waste	53	20	No	No	FeS	L = 3; S = 35	No

[a] THP = thermal hydrolysis processing; temp. = pre-treatment at 70 °C for 30–60 min; L = liquid samples; S = solid samples; EcoFloc = liquid, commercially available flocculant (Ecolab, Naperville, IL, USA) mainly composed of $FeCl_3$ in water solution. Micronox = a precipitant containing a mixture of iron oxides, iron hydroxides and other oxides. I-EXP. = an experimental biogas reactor associated with plant I. For details of polymers added to the digestates, see Table S11.

Corrected text: "The correlation calculations revealed a significant positive correlation between the CEC level and the dry-matter content (% DM)."

p. 1501 **Results and discussion – Biogas production and processing**

Original interpretation: "The results presented in Fig. 1 indicate that the careful selection of substrate composition (including dry matter content) and optimised conditioning strategies for biogas production may be considered a first important step to reduce the occurrence of potential CECs in the digestate."

Corrected text: "The results presented in Fig. 1 indicate that the careful selection of substrate composition and optimised conditioning strategies for biogas production may be considered a first important step to reduce the occurrence of potential CECs in the digestate."

p. 1503 **Results and discussion – Contaminants of emerging concern in liquid digestates**

Original interpretation: "Ibuprofen was detected in two liquid biogas digestates mainly derived from sewage sludge based substrates ($E_{(L)}$) and $A_{(L)}$) at concentrations of 36 μg L^{-1} and 26.7 μg L^{-1}, respectively."

Correction: The liquid biogas digestate $E_{(L)}$ is produced from food waste.

Corrected text: "Ibuprofen was detected in two liquid biogas digestates derived from food waste alone or in combination with sewage sludge as substrates, *i.e.* $E_{(L)}$ and $A_{(L)}$, at concentrations of 36 μg L^{-1} and 26.7 μg L^{-1}, respectively."

The Royal Society of Chemistry apologises for these errors and any consequent inconvenience to authors and readers.

Paper II

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RESEARCH ARTICLE

Confirming the presence of selected antibiotics and steroids in Norwegian biogas digestate

Astrid Solvåg Nesse[1] · Stine Göransson Aanrud[2] · Jan Ludvig Lyche[2] · Trine Sogn[1] · Roland Kallenborn[3]

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Abstract

Farms utilizing sewage sludge and manure in their agronomic plant production are recognized as potential hotspots for environmental release of antibiotics and the resulting promotion of antibiotic resistance. As part of the circular economy, the use of biogas digestates for soil fertilizing is steadily increasing, but their potential contribution to the spreading of pharmaceutical residues is largely unknown. Digestates can be produced from a variety of biowaste resources, including sewage sludge, manure, food waste, and fish ensilage. We developed a method for the detection of 17 antibiotics and 2 steroid hormones and applied the method to detect pharmaceutical residues in digestates from most municipal biogas plants in Norway, covering a variety of feedstocks. The detection frequency and measured levels were overall low for most compounds, except a few incidents which cause concern. Specifically, relatively high levels of amoxicillin, penicillin G, ciprofloxacin, and prednisolone were detected in different digestates. Further, ipronidazole was detected in four digestates, although no commercial pharmaceutical products containing ipronidazole are currently registered in Norway. A simplified risk assessment showed a high risk for soil microorganisms and indicates the tendency for antibiotic-resistant bacteria for penicillin G and amoxicillin. For prednisolone and ipronidazole; however, no toxicity data is available for reliable risk assessments.

Keywords Pharmaceuticals · Ecotoxicity · Risk assessment · Contamination · Environmental pollution

Introduction

Biogas production is increasingly used in circular bioeconomic strategies as a sustainable strategy for organic waste management. Currently, more than 18,000 biogas plants are registered in Europe (Cesaro, 2021). A variety of organic wastes such as sewage sludge and manure, as well as food and household wastes, are anaerobically digested for the production of biogas, containing mainly methane and carbon dioxide (the methane is thereafter used as an energy carrier). The nutrient-rich organic residue from that process is known as biogas digestate. While the use of biomethane for transport is well-known to reduce greenhouse gas emissions, Lyng et al. (2018) showed that the replacement of mineral fertilizer with biogas digestate is equally important for a reduced carbon footprint. Furthermore, the mineral phosphorus resources are finite with a proposed peak around 2030, and today, only 20% of all mined phosphorus is consumed in food (Childers et al. 2011). Thus, increased reuse of nutrients is important.

Unfortunately, in addition to the valuable nutrients, digestates have been shown to contain residues of legacy and emerging organic pollutants which are not fully degraded during the digestion process (Ali et al. 2019; Lindberg et al. 2005; Spielmeyer et al. 2014; Suominen et al. 2014; Widyasari-Metha et al. 2016). These anthropogenic contaminants may ultimately enter the agricultural production system if the digestates are spread on agricultural land (Chen et al. 2019).

Residues of antibiotics and steroids have been detected in sewage sludge, sewage effluents, manure, and other environmental matrices (Chang et al. 2007; Clarke and Smith 2011; Spielmeyer

Responsible Editor: Philippe Garrigues

✉ Astrid Solvåg Nesse
astrid.solvag.nesse@nmbu.no

[1] Faculty of Environmental Sciences and Natural Resource Management, Norwegian University of Life Sciences, Ås, Norway

[2] Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Ås, Norway

[3] Faculty of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Ås, Norway

2018; Verlicchi and Zambello 2015). Both antibiotics and steroid hormones, such as oestrogens and glucocorticoids, have well-known biological activity and may cause adverse environmental effects. For instance, continuous exposure of soil microbial communities to antibiotic agents by, e.g. manure, spread on agricultural land may lead to elevated levels of antibiotic resistance genes and resistant bacteria (Heuer et al. 2011). Further, the presence of antibiotics can disrupt the natural soil microbial flora and thereby adversely affect biogeochemical processes, such as nitrification, denitrification, and iron reduction (Grenni et al. 2018; Roose-Amsaleg and Laverman 2016). The exposure of aquatic organisms to glucocorticoids can lead to changes in behaviour and immunological responses, as demonstrated in previous studies (e.g. Bal et al. 2017; McNeil et al. 2016).

However, the fate of these contaminants in biogas digestate has only been sparsely investigated, due to lack of appropriate methods. Hence, we developed and validated a multi-compound quantitative trace level analytical method for the investigation of 16 antibiotics and steroid hormone residues in digestates. The choice of compounds was based on a former screening programme of the Norwegian Food Safety Authority. The list includes compounds considered important for food safety in Norway either due to frequent application in Norwegian agriculture or because they are banned for use in Norway according the EU regulation 37/2010/EC (NFSA 2015). The here developed method was applied to representative samples from 12 centralized municipal biogas plants (i.e. most of such plants in Norway) as well as two experimental reactors. Based on the detected pharmaceutical levels, a simplified risk assessment was performed by calculating expected soil concentration caused by the application of digestate and comparing those to ecotoxicity and antibiotic resistance development data.

Materials and methods

Collection of biogas digestates and information about the operating conditions

Biogas digestates were collected from twelve major municipal biogas plants in Norway (plants A to L, Tables 1 and S10), as well as two experimental units connected to research stations (plant M and plant I_{exp}, connected to plant I). Each plant sampled about 1 L of their digestate and sent it to the Norwegian University of Life Sciences for quantitative analysis. Detailed information about the operating parameters, as supplied by the biogas plants, can be found in Table S10. The feedstocks used were food waste (E, G, and K), sewage sludge (D, H, J, and I), food waste mixed with sewage sludge (A, B, F, and L), manure mixed with food waste (C), manure (M), and a manure/fish silage combination (I_{exp}). The digestates are referred to as liquid (< 5% dry matter, subscript L) or solid (20–50% dry matter, subscript S). Some plants produce both fractions; thus, the total number of digestates analysed was 18. Samples were quantified in duplicate for each digestate batch to account for method uncertainty and for the heterogeneity of the material. The same samples were previously analysed for pharmaceuticals and personal care products (PPCPs) by Ali et al. (2019).

Table 1 Overview of target compounds with their respective calibration curve range (ng mL^{-1} extract)

X-compounds	Y-compounds	Z-compounds
Range: 0.25–30 ng mL^{-1}	*Range: 2.5–300 ng mL^{-1}*	*Range: 10–600 ng mL^{-1}*
Sulfonamide antibiotics:	*Fluoroquinolone antibiotics:*	*β-lactams antibiotics:*
Sulfadiazine	Ciprofloxacin	Amoxicillin
Sulfadoxine	Difloxacin	Penicillin G
Sulfamethazine	Enrofloxacin	
Sulfamethoxazole	Sarafloxacin	*Tetracycline antibiotics:*
	Norfloxacin	Chlortetracycline
Nitroimidazole antibiotics:		Doxycycline
Ipronidazole	*Glucocorticoids:*	Methacycline
Ipronidazole-OH	Dexamethasone	Oxytetracycline
Metronidazole	Hydrocortisone	Tetracycline
Ronidazole	Prednisolone	
2-Hydroxymethyl-1-methol-5-nitro-1H-imidazole (HMMNI)		
Macrolide:		
Tiamulin		
Pyrimidine:		
Trimethoprim		

Target analytes

For the method validation process, 26 compounds from 8 classes of antibiotics and steroids were chosen as target analytes (Table 1). The target analytes have different linear concentration ranges as determined by the individual calibration curves, as well as different shelf life as standard solution. Based on the individual detector sensitivity and preservability, the compounds were divided into three groups: X, Y, and Z (Table 1). Separate solutions were prepared for each group, for both the native ^{12}C standards (STDs) and the isotope-labelled internal standards (ISTDs). A complete list of target compounds including structure information and CAS registry numbers is available in Table S1. The stock solution concentrations and compositions are summarized in Table S2.

Chemicals and solutions

Methanol (MeOH, HPLC-grade) and acetonitrile (ACN, HPLC-grade) were purchased from VWR (West Chester, PA, USA). Formic acid, ammonium acetate, disodium ethylene diamine tetra acetate (Na_2EDTA), citric acid, sodium phosphate dibasic, and phosphoric acid were purchased from Sigma Aldrich (Oslo, Norway). Only grade 1 purified water from Milli-Q water purification systems (Millipore, Bedford, MA, USA) was used for the sample preparation and analysis. All standards and internal standards were purchased according to Table S1 in the Supplementary information. Separate solutions were prepared for the X-, Y-, and Z-compound groups both for the native ^{12}C standards (STDs) and the isotope-labelled internal standards (ISTDs) (Table S2).

Sample preparation and clean-up

Based on a previously published method (Hu et al. 2010), a comprehensive sample clean-up protocol was developed and validated ensuring minimum of matrix disturbances and co-elution in the final LC–MS/MS quantification (Fig. 1). An aliquot of 2 ± 0.03 g (wet weight, ww) digestate sample was weighed into 15 mL polypropylene tubes (Fig. 1). Internal standards were added, corresponding to 7.5 ng X-compound, 75 ng Y-compounds, and 150 ng Z-compounds (Table S2). Subsequently, 3 mL of extraction solution (Table S3) was added before the sample was vortexed for 10 s, ultrasonicated for 10 min, and centrifuged at 3500 rpm for 15 min. The supernatant was transferred to a glass tube, and the extraction

Fig. 1 Flow chart for the extraction of antibiotics and corticoid steroids from biogas digestate, before quantitative analysis with UHPLC-MS/MS

Table 2 Validation results of all successfully validated compounds, with recovery, method repeatability, method accuracy, matrix effect, and efficiency of the extraction method (EEM) at level 3 of the calibration curve (i.e. 7.5 ng mL^{-1} for X-compounds, 75 ng mL^{-1} for Y-compounds, and 150 ng mL^{-1} for the Z-compounds, see Table S5). R^2 is the determination coefficient for the calibration curve

Compound	Recovery (mean ± SD)	Repeatability (CV%)	Accuracy (mean ± CV%)	R^2	Matrix effect (%)	EEM (%)	Internal standard	Linear range ng g^{-1} digestate (fw)	MDL	MQL
Acceptable range	40–115%	< 15%	± 15 ± 15%	> 0.985						
Nitroimidazoles (X-type)										
Ipronidazole	78 ± 3	3	− 2.8 ± 2	0.992	− 40	39	Ipronidazole-D_3	0.4–15	0.13	0.40
Metronidazole	103 ± 3	3	4.2 ± 4	0.998	7	88	Metronidazole-$^{13}C_2$-$^{15}N_2$	0.4–15	0.13	0.38
Ronidazole	77 ± 5	5	− 5.0 ± 5	0.994	24	79	Ronidazole-D_3	1.3–15	0.20	0.63
Sulfonamides (X-type)										
Sulfadiazine	96 ± 3	3	2.1 ± 3	0.997	87	55	Sulfadiazine-$^{13}C_6$	0.4–15	0.13	0.38
Sulfadoxine	74 ± 7	7	− 2.1 ± 7	0.993	− 12	21	Sulfadiazine-$^{13}C_6$	0.13–7.5	0.025	0.075
Sulfamethazine	111 ± 4	4	− 12 ± 4	0.995	61	62	Sulfadiazine-$^{13}C_6$	0.13–15	0.005	0.015
Macrolide (X-type)										
Tiamulin	81 ± 6	6	− 1.4 ± 6	0.994	− 56	32	Tiamulin-$^{13}C_4$	1.3–15	0.50	1.25
Fluoroquinolones (Y-type)										
Ciprofloxacin	43 ± 14	14	− 11 ± 13	0.990	− 22	9	Enrofloxacin-D_5	3.8–150	1.3	3.8
Difloxacin	111 ± 3	3	8.2 ± 4	0.996	− 39	41	Difloxacin-D_3	1.3–150	0.50	1.25
Enrofloxacin	101 ± 3	3	3.6 ± 3	0.995	− 33	23	Enrofloxacin-D_5	1.3–150	0.20	0.63
Norfloxacin	113 ± 4	4	− 2.6 ± 4	0.993	3	12	Norfloxacin-D_5	13–150	2.0	6.3
Sarafloxacin	68 ± 8	8	− 3.9 ± 8	0.994	− 23	14	Difloxacin-D_3	1.3–150	1.3	3.8
Corticosteroids (Y-type)										
Prednisolone	95 ± 4	4	0.73 ± 4	0.991	− 39	192	Prednisolone-D_8	13–150	2.0	6.3
Dexamethasone	93 ± 3	4	3.9 ± 3	0.995	− 36	188	Prednisolone-D_8	1.3–150	0.25	0.75
β-lactams (Y-type)										
Amoxicillin	58 ± 4	4	5.8 ± 4	0.995	28	15	Amoxicillin-$^{13}C_6$	25–300	9.0	25
Penicillin G	43 ± 13	13	− 0.003 ± 13	0.991	− 32	16	Penicillin G-D_7	5–300	1.5	5.0

procedure was repeated twice without ultrasonication. The combined supernatants were dried to approximately 1 mL with controlled heating at 37 °C under a stream of compressed air (analytical quality, AGA, Porsgrunn, Norway) using a Reacti-Therm III evaporator (Thermo Fisher Scientific Inc., Rockford, USA). The samples were subsequently shaken for 10 s with 4 mL of added grade 1 water. The extract, as well as 1 mL of water used to rinse the sample tube, was passed through a Bond Elut SAX column (500 mg, 3 mL) (Agilent, Santa Clara, USA) preconditioned with 2.5 mL MeOH and 2.5 mL grade 1 water, using light vacuum (water jet). The eluted extract was added to an Oasis HLB solid-phase extraction (SPE) column (200 mg, 6 mL) (Waters, Milford, USA) which was preconditioned with 5 mL MeOH and 5 mL water. The column was washed with 3 mL of SPE buffer (Table S3) and 3 mL of 5% MeOH in water. The sample was eluted with 3 mL MeOH and then evaporated to dryness at 37 °C. The sample was then reconstituted in 1 mL 20% MeOH in water and vortexed and filtered through a 0.2-µm microcentrifuge filter (Spin-X, Costar, Corning Inc. NY, USA) before the samples were transferred to 2-mL glass vials for quantitative analysis using LC–MS/MS. Triple quadrupole (QqQ) dynamic multiple reaction monitoring (dMRM) with electrospray ionization (MRM-ESI) was applied for quantitative analysis (Fig. 1).

Analysis

Compound-specific chromatographic separation and quantitative detection of the cleaned digestate extracts were conducted on an Agilent 1260 ultra-high performance liquid chromatograph (UHPLC; Agilent Technologies, Waldbronn, Germany), and the detection and quantification were done on an Agilent 6490 triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) with an Agilent Jet Stream electrospray ion source, using dynamic multiple reaction monitoring (dMRM). For instrument control and method validation and quantification, the Agilent MassHunter software (V B.07.00/Build 7.0.457.0, 2008) was used. All MRM transitions and details from the MassHunter method, as well as the ion source parameters, are described in Tables S3 and S4 in the Supplementary information section.

Method validation

For the method validation, detection limits, recovery, repeatability, accuracy, matrix effect, and efficiency of the extraction method were calculated. The details are given in the Supplementary information. Digestate are a complex matrix that, if not properly treated in advance, would affect the instrumental sensitivity and selectivity of each target analyte differently. To compensate for these variations, a matrix-matched calibration curve was chosen. The calibration curve was prepared with five concentration levels spanning the ranges given in Table 1, as well as one level with no added native compounds (level 0, see Table S5). Compounds were approved if the recovery rate was 40–115%, if the relative coefficient of variation (CV%) for the repeatability was < 15%, if the accuracy was < ± 15% with a CV% < 15%, and if the determination coefficient (R^2) of the matrix-matched calibration curve was above 0.985 (Table S6).

Simplified risk assessment

For each of the quantified pharmaceuticals, the highest concentration found in any of the biogas digestates (C_{DIG}) was used to calculate a predicted environmental concentration (PEC) in soil immediately after application of biogas digestate (Eq. 1). Application rates of digestate vary, due to, e.g. differences in nutrient and heavy metal content. Reasonable estimates according to the Norwegian Agricultural Extension Service is however 8 tonnes of solid or 45 tonnes of liquid digestates (fresh weight) per hectare (personal communication). Assuming an incorporation to a soil depth of 20 cm, and a dry soil bulk density of 1.3 kg l^{-1} (RHO_{SOIL}), this corresponded to an application rate of 3.1 or 17.3 g fresh digestate kg^{-1} dry soil, respectively.

$$PEC[\mu g \cdot kg^{-1}] = C_{DIG}[ng \cdot g^{-1}] \times Application\ rate[g \cdot kg^{-1}]/1000[ng \cdot \mu g^{-1}] \quad (1)$$

A predicted no-effect concentration (PNEC) for toxicity towards soil organisms was calculated following the standard approach of risk assessments from the European Chemicals Bureau (European Commission 2003): the lowest available EC50 or NOAEL value for soil organisms was divided by an assessment factor of 10–1000, depending on the availability of toxicity data. Tables S11 and S12 summarize the ecotoxicity data used for the PNEC calculations. For prednisolone, no soil toxicity data was available, and the $PNEC_{SOIL}$ was derived by multiplying the $PNEC_{AQUATIC}$ with the soil partitioning coefficient (K_d); see Supplementary information for details. PNEC values for selection of antibiotic-resistant bacteria (ARB) were derived from Menz et al. (2019) by Eq. 2:

$$PNEC_{ARB,SOIL}[\mu g \cdot kg^{-1}] = \frac{PNEC_{ARB,PW}[\mu g \cdot l^{-1}] \times K_d[-] \times f(pw)(v/v)}{RHO_{SOIL}[kg \cdot l^{-1}]} \quad (2)$$

where $PNEC_{ARB,PW}$ is the PNEC value in soil pore water, K_d is the soil–water distribution coefficient, and f(pw) is the fraction of pore water in the soil (0.25).

The risk quotient (RQ) was calculated as

$$RQ = PEC/PNEC \quad (3)$$

where a $RQ < 0.1$ indicates low risk, $RQ = 0.1–1$ indicates medium risk, and $RQ > 1$ indicates high risk.

Results and discussion

Method validation

Out of a total list of 26 selected target contaminants, 16 compounds were found to be valid for quantification (see Table 2). The detection and quantification thresholds for the validated compounds were highly to medium sensitive (in the pg g^{-1} to ng g^{-1} range), and their individual response curves were ranging over three orders of magnitude. The compounds are from six different compound classes, making this a multiclass quantification method appropriate for the screening in biogas digestate. Some of the compounds have lower recoveries than preferred, but since the quantification at ultra-trace level of these target chemicals (pg-range) is associated with an estimated overall method uncertainty between 40 and 50%, recovery rates between 40 and 115% were accepted. The results for all 26 compounds can be found in Table S6.

When the matrix effect is ± 20%, no significant matrix effect is assumed. However, for most of the compounds, there was significant matrix effect confirmed, either signal enhancement (> 20%) or ion suppression (< − 20%). This demonstrates the need for a matrix-matched calibration curve. The efficiency of the extraction method was below 40% and above 115% for some compounds, demonstrating the need of well-defined internal standards. This also illustrates a considerable challenge for multi-compound quantification methods for trace level analysis. The deviating extraction efficiency of the compounds is all acceptable as long as the recovery is within the acceptable range, i.e. 40–115%.

Levels of antibiotics and steroids in biogas digestate

Our survey revealed overall low levels of the target substances in the digestates. From the 16 target analytes, 8 were detected above the method quantification limit (MQL) (Table 3). Four were not detected in any of the samples, namely, ronidazole, enrofloxacin, sarafloxacin, and tiamulin. The antibiotics metronidazole, norfloxacin, difloxacin, and sulfadoxine and the glucocorticoid dexamethasone were detected in at least one biogas digestate, however, each below their respective MQLs. Despite overall low levels, a few findings were of concern. Amoxicillin, penicillin G, ciprofloxacin, and prednisolone were found at levels above 400 µg kg^{-1} dw. Also, ipronidazole was found in trace amounts in several digestates, even though the pharmaceutical is not registered for use in Norway.

β-lactams Amoxicillin and penicillin G were found at 460–960 µg kg^{-1} dw in the food waste digestates E_S and E_L and the manure digestate M, respectively (Table 3). The detection of both antibiotics was surprising, as β-lactams are expected to rapidly degrade during biological processes by, e.g. hydrolysis (Braschi et al., 2013). The hydrolytic half-life of penicillin G is 60 h at 37 °C (Chadha et al., 2003), which is comparable to the temperature of reactor M (i.e. 35 °C). Consequently, β-lactams are rarely reported in manure despite their common use in animal husbandry. Hence, none of the recent reviews on antibiotics in manure by Spielmeyer (2018) and Wohde et al. (2016) reported the presence of either amoxicillin or penicillin G. Furthermore, the detection of amoxicillin in a digestate from food waste only was unexpected. Globally, antibiotics are frequently detected in animal products and vegetables, even as high as 1500–3000 µg kg^{-1} fw (in cultivated fish, China and Turkey, reviewed by Chen et al. (2019)), and He et al. found sulfonamides and fluoroquinolones in concentrations up to 15–20 µg l^{-1} in digested restaurant food waste in China. Such levels are, however, surprising for Norway where antibiotic consumption is generally reported as low, both in animal husbandry, fish farming, and human medicine (NIPH 2019; NORM/NORM-VET 2020). Usually, antibiotics are rarely detected above the maximum residue limits in Norwegian foodstuffs of animal origin (NFSA 2019).

Penicillin G is associated with a high risk quotient both towards soil bacteria (2.2, Table 4) and for development of antibiotic-resistant bacteria (ARB, 14.6), while the evaluation of amoxicillin resulted in lower risk quotients. The difference in risk quotients for ARB selection for amoxicillin and penicillin G is mainly due to the large difference in K_d values used in Eq. 2. These were estimated from their organic carbon partitioning constants (K_{OC}) reported in a review by Cycoń et al. (2019); as for our study, no experimental K_d was available (details in Supplementary information). The K_{OC} used were 865.5 l kg^{-1} and 2.68 l kg^{-1} for amoxicillin and penicillin G, respectively. Consequently, penicillin G is predicted to sorb less to the soil and be as more bioavailable, as reflected in a lower PNEC value for penicillin G than for amoxicillin.

Fluoroquinolones Ciprofloxacin is one of the most examined and detected antibiotics worldwide (e.g. Verlicchi and Zambello 2015) and accounts for approximately 90% of the human consumption of quinolones in Norway

Table 3 Concentration of selected antibiotics and steroid hormones in the digestates [μg kg^{-1} wet weight] (μg kg^{-1} dry weight is given in parenthesis, $n=2$ except for E_L with $n=4$). Operating parameters of the biogas plants A–N can be found in Table S10. Station codes equal those of Ali et al. (2019). Subscripts S and L denote solid and liquid digestates, respectively. The digestates are presented in the order of the substrate of their respective biogas plants. *3/4 replicates were < MQL. **The concentration of ciprofloxacin in digestate D_S was above the top concentration of the calibration curve, i.e. 150 μg $kg.^{--1}$ ww. *** Only one replicate was analysed. *AMX*, amoxicillin; *PENG*, penicillin G; *NOR*, norfloxacin; *CIP*, ciprofloxacin; *DFX*, difloxacin; *SDZ*, sulfadiazine; *SMZ*, sulfamethazine; *SDX*, sulfadoxine; *MET*, metronidazole; *IPRO*, ipronidazole; *PRED*, prednisolone; *DEXA*, dexamethasone. **Substrates: *L*, manure + food waste; *M*, manure; *N*, fish silage and manure (both substrate and digestate were analysed)

Sub	Station	AMX	PENG	NOR	CIP	DFX	SDZ	SMZ	SDX	MET	IPRO	PRED	DEXA
		β-lactams		*Fluoroquinolones*			*Sulfonamides*			*Nitroimidazoles*		*Glucocorticoids*	
Food waste	E_S	121 ± 41(460)	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MQL
	E_L	28 ± 9.7(960)*	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MQL
	G_L	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MQL
	K_S	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MQL
	K_L	< MDL	< MDL	< MDL	< MDL	< MDL	3.6*** (140)	< MDL	< MDL	< MDL	< MDL	< MDL	< MQL
Sewage sludge	D_S	< MDL	< MDL	< MQL	205 ± 22** (430)	< MQL	< MDL	0.039 ± 0.002 (0.08)	< MDL	< MDL	< MDL	< MDL	< MQL
	H_S	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MQL
	J_S	< MDL	< MDL	< MQL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	0.86 ± 0.16 (2.7)	< MDL	< MQL
	I_S	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MQL
Food waste + sewage sludge	F_S	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MQL
	F_L	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	10.4 ± 1.0 (650)	< MQL
	A_S	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	0.77*** (1.6)	< MDL	< MQL
	A_L	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MQL	< MDL	< MQL
	B_S	< MDL	< MDL	< MQL	< MDL	< MDL	< MDL	0.10 ± 0.002 (0.38)	< MDL	< MDL	< MDL	< MDL	< MDL
	L_S	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MQL	< MDL	< MQL	< MDL	< MDL
	L_L	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MQL	< MDL	< MDL	< MQL
Others**	C_L	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	0.079 ± 0.011 (1.6)	< MDL	< MDL	< MDL	< MDL	< MQL
	M_L	< MQL	22 ± 7 (510)	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MQL
	I_{exp} sub	< MDL	< MDL	< MDL	< MDL	< MDL	3.1 ± 0.08	0.40 ± 0.05	< MDL	< MDL	< MDL	< MDL	< MQL
	I_{exp} dig	< MDL	< MDL	< MDL	< MDL	< MDL	3.3 ± 0.24	0.43 ± 0.04	< MDL	< MDL	< MDL	< MDL	< MQL

Table 4 Predicted environmental concentration (PEC), predicted no-effect concentration (PNEC) for soil organisms, and the corresponding risk quotients (RQ). *ARB*, antibiotic-resistant bacteria. PNEC values are based on Table S11, S12, and S15 in the Supplementary information

	PEC_{SOIL} [μg kg^{-1}]	PNEC [μg kg^{-1}] Soil organisms	RQ Soil organisms	PNEC [μg kg^{-1}] ARB selection[2]	RQ ARB selection
Amoxicillin	0.660	0.47	1.4	6.7	0.099
Penicillin G	0.380	0.17	2.2	0.026	14.6
Sulfadiazine	0.062	5.6	0.01	31	0.002
Sulfamethazine	0.0012	10	0.0001		
Ciprofloxacin	0.462	0.5	0.9	32	0.014
Ipronidazole	0.003	NA	NA		
Prednisolone	0.180	0.09[1]	2[1]		

[1]Estimated from a simulated Koc value and from aquatic toxicity data using the partitioning coefficient method

[2]Derived from PNEC values in soil pore water estimated by Menz et al. (2019) (see Supplementary information)

(Sommerschild et al., 2020). Fluoroquinolones have a high affinity for sewage sludge (Lindberg et al. 2005), and ciprofloxacin was detected in all examined sewage sludges in Norway and Sweden in concentrations ranging from 70 to 770 μg kg^{-1} dw (TemaNord, 2012). Further, fluoroquinolones are persistent towards hydrolysis and high temperatures (Thiele-Bruhn 2003), and their degradation during anaerobic digestion is limited (Golet et al. 2003; Lindberg et al. 2006; Zhang and Li 2018). In fact, the concentration may even increase during thermal hydrolysis, probably due to release of intracellular antibiotics (Zhang and Li 2018). Ciprofloxacin can therefore be expected to be found in sewage sludge digestate, and the amount of 430 μg kg^{-1} dw found in digestate D_S in our study corresponds well with the range detected in sewage sludge by TemaNord (2012). A positive result was thus the lack of ciprofloxacin in 7 out of 8 biogas plants receiving sewage sludge, perhaps explained partly also by a decline in ciprofloxacin prescriptions of approximately 50% since 2012 as a strategy to prevent ciprofloxacin resistance (Sakshaug et al. 2017; Sommerschild et al. 2020).

In soils, fluoroquinolones are strongly sorbed to clay particles and organic matter, limiting their bioavailability and thus reducing their impact on soil biota and processes such as nitrogen transformation (Rosendahl et al., 2012). On the other side, the low availability combined with low hydrolysis and thermal and biological degradation (Al-Ahmad et al. 1999; Alexy et al. 2004; Thiele-Bruhn 2003) leads to their persistence in soil. Thus, they can accumulate when repeatedly added, as shown by Dalkmann et al. (2012), who found accumulation of ciprofloxacin in soils irrigated with wastewater. Further, Girardi et al. (2011) found that ciprofloxacin could inhibit soil respiration despite the formation of non-extractable residues. The risk quotient for ciprofloxacin was close to 1, indicating a moderate risk towards soil organisms.

It should be noted that ciprofloxacin was measured at a concentration more than twice as high as the upper boundary of the calibration curve. As the measured concentration of 430 μg kg^{-1} fw is uncertain, the upper boundary of 205 μg kg^{-1} fw was used for PEC_{SOIL} calculations. Thus, the risk quotient is probably underestimated. Considering its effect on soil organisms, persistence in soil, and the frequent detection in other studies, further investigations on the levels of ciprofloxacin in biogas digestates are needed.

Sulfonamides Sulfonamides, represented by sulfadiazine and sulfamethazine, were found in several digestates. The concentrations were low, leading to low risk quotients. Neither sulfadiazine nor sulfamethazine was found in sewage sludge in the Nordic countries in 2012 (TemaNord 2012), probably because of their high hydrophobicity and low solid–liquid partition coefficients (K_d) and because their negative charge at high pH hinders electrostatic sorption to the negatively charged surfaces of sludge (Göbel et al. 2005; Zhang and Li 2018). Sulfonamides are also used in veterinary medicine, but the detection frequency in German digestates from swine manure has been low (Spielmeyer et al. 2014).

Nitroimidazoles Ipronidazole was found at trace levels in four digestates from three different plants. Its presence is nevertheless surprising and concerning, as there are no registered pharmaceuticals in Norway for humans or animals containing ipronidazole (Østensen, H., personal communication, 16.07.2021). The digestates were based on sewage sludge alone (J_S) or in combination with food waste (A_S, A_L, and B_S) suggesting that human use is the origin of the pharmaceutical. For ipronidazole, there was no toxicological data available, and the risk quotient was not calculated.

Steroids The glucocorticoid hormone prednisolone was found in one digestate sample only, while dexamethasone was found in trace amounts in almost all digestates. Presently, no toxicological information on prednisolone

is reported for soil organisms, but studies on aquatic organisms have confirmed endocrine disrupting effects (Bal et al. 2017). Based on available toxicity data and K_d calculated from a modelled K_{OC} (see details in Supplementary information), a $PNEC_{SOIL}$ of 0.09 μg kg^{-1} was calculated for prednisolone, yielding a risk quotient of 2. This number is only indicative but confirms the need of conducting in-depth toxicity tests for glucocorticoids and steroids on soil organisms as well as aquatic organisms. Dexamethasone has received increased attention as the World Health Organization is recommending dexamethasone to treat severe to critical COVID-19 cases (WHO 2020). This synthetic steroid is more effective compared to natural steroids but also more persistent in the environment due to the fluorine moiety in the molecule. As dexamethasone only was found in trace amounts, it was not done a risk evaluation of this compound.

Concluding remarks

Food waste is an understudied matrix with regard to pharmaceutical pollution, as manure and sewage sludge are assumed to be more important entry routes to the soil. However, our results indicate that food waste can be an important entry route to the environment as well. Two other studies on pollutants in digestate from a variety of feedstocks (including food waste) also failed to identify a clear relationship between biogas feedstock and the level of pharmaceuticals and personal care products (PPCP) (Ali et al. 2019, using the same sample set as here) and POPs (Suominen et al. 2014).

As confirmed by our results, significant levels of pharmaceutical residues were detected in Norwegian biogas digestates despite well-established retainment technologies and national regulations for pharmaceuticals in veterinary and human medicine. Hence, it is likely that pharmaceutical residues are common in many biogas digestates in other countries as well. If untreated, the presence of antibiotics in biogas digestates may lead to increased antibiotic resistance, harm towards soil organisms, leaching to water bodies, and potential exposure of human consumers, when digestates are applied on agricultural land.

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Author contribution Astrid S. Nesse collected digestate, prepared the original draft, performed data analysis, prepared the graphical abstract, and edited the Supplementary information. Stine G. Aanrud developed and adjusted the method for analysis of antibiotics and hormones in biogas digestate, wrote the original draft for part S1 to S8 in the Supplementary information, and edited the manuscript. Jan L. Lyche advised on how to evaluate the ecotoxicity data and edited the manuscript. Trine A. Sogn contributed to the study conception and design and edited the manuscript. Roland Kallenborn was project leader, quality assured the method for analysis of antibiotics and hormones, and edited the manuscript. All authors read and approved the final manuscript.

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Data availability All the research data used in the study are available in Table 1.

Declarations

Ethics approval The research did not involve human or animal participants, and there was no release of harmful substance to the environment as a result of the study. The authors followed the rules for good scientific practice, as described in the author guidelines.

Consent to participate Not applicable, as there were no human participants in the study.

Consent for publication All authors gave their explicit consent to publish the manuscript before it was uploaded to ESPR.

Conflict of interest The authors declare no competing interests.

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Supplementary information

Confirming the presence of selected antibiotics and steroids in Norwegian biogas digestate

Astrid S. Nesse*[1], Stine G. Aanrud[2], Jan L. Lyche[2], Trine A. Sogn[1], Roland Kallenborn[3]

[1]Norwegian University of Life Sciences, Faculty of Environmental Sciences and Natural Resource Management

[2]Norwegian University of Life Sciences, Faculty of Veterinary Medicine

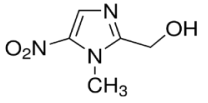
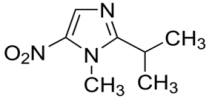
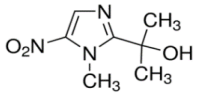
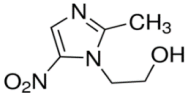
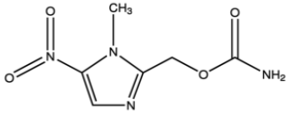
[3]Norwegian University of Life Sciences, Faculty of Chemistry, Biotechnology and Food Science

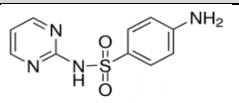
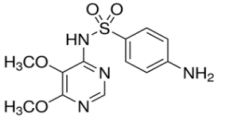
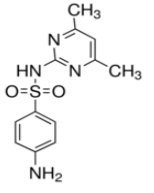
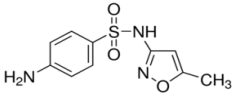
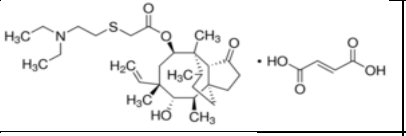
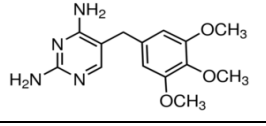
* email: astrid.solvag.nesse@nmbu.no

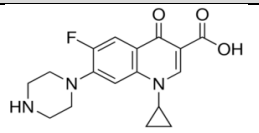
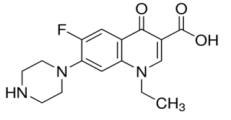
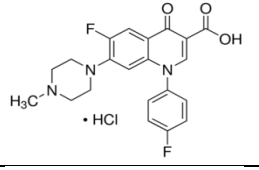
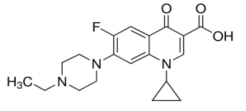
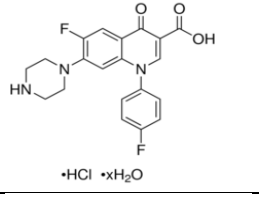
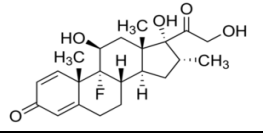
Overview of contents

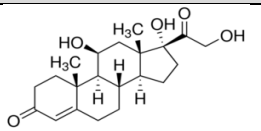
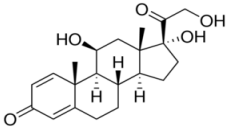
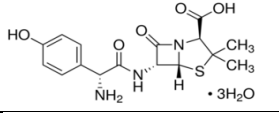
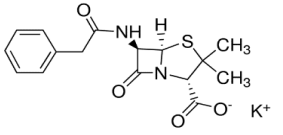
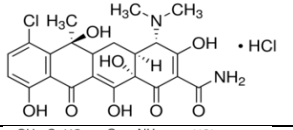
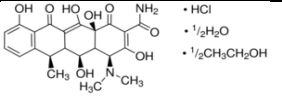
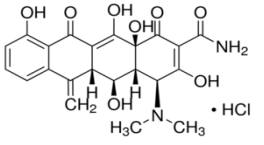
S1. Target substances and internal standards

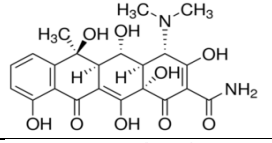
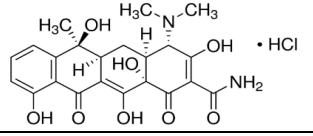
Table S1. Chemical structure and providing company, IUPAC name, and CAS-nr. for all tested compounds.

Compound (Abbreviation)	Molecular formula	Structure	CAS number	Description	Supplier
HMMNI (HMMNI)	$C_5H_7N_3O_3$		936-05-0	Nitroimidazole, metabolite	Sigma Aldrich, Oslo, Norway
Ipronidazole (IPRO)	$C_7H_{11}N_3O_2$		14885-29-1	Nitroimidazole	Sigma Aldrich, Oslo, Norway
Ipronidazole-OH (IPOH)	$C_7H_{11}N_3O_3$		35175-14-5	Nitroimidazole	Sigma Aldrich, Oslo, Norway
Metronidazole (MET)	$C_6H_9N_3O_3$		443-48-1	Nitroimidazole	Sigma Aldrich, Oslo, Norway
Ronidazole (RDZ)	$C_6H_8N_4O_4$		7681-76-7	Nitroimidazole	Sigma Aldrich, Oslo, Norway

Compound (Abbreviation)	Molecular formula	Structure	CAS number	Description	Supplier
Sulfadiazine (SDZ)	$C_{10}H_{10}N_4O_2S$		68-35-9	Sulfonamide	Sigma Aldrich, Oslo, Norway
Sulfadoxine (SDX)	$C_{12}H_{14}N_4O_4S$		2447-57-6	Sulfonamide	Sigma Aldrich, Oslo, Norway
Sulfamethazine (SMZ)	$C_{12}H_{14}N_4O_2S$		57-68-1	Sulfonamide	Sigma Aldrich, Oslo, Norway
Sulfamethoxazole (SXZ)	$C_{10}H_{11}N_3O_3S$		723-46-6	Sulfonamide	Sigma Aldrich, Oslo, Norway
Tiamulin fumarate (TMN)	$C_{32}H_{51}NO_8S$		55297-96-6	Macrolide	Sigma Aldrich, Oslo, Norway
Trimethoprim (TMP)	$C_{14}H_{18}N_4O_3$		738-70-5	Pyrimidine	Sigma Aldrich, Oslo, Norway

Compound (Abbreviation)	Molecular formula	Structure	CAS number	Description	Supplier
Ciprofloxacin (CIP)	$C_{17}H_{18}FN_3O_3$		85721-33-1	Fluoroquinolone	Sigma Aldrich, Oslo, Norway
Norfloxacin (NOR)	$C_{16}H_{18}FN_3O_3$		70458-96-7	Fluoroquinoloe	Sigma Aldrich, Oslo, Norway
Difloxacin HCl (DFX)	$C_{21}H_{19}F_2N_3O_3$ *HCl		91296-86-5	Fluoroquinolone	Sigma Aldrich, Oslo, Norway
Enrofloxacin (ENR)	$C_{19}H_{22}FN_3O_3$		93106-60-6	Fluoroquinolone	Sigma Aldrich, Oslo, Norway
Sarafloxacin HCL xH_2O (SFX)	$C_{20}H_{17}F_2N_3O_3$ *HCl *H_2O		91296-87-6 (anhydrous)	Fluoroquinolone	Sigma Aldrich, Oslo, Norway
Dexamethazone (DEXA)	$C_{22}H_{29}FO_5$		50-02-2	Corticosteroide	Sigma Aldrich, Oslo, Norway

Compound (Abbreviation)	Molecular formula	Structure	CAS number	Description	Supplier
Hydrocortisone (HYCO)	$C_{21}H_{30}O_5$		50-23-7	Corticosteroide	Sigma Aldrich, Oslo, Norway
Prednisolone (PRED)	$C_{21}H_{28}O_5$		50-24-8	Corticosteroide	Sigma Aldrich, Oslo, Norway
Amoxicillin trihydrat (AMX)	$C_{16}H_{19}N_3O_5S$ *$3H_2O$		61336-70-7	β-lactam	Sigma Aldrich, Oslo Norway
Penicillin-G Potassium salt (PENG)	$C_{16}H_{17}KN_2O_4S$		113-98-4	β-lactam	Sigma Aldrich, Oslo, Norway
Chlortetracycline HCl (CTC)	$C_{22}H_{23}ClN_2O_8$ *HCl		64-72-2	Tetracycline	Sigma Aldrich, Oslo, Norway
Doxycyline hyclate (DC)	$C_{22}H_{24}N_2O_8$ *HCl * 0,5 H_2O * 0,5 C_2H_6O		24390-14-5	Tetracycline	Sigma Aldrich, Oslo, Norway
Methacycline HCl (MC)	$C_{22}H_{22}N_2O_8$ *HCl		3963-95-9	Tetracycline	Sigma Aldrich, Oslo, Norway

Compound (Abbreviation)	Molecular formula	Structure	CAS number	Description	Supplier
Oxytetracycline (OTC)	$C_{22}H_{24}N_2O_9$		79-57-2	Tetracycline	Sigma Aldrich, Oslo, Norway
Tetracycline HCl (TC)	$C_{22}H_{24}N_2O_8$ *HCl		64-75-5	Tetracycline	Sigma Aldrich, Oslo, Norway

The internal standards HMMNI-D_3, norfloxacin-D_5, enrofloxacin-D_5 HCl, difloxacin-D_3 HCl $3xH_2O$, cortisol-D_4, trimethoprim -D_9, sulfadiazine phenyl-$^{13}C_6$, ronidazole-D_3, metronidazole $^{13}C_2$-$^{15}N_2$, and ipronidazole-D_3 were bought from Sigma Aldrich, Oslo, Norway. The internal standards tetracycline-D_6 (80%), benzyl-penicillin-D_7 potassium salt, amoxicillin $^{13}C_6$, prednisolone-D_8 (major), and tiamulin-$^{13}C_4$ fumarate were bought from Toronto Research Chemicals, Toronto Canada.

S2. Solutions prepared for extraction

Three solutions were prepared for the extraction of target compounds from the digestate. The ratios in the recipes are given on a volume basis. For an overview of X-, Y-, and Z-compounds, see Table S2. In addition, the ISTD stock solutions in Table S3 were prepared. The STD stock solutions in Table S3 were prepared for the calibration curves.

Extraction solution	1% formic acid in 50:50 ACN:MeOH
McIlvaine buffer	82% 0.2M Na_2HPO_4 and 18% 0.1M citric acid (pH 7)
SPE-buffer	1% 0.1M EDTA, 1% McIlvaine buffer, 2% MeOH, and 0.05% H_3PO_4 in grade 1 water.

Table S2. Stock solutions for the preparation of the calibration curves. STD stock A2 have ten times higher concentration of X- and Z-type compounds compared to STD stock A1, to cover the concentration span of the calibration curve.

STD stock A1 and STD stock A2		**STD stock B**
A1: 10 ng mL^{-1} / A2: 100 ng mL^{-1}	*A1: 100 ng mL^{-1} / A2: 1000 ng mL^{-1}*	*1000 ng mL^{-1}*
HMMNI	Ciprofloxacin	Amoxicillin
Ipronidazole	Sarafloxacin	Penicillin G
Ipronidazole-OH	Difloxacin	Doxycycline
Metronidazole	Enrofloxacin	Methacycline
Ronidazole	Norfloxacin	Oxytetracycline
Sulfadiazine	Dexamethasone	Chlortetracycline
Sulfadoxine	Prednisolone	Tetracycline
Sulfamethazine	Hydrocortisone	
Sulfamethoxazole		
Tiamulin		
Trimethoprim		
ISTD stock A		**ISTD stock B**
100 ng mL^{-1}	*1 µg mL^{-1}*	*1 µg mL^{-1}*
HMMNI-D_3	Cortisol-D_4	Amoxicillin-$^{13}C_6$
Ipronidazole-D_3	Difloxacin-D_3	Penicillin G-D_7
Metronidazole-$^{13}C_2$-$^{15}N_2$	Enrofloxacin-D_5	Tetracycline-D_6
Ronidazole-D_3	Norfloxacin-D_5	
Sulfadiazine-$^{13}C_6$	Prednisolone-D_8	
Tiamulin-$^{13}C_4$		
Trimethoprim-D_9		

S3. Ultra High-Performance Liquid Chromatograph / Triple Quadrupole mass spectrometry based quantification

Table S3. Ion source parameters

Parameter	Value	Unit
Gas Temp:	180	°C
Gas Flow	19	l/min
Nebulizer	35	psi
Sheath Gas Temp	350	°C
Sheath Gas flow	12	l/min
Capillary	3000	V
Nozzle Voltage	0	V
High Pressure RF	150	V
Low Pressure RF	70	V

Table S4. Chosen MS-parameters and multiple reaction monitoring transitions (MRMs) tested for validation, with compound type. Retention time (RT) and delta retention time (ΔRT) are given in minutes. Validated quantifier transitions are marked bold with grey background. Validated qualifier transitions are marked in bold. For internal standards, the monitoring transition validated for the analytes' quantifier transition are marked with grey background. Collision energy (CE) are given in Volt. Cell Accelerator voltage (CAV) is the voltage gradient in the collision cell.

Type	Compound Name	RT (min)	ΔRT	Precursor Ion	Product Ion	CE	CAV
X	HMMNI	1.9	1	158.0	139.8	10	2
				158.0	55.2	20	3
X	HMMNI-D_3 (ISTD)	1.9	1	161.0	143.0	10	2
				161.0	58.0	19	2
X	Ipronidazole	6.2	1	**170.0**	**108.9**	26	5
				170.0	**124.1**	20	3
X	Ipronidazole-D_3 (ISTD)	6.2	1	173.0	112.0	29	2
				173.0	127.0	18	2
X	Ipronidazole-OH	5.0	1	186.0	121.0	30	3
				186.0	168.0	10	7
X	Metronidazole	2.1	1	**172.0**	**82.0**	26	7
				172.0	**128.0**	12	4
X	Metronidazole-$^{13}C_2$-$^{15}N_2$ (ISTD)	2.1	1	176.0	86.0	27	3
				176.0	132.0	13	3
X	Ronidazole	2.1	1	**201.0**	**55.1**	30	3
				201.0	**139.9**	10	2
X	Ronidazole-D_3 (ISTD)	2.1	1	204.0	58.0	21	2
				204.0	113.0	15	2
				204.0	143.0	7	2
X	Sulfadiazine	2.0	1	**251.0**	**91.8**	23	7
				251.0	107.6	22	5
				251.0	**156.0**	11	2
X	Sulfadiazine-$^{13}C_6$ (ISTD)	2.0	1	257.0	98.0	27	2
				257.0	114.0	25	2
				257.0	162.0	14	3
X	Sulfadoxine	5.5	1	**311.0**	**107.7**	26	5
				311.0	**156.0**	10	8
X	Sulfamethazine	3.9	1	279.1	91.9	32	6
				279.1	**123.9**	23	3
				279.1	**185.9**	15	6
X	Sulfamethoxazole	5.1	1	254.0	92.0	27	4
				254.0	108.0	22	4
				254.0	156.0	11	2
X	Tiamulin	9.0	1	**494.0**	**118.8**	41	4

Type	Compound Name	RT (min)	ΔRT	Precursor Ion	Product Ion	CE	CAV
				494.0	**192.0**	20	5
X	Tiamulin-$^{13}C_4$ (ISTD)	9.0	1	498.0	119.0	45	4
				498.0	196.0	20	4
X	Trimethoprim	3.5	1	291.1	122.9	35	4
				291.1	230.0	21	3
				291.1	260.9	23	2
X	Trimethoprim-D_9 (ISTD)	3.5	1	300.0	123.0	24	2
				300.0	234.0	25	2
				300.0	264.0	28	2
Y	Ciprofloxacin	5.0	1.5	**332.1**	**230.9**	38	4
				332.1	**287.9**	16	7
				332.1	314.0	19	6
Y	Cortisol-D_4 (ISTD)	9.4	1	367	121.0	26	4
				367	327.2	16	2
				367	349.0	14	2
Y	Dexamethasone	9.7	1	**393.1**	**146.9**	33	8
				393.1	**373.0**	3	4
Y	Difloxacin	5.6	1	**400.0**	**298.9**	28	7
				400.0	**355.9**	18	5
				400.0	381.9	28	4
Y	Difloxacin-D_3 (ISTD)	5.6	1	403.0	299.0	34	2
				403.0	359.0	20	2
				403.0	385.0	23	4
Y	Enrofloxacin	5.2	1	**360.0**	**245.0**	25	3
				360.0	315.8	18	6
				360.0	**341.9**	19	5
Y	Enrofloxacin-D_5 (ISTD)	5.2	1	365.0	245.0	30	2
				365.0	321.0	21	2
				365.0	347.0	23	3
Y	Hydrocortisone	9.4	1	363.0	97.0	26	5
				363.0	121.0	20	3
				363.0	327.2	13	5
Y	Norfloxacin	4.8	1.5	**320.0**	**189.0**	53	6
				320.0	**230.7**	48	3
				320.0	301.9	25	6
Y	Norfloxacin-D_5 (ISTD)	4.8	1.5	325.0	238.0	28	2
				325.0	281.0	18	2
				325.0	307.0	20	3
Y	Prednisolone	9.3	1	**361.0**	**147.0**	30	2
				361.0	325.0	10	2
				361.0	**343.0**	5	3
Y		9.3	1	369.0	151.0	35	3

Type	Compound Name	RT (min)	ΔRT	Precursor Ion	Product Ion	CE	CAV
	Prednisolone-D_8 (ISTD)			369.0	332.0	8	3
				369.0	351.0	7	2
Y	Sarafloxacin	6.0	1	**386.0**	**342.0**	17	5
				386.0	**367.7**	20	4
Z	Amoxicillin	1.6	1	366.0	113.9	22	3
				366.0	**134.0**	29	3
				366.0	208.0	8	4
Z	Amoxicillin-$^{13}C_6$ (ISTD)	1.6	1	372.0	114.0	20	4
				372.0	213.0	16	2
				372.0	355.0	6	2
Z	Chlortetracycline	7.2	2	479.0	154.0	30	2
				479.0	444.0	20	2
				479.0	462.0	15	2
Z	Doxycycline	8.4	2	445.0	98.0	46	6
				445.0	321.0	33	6
				445.0	428.0	17	2
Z	Methacycline	8.1	2	443.0	200.7	32	6
				443.0	425.8	16	3
Z	Oxytetracycline	4.8	2	461.0	200.6	33	6
				461.0	425.8	18	3
				461.0	443.3	12	2
Z	Pencillin G	9.0	1	**335.0**	**114.0**	27	6
				335.0	**160.0**	10	7
				335.0	176.0	12	8
Z	Penicillin G-D_7 (ISTD)	9.0	1	342.0	114.0	35	3
				342.0	160.0	10	3
				342.0	183.0	11	2
Z	Tetracycline	4.6	2	445.0	153.9	28	2
				445.0	409.9	17	3
				445.0	427.0	10	5
Z	Tetracycline-D_6 (ISTD)	4.6	2	451.0	160.0	30	4
				451.0	416.0	18	3
				451.0	433.0	12	2

S4. Quality control

Detection limits

The method detection limit (MDL) and method quantification limit (MQL) were determined by preparing and injecting three spiked blank matrix samples with varying concentration below the lowest concentration in the calibration curve (i.e., below level 1, Table 3), measuring signal and noise, and determining when signal to noise (S/N) was above 3 for MDL or above 10 for MQL.

Matrix matched calibration curve

) The concentration levels of the matrix matched calibration curve are found in Table S6. To make the matrix matched calibration curve, STDs and ISTDs were added to digestate samples prior to the sample preparation procedure (Figure S1), with two replicates of each level. Four additional replicates were made at level 3, to have six replicates for the calculations of recovery, repeatability, accuracy, and efficiency of extraction method.

In addition, a blank matrix sample (n=1) and a method blank (n=1) was prepared. The blank matrix sample was prepared by extracting digestate with no added native standards (STDs) or internal standards (ISTDs). The purpose was to examine whether any matrix components would give artificial peaks, especially for the ISTDs. Method blank was prepared by adding ISTDs to 2 mL grade 1 water prior to sample preparation. The method blank was used to control whether the sample preparation procedure would produce any peak for the analytes.

Table S5. Concentration levels of target compounds (STD) and isotope labelled internal standards (ISTD) in the matrix matched calibration curve. The ISTD were added in equal concentration to all six levels.

	STD (ng mL^{-1})						ISTD (ng mL^{-1})
Level	**0**	**1**	**2**	**3**	**4**	**5**	
X-compounds	0	0.25	2.5	7.5	15	30	7.5
Y-compounds	0	2.5	25	75	150	300	75
Z-compounds	0	10	50	150	300	600	150

As the calibration curve spans over several order of magnitudes in concentration, five solutions of STDs and ISTDs containing X+Y or Z compounds (Table S2) in different concentrations were used to prepare the matrix matched calibration curve. The Z-compounds are kept in separate solutions, as they need to be prepared fresh before every analysis day due to their short shelf life.

Recovery, repeatability, and accuracy

Recovery was calculated according to equation 1, where *signal (I)STD* refers to the actual signal level and *conc* refers to the expected signal level (i.e., level 3, Table S6). *Matrix* refers to the six replicates at level 3 in the matrix matched calibration curve, *blank* refers to the blank matrix sample (n = 1). The *solvent* sample (n = 1) was prepared by adding STDs corresponding to level 3 (Table S6) to 20% MeOH. The standard deviation for the recovery are reported as well (Table S7).

$$Recovery\ (\%) = \sum_{i=1}^{n=6} \frac{\left(\frac{signal\ STD\ (matrix)_i}{signal\ ISTD(matrix)_i} - \frac{signal\ (blank)}{signal\ ISTD(blank)}\right) * conc(solvent)}{\frac{signal\ STD\ (solvent)}{signal\ ISTD(solvent)} * conc(sample)} * \frac{100}{n} \qquad \text{(eq. 1)}$$

Repeatability is reported as the relative coefficient of variation (CV%) of the six matrix samples at level 3 according to equation 2 and 3, where x is the signal of the six matrix matched samples at level 3 and N is six.

$$\sigma = \sqrt{\frac{1}{N-1} \sum_{i=1}^{N} (x_i - \bar{x})^2} \qquad \text{(eq. 2)}$$

$$CV\% = \frac{\sigma}{\bar{x}} * 100\% \qquad \text{(eq. 3)}$$

Accuracy was calculated as the average difference between calculated concentration and expected concentration for each of the six level 3 samples and are reported in % of expected concentration (equation 4). The relative coefficient of variation (CV%) was calculated and are reported in the results as well (Table S7).

$$Accuracy\ (\%) = \sum_{i=1}^{n=6} signal\ STD(matrix)_i - conc(sample) * 100\% \,/\, n \qquad \text{(eq. 4)}$$

The calculations for recovery, repeatability, and accuracy were done for each monitored transition for all target analytes.

S5. Result validation

The initially chose target analytes were validated if they passed four criteria: (1) Recovery rate in the range 40 to 115%, (2) The relative coefficient of variation (CV%) for the repeatability was below 15%, (3) The accuracy was below 15% with a CV < 15%, and (4) The determination coefficient (R^2) of the matrix matched calibration curve was above 0.985.

Several target substances did not meet the four quality criteria thresholds. All compounds marked in grey (Table S7) were rejected for further validation. This includes HMMNI (1-methyl-5-nitro-1H-imidazol-2-yl-methanol), for which no validated qualifier MRM-transition was found in the digestate, as well as hydrocortisone, which had a significant matrix-associated contamination in combination with low recovery rates during the matrix spiking experiment.

The quantification frame defined by linearity range, method detection limit (MDL), and method quantification limit (MQL) for the individual analytes is summarized in Table 5, where an overview over all approved compounds are given. In general, highly sensitive thresholds were achieved (pg g^{-1} to ng g^{-1} range) and individual linear response ranges over three orders of magnitudes are reached.

Table S6. Validation results with recovery, method repeatability, and method accuracy at level 3 (7.5 ng mL^{-1} for X-compounds, 75 ng mL^{-1} for Y-compounds and 150 ng mL^{-1} for Z-compounds, see Table 3), as well as the determination coefficient (R^2) for the calibration curve. Targets marked with grey did not meet the quality control criteria. *Tetracycline, with an accepted linear range from 150 – 300 ng/mL was not approved as validated.

Type	Compound	Recovery (mean ± sd)	Repeatability (CV%)	Accuracy (mean ± CV%)	R^2
Acceptable range		40-115%	<15%	±15 ±15%	>0.985
X	2-Hydroxymethyl-1-methyl-5-nitro-1*H*-imidazole (HMMNI)	135±15	18	7.1±1	0.994
X	Ipronidazole	78±3	3	-2.8±2	0.992
X	Ipronidazole-OH	214±7	7	4.7±7	0.996
X	Metronidazole	103±3	3	4.2±4	0.998
X	Ronidazole	77±5	5	-5.0±5	0.994
X	Sulfadiazine	96±3	3	2.1±3	0.997
X	Sulfadoxine	74±7	7	-2.1±7	0.993
X	Sulfamethazine	111±4	4	-12±4	0.995
X	Sulfamethoxazole	40±10	10	38±14	0.924
X	Tiamulin	81±6	6	-1.4±6	0.994
X	Trimethoprim	105±17	17	27±22	0.950
Y	Ciprofloxacin	43±14	14	-11±13	0.990
Y	Dexamethasone	93±3	4	3.9±3	0.995
Y	Difloxacin	111±3	3	8.2±4	0.996
Y	Enrofloxacin	101±3	3	3.6±3	0.995
Y	Hydrocortisone	26±10	9	5.4±10	0.988
Y	Norfloxacin	113±4	4	-2.6±4	0.993
Y	Prednisolone	95±4	4	0.73±4	0.991
Y	Sarafloxacin	68±8	8	-3.9±8	0.994
Z	Amoxicillin	58±4	4	5.8±4	0.995
Z	Penicillin G	43±13	13	-0.003±13	0.991
Z	Chlortetracycline	42±82	70	-42±41	0.965
Z	Doxycycline	71±18	18	19±21	0.976
Z	Methacycline	180±28	28	22±35	0.952
Z	Oxytetracycline	153±15	15	0.23±15	0.991
Z	Tetracycline*	105±12	12	10±13	0.994

For this method, the individual isotope labelled internal standards were chosen based on structural similarities (Table 5). Whenever possible an identical isotope labelled standard was chosen for target quantification. All the corticosteroids were tested both with prednisolone-D_8 and cortisol-D_4. For hydrocortisone, cortisol-D_4 was chosen as internal standard. Both trimethoprim and sulfamethoxazole were analysed with sulfadiazine-$^{13}C_6$ as internal standard.

S6. Matrix effect of the validated analytes

For the sixteen accepted compounds, matrix effects (ME%) were tested by spiking known concentrations of target compounds in uncontaminated biogas digestate. Five replicates of both blank matrix (i.e., extracted digestate with no added STDs or ISTDs) and 20% MeOH (solvent) were prepared. Each of the samples were spiked with STDs corresponding to level 3 in the matrix matched calibration curve to make five replicates of matrix matched samples (MM_S, equation 4) and solvent matched samples (MS_S), respectively. In addition, non-spiked samples of both blank matrix and solvent were prepared (MM_0 and MS_0, n = 1). After analysis, the arithmetic means of the measurements were inserted in equation 4 to calculate the matrix effect.

$$ME\ (\%) = \left[\frac{(\overline{MM_S} - MM_0)}{(\overline{MS_S} - MS_0)} - 1\right] * 100 \qquad \text{(eq. 5)}$$

Efficiency of the extraction method (EEM) was calculated in the same manner as recovery (equation 5), except that the signal of the ISTDs were not included.

$$EEM\ (\%) = \sum_{i=1}^{n=6} \frac{(signal\ (matrix)_i - signal(blank)) * conc(solvent)}{signal(solvent) * conc(matrix)} * \frac{100}{n} \qquad \text{(eq. 6)}$$

Table S9 shows the matrix effect of the validated compounds. When the matrix effect is positive, it indicates that the compound experiences a signal enhancement due to the digestate matrix, while a negative value indicates ion suppression. Some small amount of matrix effect is expected, and within a range of ±20% it is acceptable to omit matrix effect compensating measures. As can be seen from Table S9, the matrix effect was considerable for most of the validated compounds. Therefore, a matrix matched

calibration curve was applied for the quantification of the individual target substance to compensate for matrix associated responses on the quantification signal in the LC/MS-MS quantification method.

The efficiency of the extraction method (EEM) was calculated by comparing the signal in spiked matrix samples (spiked before sample work up) with the signal in spiked solvent (no sample work up). The EEM expresses the combined effect of the matrix effect and the extraction efficiency. For the validated compounds, EEM varies from 9 to 192%. This demonstrates some of the difficulty in validating multi-compound methods and highlight the need to include a matrix matched calibration curve where the analytes are added before sample preparation.

S7. Chromatography

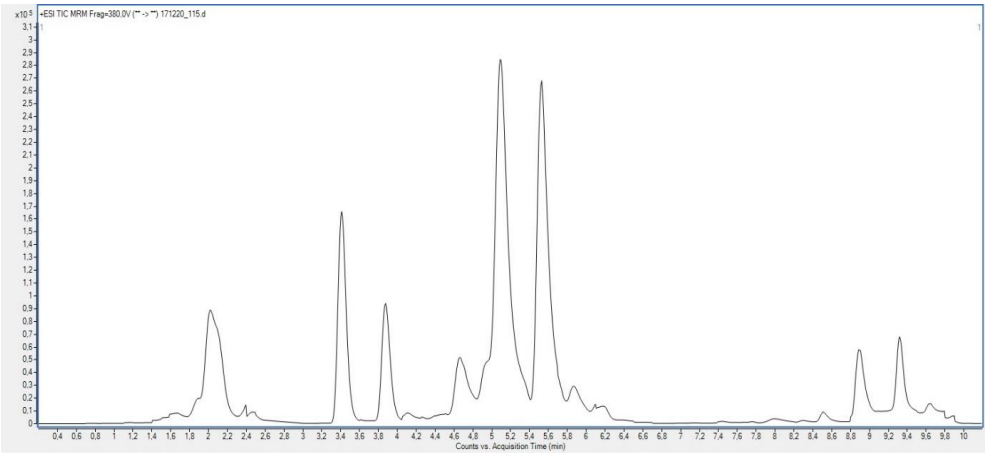


Figure S1. Total ion chromatogram of 15 ng mL^{-1} for X-compounds, 150 ng mL^{-1} for Y-compounds and 300 ng mL^{-1} for Z-compounds

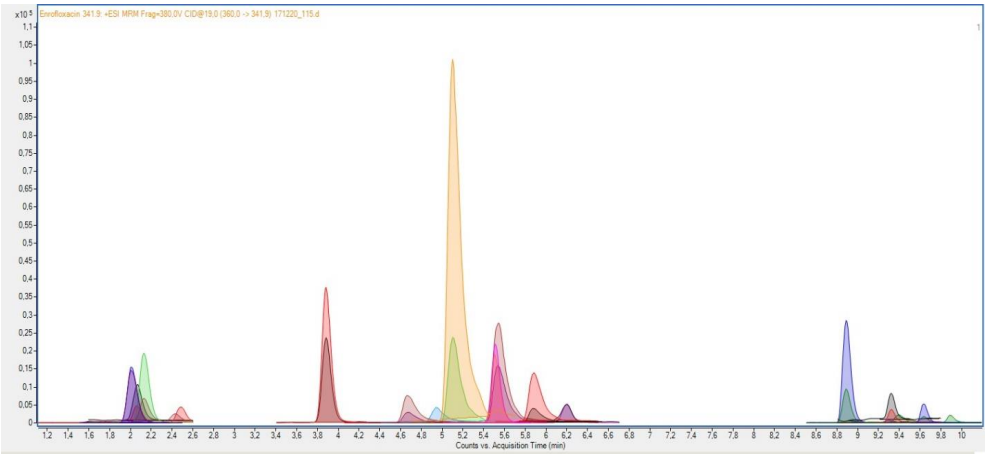


Figure S2. MRM-transitions for all successfully validated compounds at 15 ng mL^{-1} for X-compounds, 150 ng mL^{-1} for Y-compounds and 300 ng mL^{-1} for Z-compounds. The quantitative and qualitative MRM transition chosen in the validation is shown.

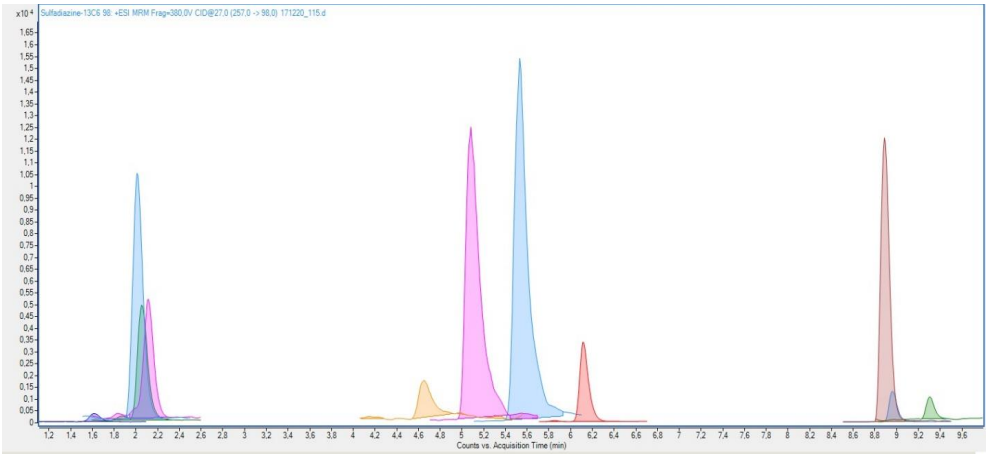


Figure S3. MRM-transitions for all successfully validated internal standards at 7.5 ng mL^{-1} for X-compounds, 75 ng mL^{-1} for Y-compounds and 150 ng mL^{-1} for Z-compounds. The transition chosen to calculate concentrations for each internal standard is shown.

S8 Operating conditions at the biogas plants

Table S7. Operating conditions at the 14 biogas plants. Reactor T: Temperature in the biogas reactor. Retention time: Retention time in the biogas reactor. THP: Thermal hydrolysis, i.e. the slurry is treated at 160°C at 3.5 bar prior to anaerobic digestion. The labelling of the biogas plants follow that of Ali et al. (2019), but are here ordered after substrate.

Plant	Solid / Liquid	Substrate	Reactor T (°C)	Retention time (days)	Thermal Pre treatment	% dry matter
E	L + S	Food waste	39-41	35	THP	3.0 / 26.6
G	L	Food waste	39-40	20	THP	3.5
K	L + S	Food waste	52-53	15-20	No	2.5 / 34.8
D	S	Sewage sludge	37	20	No	47.4
H	S	Sewage sludge	54	12-15	70°C	26.1
J	S	Sewage sludge	40	20-25	THP	33.1
I	S	Sewage sludge	55	14	No	31.6
F	L + S	45% food waste, 55% sewage sludge	62	20	70°C	1.6 / 21.2
A	L + S	45% food waste, 53% sewage sludge and 2% fish silage	40	12-16	THP	1 / 27
B	S	85% sewage sludge, 15% food waste	38	40	THP	26.2
L	S + L	60% sewage sludge, 12% septic, 28% food waste and fats	40	20	THP	4.8 / 38.9
C	L	27% manure (swine and cattle), 72% food waste	38-40	30-35	70°C	4.8
M	L	Slurry from organic milk cows	35	30	No	4.3
I_{exp}	Substrate and digestate	20% sludge from young fish, 80% manure	40	20	No	

S9 Ecotoxicity tests and calculation of predicted no effect concentration (PNEC) values

Table S8. Inhibition of soil organisms by antibiotics. NOAEL = No observed adverse effect level. EC50 = Effect concentration 50, i.e. the measured endpoint is reduced by 50% compared to the control. IC50 = Inhibiting concentration 50. ED50 = Effective dose 50. No soil toxicity studies were found for ciprofloxacin and prednisolone. Aquatic studies for these compounds can be found in table S4.

Substance	Endpoint	Days /hours		Test organism	Concentration (µg kg^{-1} dw)	AF	PNEC (µg kg^{-1} dw)	Reference
Amoxicillin	Metabolic activity	8 h	EC50	*Bacillus amyloliquefaciens*	466	1000	0.466	Menz et al. (2018)
	Metabolic activity	8 h	EC50	*Pseudomonas putida*	61 500			Menz et al. (2018)
Penicillin G	Metabolic activity	8 h	EC50	*Bacillus amyloliquefaciens*	171	1000	0.171	Menz et al. (2018)
	Metabolic activity	8 h	EC50	*Pseudomonas putida*	279 000			Menz et al. (2018)
Sulfadiazine	Metabolic activity	8 h	EC50	*Bacillus amyloliquefaciens*	>1 000 000			Menz et al. (2018)
	Metabolic activity	8 h	EC50	*Pseudomonas putida*	5 610	1000	5.61	Menz et al. (2018)
	Iron reduction	5 d	ED50	Soil microorganisms	47 553			Thiele-Bruhn (2005)
	Root elongation	2 d	IC50	*Wheat*	28 100			Jin et al. (2009)
	Root elongation	3 d	IC50	*Chinese cabbage*	31 300			Jin et al. (2009)
	Root elongation	5 d	IC50	*Tomato*	92 900			Jin et al. (2009)
Sulfamethazine	Iron reduction	5 d	ED50	Soil microorganisms	75 149			Thiele-Bruhn (2005)
	Soil respiration	2 d	EC10	Soil microorganisms	13 000			Liu et al. (2009)
	Root length	20 d	NOAEL	Rice	1 000	100	10	Liu et al. (2009)
	Root length	20 d	NOAEL	Cucumber	100 000			Liu et al. (2009)
	Seedling height	20 d	NOAEL	Rice	70 000			Liu et al. (2009)
	Seedling height	20 d	NOAEL	Cucumber	100 000			Liu et al. (2009)
Ciprofloxacin	Nitrification	28 d	NOAEL	Soil microorganisms	100 000			Parente et al. (2018)
	Cumulative respiration	28 d	NOAEL	Soil microorganisms	1 000 000			Parente et al. (2018)
	Leaf growth	9-16 d	NOAEL	*Lolium perenne* (ryegrass)	10 000 000			Parente et al. (2018)
	Root growth	9-16 d	NOAEL	*Lolium perenne* (ryegrass)	10 000 000			Parente et al. (2018)
	Stem growth	9-16 d	NOAEL	*Raphanus sativus* (radish)	1 000 000			Parente et al. (2018)
	Root growth	9-16 d	NOAEL	*Raphanus sativus* (radish)	1 000 000			Parente et al. (2018)
	Leaf growth	9-16 d	NOAEL	*Allium cepa* (onion)	5 000 000			Parente et al. (2018)
	Root growth	9-16 d	NOAEL	*Allium cepa* (onion)	1 000 000			Parente et al. (2018)
	Micronucleus induction	3 d	NOAEL	*Vicia faba*	50	100	0.5	Khadra et al. (2012)

Table S9. Inhibition of aquatic organisms by prednisolone. LOEC = Lowest observed effect concentration. EC50 = Effective concentration 50, i.e. the measured endpoint is reduced by 50%. LC50 = Lethal concentration 50, i.e. 50% of the population dies.

Endpoint	Days		Test organism	Concentration ($\mu g\ l^{-1}$)	AF	PNEC ($\mu g\ l^{-1}$)	Reference
Mortality		LC50	*Brachionus calcyciflorus*	22 290			DellaGreca et al. (2004)
Mortality		LC50	*Thamnocephalus platyrus*	23% at 140 000			DellaGreca et al. (2004)
Immobilization		EC50	*Daphnia magna*	>85 000			DellaGreca et al. (2004)
Cell density		EC50	*Pseudokirchneriella subcapita*	>160 000			DellaGreca et al. (2004)
Growth inhibition		EC50	*Ceriodaphnia duba*	230			DellaGreca et al. (2004)
Developmental abnormalities	118, F2 generation.	EC10 ≈ NOEC	*Physa acuta*	1.6	100	0.016	Bal et al. (2017)

Calculation of Predicted No Effect Concentration (PNEC)

The calculation of PNEC follows the procedure from the Technical Guidance Document on Risk Assessment from the European Chemicals Bureau (2003), the procedure is described in the following:

1. Collect terrestrial ecotoxicity data and convert into µg kg^{-1} dry weight soil.

2. In cases where there are no terrestrial ecotoxicity data, or there are data on only one organism, aquatic ecotoxicity data are collected as well. This is the case for prednisolone (no data).

3. $PNEC_{soil}$ can be calculated in two different ways:

A. PNEC calculation by the use of assessment factors.

This method applies to compounds when toxicity data are available for a producer, a consumer and/or a decomposer in soil.

$$\frac{EC50\ or\ NOAEL\ or\ LC50\ [\mu g\ kg^{-1}]}{AF} = PNEC_{SOIL}[\mu g\ kg^{-1}] \qquad \text{(eq. 7)}$$

Where AF is the assessment factor appropriate for the toxicity data. The AF is meant to reflect the uncertainty with predicting ecosystem effects from laboratory tests on a limited number of species, often for a limited duration.

Table S10. Overview of the assessment factors. The table is adapted from table 20 in the Technical Guidance Document on Risk Assessment of the European Commission (2003).

Information available	**Assessment factor**
LC50 / EC50 short term toxicity test(s)	1000
NOAEL for one long-term toxicity test	100
NOAEL for additional long-term toxicity tests of two trophic levels	50
NOAEL for additional long-term toxicity tests for three species of three trophic levels	10

Example of calculation: For sulfadiazine, there are only short-term acute toxicity tests. Therefore, an assessment factor of 1000 is used: $PNEC_{soil}$ = 5610 µg kg^{-1} dw / 1000 = 5.61 µg kg^{-1}.

B. PNEC calculation by the use of the equilibrium partitioning method

When there are no terrestrial toxicity data, or only data from one species, the equilibrium partitioning

method can be used. $PNEC_{WATER}$ is calculated as in section A, with the unit µg l^{-1}. Thereafter, the $PNEC_{WATER}$ is converted into $PNEC_{SOIL}$ by equation 1 (adapted from equation 72 in European Commission (2003)).

$$PNEC_{soil}[\mu g\ kg^{-1}] = \frac{K_d[-]}{RHO_{soil}[kg * l^{-1}]} * PNEC_{water}[\mu g\ l^{-1}] \qquad \text{(eq. 8)}$$

Where K_d is the partition coefficient soil water, and RHO_{SOIL} is the bulk density of soil. K_d is calculated by equation 2, as recommended by the European Commission (2003).

$$K_d = K_{OC} * f_{OC} * 10 \qquad \text{(eq. 9)}$$

For prednisolone, K_{OC} is modelled to 36.36 by EPISUITE. The European Commission (2003) use 0.02 as a standard value for f_{OC}. K_d for prednisolone is then:

$$K_d = 36.36 * 0.02 * 10 = 7.272$$

The calculation of K_d from K_{OC} is primarily meant to be used on neutral species, as the main sorption mechanism for these in soil is partitioning into organic matter. Prednisolone can ionise, and therefore the K_d is multiplied with 10 to account for electrostatic sorption as recommended by European Commission (2003). Further, $PNEC_{SOIL}$ is calculated by equation 1. RHO_{SOIL} is set to 1.3 kg l^{-1}, as a typical bulk density of soil.

$$PNEC_{SOIL} = \frac{0.016\ \mu g\ l^{-1} * 7.272}{1.3\ kg\ l^{-1}} = 0.224\ \mu g\ kg^{-1} dw$$

Note that the calculation of $PNEC_{SOIL}$ for prednisolone has significant uncertainties, due to lack of soil ecotoxicity data and measurements of K_d.

Calculation of PNEC values for selection antibiotic resistant bacteria

Menz et al. (2019) calculated PNEC values for selection of antibiotic resistant bacteria from *in vitro* antimicrobial susceptibility data (MICs). For the detailed calculation procedure, please read Menz et al. (2019). These PNEC values are given as µg l^{-1} soil pore water. To compare with the PEC_{SOIL} values

calculated in the present study, the PNEC values must be converted into µg kg^{-1} dry soil. The equilibrium partitioning method suggested for aquatic ecotoxicity data (see section 3B under *Calculation of PNEC*) are used, with the addition of a term for the fraction of pore water in soil (equation 3):

$$PNEC_{soil}[\mu g\ kg^{-1}] = \frac{K_d[-]}{RHO_{soil\,[kg*l^{-1}]}} * PNEC_{pore\ water}[\mu g\ l^{-1}] * f_{pw} \qquad \text{(eq. 10)}$$

Where pw = pore water. As in other parts of the current study, RHO_{SOIL} is set to 1.3 kg l^{-1}. The fraction of pore water, f_{PW} is set to 0.25 (v/v). For the calculation of PNEC, the Kd, and K_{OC} values given in Table S5 were used. For amoxicillin and penicillin G, K_d are estimated from K_{OC} by equation 2. For sulfadiazine and ciprofloxacin, K_d is estimated as the average of the minimum and maximum K_d values reported in Table S5. Further, $PNEC_{SOIL}$ is estimated by equation 3 as in table S6.

Table S11. Log K_{OW}, K_d and K_{OC} as reported by Cycoń et al. (2019)

	Kd (l kg^{-1})	K_{OC} (l kg^{-1})
Amoxicillin	-	865.5
Penicillin G	-	2.68
Sulfadiazine	1.40-14	37-125
Ciprofloxacin	427-4,844	1,127-61,000

Table S12. Estimation of $PNEC_{SOIL}$ for selection of antibiotic resistant bacteria from experimental data provided by Cycoń et al. (2019).

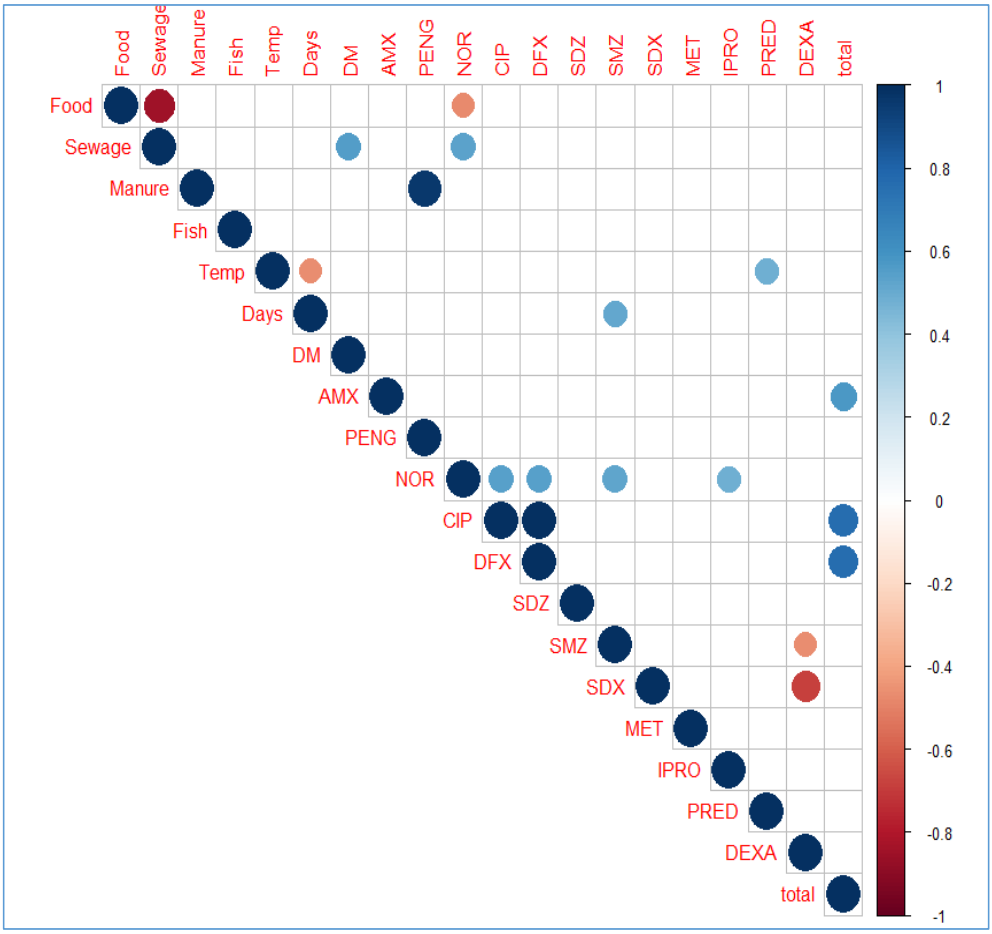
	$PNEC_{PW}$, Menz et al. (2019)	K_{OC}	Kd	$PNEC_{SOIL}$
Amoxicillin	0.20-0.25	865.5	865.5*0.02*10 = 173.1	(0.20-0.25)*173.1*0.25/1.3 = 6.7-8.3
Penicillin G	0.25	2.68	2.68*0.02*10 = 0.536	0.25*0.536*0.25/1.3 = 0.026
Sulfadiazine	21		(1.4+14)/2 = 7.7	21*7.7*0.25/1.3 = 31
Ciprofloxacin	0.064		(427+4,844)/2 = 2624.5	0.064*2624.5*0.25/1.3 = 32

S10. Description of the performed statistics

Correlation analysis

Pearson correlation analysis were used to explore the numerical part of the data set, i.e. the levels of pharmaceuticals (Table 1) and the following operating conditions (Table S1): Substrate (fraction of food waste, sewage sludge, manure, fish sludge), Retention time, Reactor temperature, Dry matter of the digestate. The qualitative operating conditions pre-treatment, polymer addition and precipitant addition were left for analysis of variance. Values below LOQ was replaced with ½ LOQ, while values below the detection limit (LOD) was replaced with ½ LOD.

Figure S4. Pearson correlation analysis. Blue circles means there is a positive correlation, red circles means there is a negative correlation. Only significant correlations ($p < 0.05$) are shown. The size of the circle and the darkness of the colour increases with the correlation coefficient (colour legend to the right). Total: sum of pharmaceuticals in each digestate.



There is a positive correlation between the fluoroquinolones norfloxacin, ciprofloxacin, and difloxacin. For the other pharmaceuticals there are no correlations within the groups. Note that the correlations are strongly affected by individual observations. For example, the Penicillin G concentration is strongly positively correlated with the manure content of the substrate. This can be explained as the only digestate where Penicillin G was present was the only digestate using 100% manure as substrate.

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Paper III

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Article

Uptake of Ultrashort Chain, Emerging, and Legacy Per- and Polyfluoroalkyl Substances (PFAS) in Edible Mushrooms (*Agaricus spp.*) Grown in a Polluted Substrate

Astrid Solvåg Nesse,* Agnieszka Jasinska, Aasim Musa Ali, Oskar Sandblom, Trine A. Sogn, and Jonathan P. Benskin

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ABSTRACT: Uptake of 19 per- and polyfluoroalkyl substances (PFAS), including C3−C14 perfluoroalkyl carboxylic acids (PFCAs), C4, C6, and C8 perfluoroalkyl sulfonates (PFSAs), and four emerging PFAS, was investigated in two mushroom species (*Agaricus bisporus* and *Agaricus subrufescens*) cultivated in a biogas digestate-based substrate. Accumulation of PFAS in mushrooms was low and strongly chain-length dependent. Among the different PFCAs, bioaccumulation factors (log BAFs) decreased from a maximum of −0.3 for perfluoropropanoic acid (PFPrA; C3) to a minimum of −3.1 for perfluoroheptanoate (PFHpA; C7), with only minor changes from PFHpA to perfluorotridecanoate (PFTriDA; C13). For PFSAs, log BAFs decreased from perfluorobutane sulfonate (PFBS; −2.2) to perfluorooctane sulfonate (PFOS; −3.1) while mushroom uptake was not observed for the alternatives 3H-perfluoro-3-[(3-methoxy-propoxy)propanoic acid] (ADONA) and two chlorinated polyfluoro ether sulfonates. To the best of our knowledge, this is the first investigation of the uptake of emerging and ultra-short chain PFAS in mushrooms, and generally the results indicate very low accumulation of PFAS.

KEYWORDS: *bioaccumulation factor, PFAS, fungi, mushroom, organic pollutants, biogas digestate, Agaricus subrufescens, Agaricus bisporus, circular economy*

INTRODUCTION

End-of-life material recycling is a critical step toward achieving a circular economy and ultimately reducing raw material demand and waste production, including greenhouse gas emissions.[1] As an example, organic waste can be utilized for biogas production, a process in which methane is produced from the breakdown of organic matter by anaerobic microorganisms. Liquid and solid digestate generated by this process can be utilized as fertilizer in agronomic plant production in order to improve nutrient circularity.[2] However, an important consideration when using digestates for agricultural fertilizer is the occurrence of micropollutants originating from the waste feedstocks which may persist during the digestion process. Occurrence of micropollutants in digestate-based fertilizer risks contamination of amended soil[3] and accumulation in crops[4−6] which can ultimately lead to human exposure, either by direct ingestion or leaching into groundwater and adjacent water bodies used for drinking water.[7]

One group of organic pollutants of high global concern which occur in organic wastes is the per- and polyfluoroalkyl substances (PFAS).[8−10] PFAS encompass a diverse group of over 9000 substances that contain at least one perfluoromethyl ($-CF_3$) or perfluoromethylene ($-CF_2-$) group.[11] The considerable strength of the C−F bond, combined with unique lipophobic and hydrophobic properties imparted by highly fluorinated aliphatic chains, has led to widespread use of PFAS in consumer products and industrial processes.[12] The vast majority of PFAS are expected to persist in the environment or degrade to environmentally persistent end-products (i.e., perfluoroalkyl acids; PFAAs), the latter of which are highly mobile in water.[13,14] Due to the risks associated with these substances, perfluorooctanesulfonic acid (PFOS), perfluorooctanoic acid (PFOA), perfluorohexanesulfonic acid (PFHxS), and long-chain perfluoroalkyl carboxylic acids (PFCAs; including related compounds) have been listed or proposed for listing as persistent organic pollutants (POPs) under the United Nations Stockholm Convention.[15,16] Despite this initiative, alternative PFAS, for example with shorter perfluorinated chains or ether linkages within the fluorinated chain, continue to be produced.[17] Short chain PFAS generally display reduced bioaccumulation potential in humans and wildlife but are equally as persistent as legacy PFAS.[14,18] Moreover, uptake in crops has been shown to be largely dependent on sorption (either to soil, root-based lipids, or during the flow from leaves to fruit),[19,20] resulting in greater accumulation of more hydrophilic/shorter chain length PFAS in plants.[5,19]

Mushrooms are a popular and nutritious food,[21] which can be grown on a variety of organic waste products, including

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animal manure and biogas digestate.[22,23] Due to their unique enzymatic machinery, mushrooms have received considerable attention as tools for bioremediation of different pollutants and agro-industrial wastes.[24] Previous research investigating the uptake and degradation of contaminants by mushrooms has focused mostly on heavy metals[25−27] and certain groups of organic contaminants such as polycyclic aromatic hydrocarbons[28,29] and pesticides,[30] but to the best of our knowledge, only a single study has investigated uptake of PFAS. In that work, Golovko et al.[31] measured chain-length dependent uptake of 10 legacy PFAS in the edible oyster mushroom (*Pleurotus ostreatus*) using two different substrates (one with biogas digestate) containing 100 ng PFAS/g wet weight. Uptake was more efficient for perfluoroalkyl sulfonic acids (PFSAs) compared to PFCAs and was significantly reduced with increasing chain length. Moreover, uptake of PFAS was dependent on the PFAS concentration in the substrate, rather than the substrate composition itself.

In the current study, we build on the prior work of Golovko et al.[31] by evaluating the uptake of 14 legacy, three emerging, and two ultrashort chain PFAS in two mushroom species (*Agaricus bisporus* and *Agaricus subrufescens*) cultivated in biogas digestate generated through an anaerobic digestion of food waste and manure to produce biogas. The objectives of this study were twofold: first, we sought to investigate the fate of ultra-short- and emerging replacement PFAS, for which there are a paucity of data. Second, we aimed to investigate inter-species differences in the uptake of PFAS in mushrooms. These data are the first to investigate uptake of emerging and ultra-short chain PFAS in mushrooms and provide new insights into the risks associated with using waste feedstocks for agricultural fertilizer.

MATERIALS AND METHODS

Standards and Reagents. Authentic and isotopically labeled PFAS standards were purchased from Sigma, Wellington Laboratories, or Shanghai Syncia Co., Ltd. A full list of standards, including acronyms, is provided in Table S1. Methanol (HPLC-grade) was purchased from VWR. The water used throughout this work was either deionized, distilled, or grade 1 Milli-Q water, depending on the location of use (Stockholm University, NMBU, or Lindum).

Dose Preparation. The mushroom substrate was spiked with 13 PFCAs (C2/trifluoroacetate [TFA] through C14/perfluorotetradecanoate [PFTeDA]), 3 PFSAs (PFBS, PFHxS, and PFOS), and the PFAA replacements ADONA (4,8-dioxa-3H-perfluorononaoic acid), F-53B (consisting of 9-chlorohexadecafluoro-3-oxanone-1-sulfonic acid [9Cl-PF3ONS] (major component) and 11-chloroeicosafluoro-3-oxaundecane-1-sulfonic acid [11Cl-PF3OUdS] (minor component)), and 2,3,3,3-tetrafluoro-2-(heptafluoropropoxy)propanoic acid [HFPO-DA/Gen-X] (Table S1). The doses of 6 mg of each PFCA and PFSA, 1.08 mg of F-53B, and 0.54 mg of ADONA, and Gen-X were dissolved in 100 mL of methanol at Stockholm University. For control, 100 mL of methanol was used. The two solutions were diluted to 2000 mL in grade 1 Milli-Q water at the Norwegian University of Life Sciences. Unfortunately, due to analytical challenges associated with TFA and Gen-X (the latter of which was due to degradation in acetonitrile[32]), results for these substances are not reported.

Experimental Setup. An overview of the experimental setup is provided in Figures 1 and S1. Briefly, two batches of mushroom substrate ("substrate") were prepared, one control batch and one spiked with PFAS. Both control and spiked batches consisted of 11 subunits: four containing *A. subrufescens*, four with *A. bisporus*, and three without any mushrooms. Both whole mushrooms (i.e., the aboveground part of the mushroom, including the stem) and substrate were sampled at the same time for PFAS analysis, 1−2 times for each unit (depending on the number of "flushes" or "harvests," i.e., the number of times the mushroom hats emerged from the substrate). Mushroom yield (g fresh weight) was also determined at each harvest (Table S2).

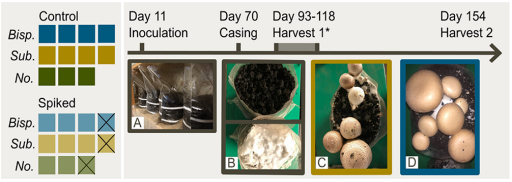

Figure 1. Mushroom growth. Twenty-two bags (i.e., experimental units) were filled with mushroom substrate, three of the spiked bags went rotten during the experiment (crossed out in the figure). (A) Bags filled with the substrate. (B) Mycelia has overgrown the substrate and casing is applied. (C) Emerging *A. subrufescens* pins, and mushroom hats. (D) Mature *A. bisporus* ready for harvest. *A few of the bags were harvested twice (i.e., harvests 1 and 2) during this period.

Preparation of the Mushroom Substrate. Each batch of substrate was prepared from 33 kg of biogas digestate mixed with control or spiking solution (digestate dry matter (DM): 4.3%), 10.5 kg of wheat straw, 300 g of chalk ($Ca(OH)_2$), 300 g of gypsum ($CaSO_4$), and 1 kg of activated garden compost for inoculation, to a total DM of 25%. The digestate was made from a feedstock of 73% household food waste and 27% manure and contained small amounts of native PFAS (see Results and Discussion). The substrate was composted (Phase I) for 9 days and pasteurized (Phase II) for 2 days (Figure S1). On days 0, 3, 6, 9, and 11, the substrate was mixed and sampled. On day 6, the control batch became too wet and about 1 L of liquid had gathered at the bottom of the drum. The liquid was removed as the compost would have become too wet if it was mixed into the compost again. This did not occur in the spiked compost. The preparation procedure is shown in Figure S1 along with temperature data for the two batches (mean of three temperature loggers in each batch). Further descriptions of the spiking procedure and equipment are found in the SI and Stoknes et al.[22]

Mushroom Cultivation. ***Inoculation.*** After pasteurization, each batch was split into 11 units. Four were inoculated with *A. subrufescens*, four with *A. bisporus*, and three were not inoculated (Figure 1). For inoculation, 90 g of granular spawn of the strains M7700 (*A. subrufescens*) and M7243 (*A. bisporus*) (Mycelia, Deinze, Belgium) were used (3% of the fresh substrate weight). Each unit was kept in a sealed 50 micron polypropylene bag of 7 L, with four linear ventilation filters (type PP50/SEU4/V40-51, SacO2 Microsac, Deinze, Belgium), at 25 °C (Figure 1A). ***Casing.*** After the spawn overgrew the substrate (day 70, Figure 1B), the bags were opened and a 5−8 cm layer of casing (dark peat mixed with $Ca(OH)_2$ and gypsum) was applied to initiate pinning (i.e., the emergence of fruiting bodies). The opened bags were moved to a cultivation chamber holding 25−30 °C and an air humidity of 70−75%. ***Pinning/fructification.*** After 9 days, the mycelia had overgrown the surface of the casing and pinning (Figure 1) was initiated by moving *A. bisporus* bags to a chamber holding 17−18 °C. The tropical *A. subrufescens* and the no mushroom bags were kept in the first chamber, but the temperature was lowered to 20 °C for 5 days. The CO_2 concentration was kept below 1000 ppm in both chambers. ***Harvesting.*** The whole mushrooms were picked from the individual bags as they obtained maturity (Figure 1D), collecting all mushrooms from a single bag at once. After harvesting, the mushrooms again produce mushroom hats which were harvested the second time. Details of mushroom cultivation, sampling, and homogenization of samples are provided in the SI.

Sample Characterization. Immediately after sampling, pH (determined using a ratio of 3:10 substrate: water, Milwaukee 802 pH/EC/DS meter), DM (105 °C, duplicate samples), and loss of ignition (550 °C) were determined. At harvests 1 and 2, pH was

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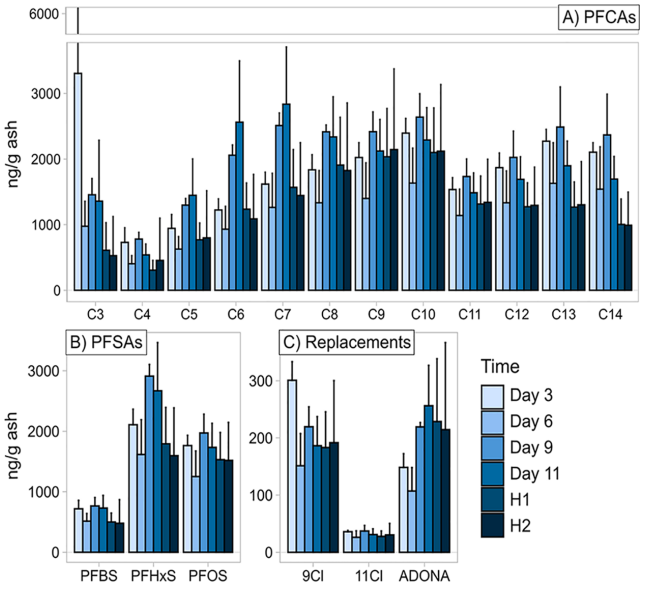

Figure 2. PFCAs (A), PFSAs (B), and replacements ("9Cl" = 9Cl-PF3ONS, "11Cl" = 11Cl-PF3OUdS) (C) in the spiked substrate (ng g^{-1} ash). Note that the y-axis has been cut due to the large standard deviations of C3 at day 3. H1 = Harvest 1, H2 = Harvest 2.

measured directly in the substrate with a "stick-in" pH meter (Table S3; G071505, BIOGRÓD) as it was not possible to sample enough material for standard pH analysis.

For analysis of PFAS, samples of substrate (oven dried) and mushrooms (fresh) were fortified with 10 ng of individual isotopically labeled internal standards (complete list provided in Tables S4 and S5) prior to extraction. Details of the extraction procedure are provided in the Supporting Information. Briefly, the substrate was extracted with methanol, while the mushrooms were extracted with acetonitrile. Both types of extracts were subjected to a dispersive carbon clean-up and then fortified with 10 ng of individual recovery standards and aqueous ammonium acetate prior to instrumental analysis. Two different instrumental methods were employed: PFPrA, PFBA, and PFPeA were analyzed by LC-high resolution mass spectrometry (HRMS) using a hybrid reversed phase/ion exchange column (Table S4).[33] The remaining PFAS were analyzed by LC–MS/MS using a reversed phase column (Table S5). Further details on extraction, instrumental analysis, and quality control are provided in the SI, including results of matrix spike/recovery experiments involving both mushroom and fish muscle matrix (Tables S6 and S7) as well as analysis of NIST standard reference material (Table S8).

Data Analysis. All statistics were performed in R Studio, version 4.1.2.[34] Graphics were prepared in R Studio and Inkscape version 1.1.[35] To assess differences in uptake between mushroom species, treatment (control/spiked), and harvest time for the spiked mushrooms, a mixed effect model was used. The experimental units were treated as random effects to avoid temporal autocorrelation. Due to the high number of nondetects in the mushroom samples for most compounds, the mixed effect model analysis was applied only for C8. Handling of data below the limit of quantification (LOQ) is described in detail in the SI.

For those replicates of the spiked treatment in which PFAS were detected, the bioaccumulation factors (BAFs) were calculated for each experimental unit at both harvests using the concentration in the mushroom divided by the concentration in the substrate (dry weight basis). For those replicates where the uptake was below the LOQ, worst case BAFs were calculated by dividing the mushroom-LOQ (calculated on dry weight basis) by the substrate concentration. Linear regression was used to determine the relationship between PFAS chain length and the logarithm of the BAFs. Assumptions were checked by inspection of the plots for "Residuals vs. Fitted" and "Normal Q–Q" (see SI for details).

RESULTS AND DISCUSSION

Substrate Concentration. PFAS concentrations in substrates are reported on both a dry weight (Tables S9 and S10) and ash weight (Tables S11 and S12) basis. Ash-weight concentrations are generally considered more accurate due to the continuous degradation of the substrate, which would lead to an apparent upconcentration of PFAS with time for dry-weight concentrations. Analysis of PFAS in the spiked substrate revealed concentrations ranging from 18 to 83% of nominal (approximately 4000 ng g^{-1} ash for PFCAs and PFSAs) at day 3 (Figure 2). The possible occurrence of PFAA-precursors in the substrate (which, if present, could contribute to observed PFAA concentrations) was ruled out after observing that the PFAS concentrations in the control substrate were below 0.5% of that in the spiked substrate for all compounds at harvests 1 and 2. Removal of PFAS from the substrate via mushroom uptake was also limited based on the low concentrations observed in mushrooms (discussed further below).

Mushroom Uptake of PFAS. PFAS concentrations determined in mushrooms are reported on both a fresh weight (Table S13) and dry weight (Table S14) basis. Overall, uptake of PFAS in both species of mushrooms on harvests 1 and 2 was limited with concentrations up to 14 ng g^{-1} dw in *A. subrufescens* and 28 ng g^{-1} dw in *A. bisporus* (for C3). Similar to observations in plants, uptake was strongly chain-length dependent with ultra-short chain PFAS displaying a much greater propensity for accumulation compared to long chain PFAS. PFAS concentrations in the mushrooms generally decreased from C3 to C7 before stabilizing at a concentration of 0.38–1.1 ng g^{-1} dw from C8 to C13 (no uptake of C12 and C14; Table 1). Note that C3, C4, C12, and C14, which were

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Table 1. Concentration (ng g^{-1} dw) and Total Uptake (ng) of PFAS in the Mushroom Hats Grown in the Spiked Substrate[a]

	concentration (ng g^{-1} dw)				total uptake (ng)			
	harvest 1		harvest 2		harvest 1		harvest 2	
compound	sub (n = 3)	bisp (n = 3)	sub (n = 2)	bisp (n = 2)	sub	bisp	sub	bisp
C3	4.2*	7.9*	14 (6.5)	28 (21)	74*	21*	95	117
C4	<LOQ	2.4*	<LOQ	9.0 (5.5)		6.3*		35
C5	<LOQ	0.46*	<LOQ	3.7 (4.4)		1.2*		18
C6	<LOQ	1.2 (1.1)	0.78 (0.12)	2.0 (1.2)		5.1	5.7	7.7
C7	<LOQ	0.91 (0.79)	0.50 (0.03)	0.47 (0.47)		4.1	3.7	2.1
C8	0.45 (0.11)	1.0 (0.55)	0.48 (0.04)	0.50 (0.15)	7.5	4.8	3.5	1.8
C9	<LOQ	0.80 (0.62)	<LOQ	0.43 (0.33)		3.7		1.8
C10	0.09*	0.79 (0.64)	0.34 (0.29)	0.60 (0.64)	1.7*	3.6	2.7	2.8
C11	<LOQ	0.71 (0.48)	0.38 (0.21)	0.59 (0.49)		3.3	3.0	2.5
C13	<LOQ	1.1 (0.94)	0.38 (0.21)	0.52 (0.39)		4.6	3.0	2.2
PFBS	<LOQ	0.77 (0.80)	0.46 (0.03)	1.2 (0.53)		3.0	3.4	4.5
PFHxS	0.09*	1.1 (0.83)	0.50 (0.38)	0.23 (0.13)	1.7*	5.3	4.0	0.9
PFOS	<LOQ	0.46 (0.28)	0.31 (0.25)	0.35 (0.30)		2.3	2.5	1.5

[a]Standard deviation is given in parenthesis. *There was uptake in only one replicate, the concentration/uptake divided on no. of replicates are given.

detected less frequently compared to the other PFAS, had the highest LOQs (Table S13). PFSA concentrations were in the same range as the long-chained PFCAs. A similar pattern was reported for uptake of C2−C6, C8, and PFOS in hydroponically grown wheat (*Triticum acstivnm* L.) where the concentration of C2 in the shoots was 13-fold higher than C3, which again was fourfold higher than for any of the other PFAS.[36] Zhang et al.[36] explained the considerable uptake of ultra-short chain PFAS by their high water solubility and small molecular size, leading to easier passage of the Casparian strip and translocation within the plant.

There was no observable uptake of F-53B components (9Cl-PF3ONS or 11Cl-PF3OUdS) or ADONA in any of the mushrooms perhaps due to the lower nominal concentration of these compounds (~360 ng g^{-1} ash) compared to the PFAAs (~4000 ng g^{-1} ash). Moreover, several plant uptake experiments have demonstrated that transport of F-53B from plant roots to shoots is limited with shoot concentrations less than 10% of those in roots (summarized by Zhang et al.[37]). However, in cattails, a frequently used bioremediation plant, the uptake of F-53B was higher.[38] In both experiments performed by Zhang et al.,[37,38] the shoot concentration of ADONA was several times higher than the F-53B concentration. Zhang et al.[37] explained the difference by the fraction of water extractable compounds, which was about 2.3% for F-53B and 14% for ADONA in a soil with 29% organic carbon.

While the present work did not systematically investigate the influence of dose on PFAS uptake, some observations on the effect of dose could be made based on the occurrence of PFOA in control (i.e., unspiked) experiments. The concentration of C8 in spiked mushrooms, as estimated by a mixed effect model, was about twice as high as that in the control mushrooms (Table 2, p = 0.059). Considering that the level of C8 in the control substrate was below 0.5% of the concentration in the spiked substrate, the uptake of C8 by the control fungi was surprisingly high. C8 was found in nearly all replicates of both the control fungi and the spiked *A. subrufescens*, while other PFAS were taken up infrequently (Tables 1, 2, and S14). Furthermore, the level of C8 was similar to that of other PFAS in the control substrate, which makes it surprising that this particular compound should be taken up, while the other carboxylates generally were not.

Table 2. Linear Mixed Model Test on the Effect of Treatment (Control or Spiked), Time (Harvest 1 or 2), and Mushroom Species (*A. subrufescens* or *A. bisporus*) on the Concentration of PFOA (C8) in the Mushrooms (ng g^{-1} dw)[a]

	estimate	df	p-value
intercept	0.27 ± 0.13	10.27	0.056
treatment: spiked	0.32 ± 0.15	10.04	0.059
time: harvest 2	−0.04 ± 0.06	6.56	0.57
species: bisporus	0.22 ± 0.15	10.4	0.17

[a]The experimental units were included as a random factor. The intercept shows the estimate of the C8 concentration in control *A. subrufescens* mushrooms at harvest 1. The three bottom rows show the estimated additional effect of changing from control to spiked treatment, from harvest 1 to harvest 2, and from *A. subrufescens* to *A. bisporus*. df = degrees of freedom.

For the remaining PFAS, no statistical tests were performed as the number of observations was considered too low. However, inspection of the figures in Table 1 reveals that most PFAS were detected more often and (for some compounds) in higher concentrations in *A. bisporus* compared to *A. subrufescens*. PFAS were also taken up more frequently at harvest 2 compared to harvest 1. The uptake was 10−40-fold lower in both species from the present study compared to the oyster mushroom uptake assessed by Golovko et al.[31] despite a higher spiking level (443 and 286 ng g^{-1} dw for each PFCA and PFSA, *A. spp.* and *P. ostreatus*, respectively). Different species of plants have also displayed differential PFAS accumulation with BAFs varying by up to eight orders of magnitude.[20] Even different varieties of the same plant species may have variable uptake of PFAS.[39,40]

Experimental setup and growth conditions can influence the uptake of PFAS. From day 84 until the end of the experiment at day 154, *A. subrufescens* was maintained at 25 °C, while *A. bisporus* was cultivated at 17−18 °C. Since temperature influences the degradation rate of organic matter, the substrate composition for the two species may have become different, influencing the sorption and bioavailability of the target compounds. Likewise, the substrate of *P. ostreatus* was of a different composition from the *A. spp.* substrate; alder sawdust

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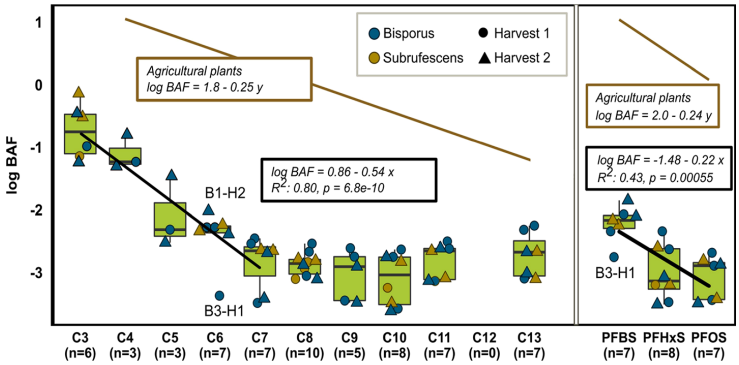

Figure 3. Bioaccumulation factors (BAFs) for PFAS in the mushroom hats, which were grown in spiked compost, and linear regression of log BAFs as a function of carbon chain length (x). Linear regression was based on combined data for both harvests (H1 and H2) and both species (S and B). There were a total of 10 datapoints for each PFAS (six for H1 and four for H2). When fewer datapoints are shown, it is because no uptake was detectable. Note that the nondetects are not included in the regression. Outliers are labeled: B1-H2: *A. bisporus*, replicate 1, harvest 2. B3-H1: *A. bisporus*, replicate 3, harvest 1. The regression lines for agricultural plants are taken from a review by Lesmeister et al.,[20] where y is the number of perfluorinated carbons. Thus, $x = y$ for the PFSAs and $x = y + 1$ for the PFCAs. The p-values are for the regression slopes.

combined with either biogas digestate or wheat bran and calcium sulphate. The substrate preparation for the oyster mushroom experiment by Golovko et al.[31] was shorter (8 h pasteurization), and the duration of the experiment was shorter as well, i.e., 28−25 days from inoculation to harvest (Golovko et al., personal communication).

Bioaccumulation of PFAS in Mushrooms. The distinct chain-length dependency for C3 to C7 PFAS concentrations in mushrooms was reflected in calculated mean log BAFs, which decreased by 0.54 for each additional CF_2-moiety among PFCAs (Figure 3; Table S15). From C7 to C13, the BAFs were almost equal. The PFSAs showed a similar pattern with a higher BAF for PFBS compared to PFHxS and PFOS. However, the median BAF was higher for PFOS than for PFHxS and linear regression explained only 43% of the variance (Figure 2). Similarly, Golovko et al. observed a similar mushroom concentration of PFHxS and PFOS, while the concentration of PFBS was about twice as high.[31]

The chain-length dependency can be explained by the bioavailability of PFAS since sorption of PFCAs and PFSAs generally increases with chain-length.[41−43] In the study by Pereira et al.,[41] less than 10% of the short chained PFAS such as C4, C5, and PFBS was sorbed in an organic soil layer (45% organic carbon), while an average of 99−100% of C10−C12, C14, and PFOS were sorbed. Similarly, Milinovic et al.[44] assessed sorption to a peat soil (39% organic carbon) and found that up to 95−97% of PFOS, 70−81% of PFOA (C8), and 28−40% of PFBS were sorbed. In the study by Nguyen et al.,[43] it was found that short-chained PFAAs such as C4−C6 PFCAs and C4−C5 PFSAs, as well as ADONA, were highly mobile in 10 different mineral soils (pH 6.2−7.7, 0.08−4.9% organic carbon), as seen by their negative log K_d values. These short-chained acids also appear to be less affected by changes in pH than the longer-chain length substances most probably because they already preferred the aqueous phase.[43]

The chain-length dependent uptake of PFAS has also been reported for oyster mushrooms and for agricultural plants. Golovko et al.[31] found a decrease in oyster mushroom hat concentration of 1.68 and 5.4 ng g^{-1} dw for each additional CF_2-moiety, for the PFCAs and PFSAs, respectively. In plants, a linear regression based on the median of 1800 BAFs for PFCAs and 500 for PFSAs showed a decrease in the BAFs from 0.24 to 0.25 $\log_{10}$ units for each additional CF_2-moiety (Figure 3, Lesmeister et al.[20]). Different plant studies indicate that the chain-length dependent uptake of PFAS arises not only due to the bioavailability in the growth medium but also from a selective transport within the plant. Even in hydroponic studies, where sorption does not restrict uptake, shoot-BAFs show a chain-length dependency.[20,45] The transport mechanism of polar organic chemicals such as PFAS in fungi is, however, unknown. Available literature on mechanisms of chemical uptake in fungi has mainly focused on metals and nutrients (e.g., iron[46,47]), textile dyes,[48,49] and hydrophobic organic compounds connected to oil spills and fuel, such as alkanes and PAHs (e.g.,[50−53]).

The higher accumulation of PFCAs compared to PFSAs with equal number of CF_2-moieties, which is often observed in plants (e.g.,[54]), was not seen in the present study. For example, in the spiked *A. bisporus*, at both harvests, the log BAFs differ by only 0.5−9% when comparing the pairs PFBS/C5, PFHxS/C7, and PFOS/C9 (for the same species, time, and treatment).

Only PFBS in one experimental unit had a BAF above 1 (log BAF above 0), which may indicate a potential for bioaccumulation in a scientific context as the concentration is higher in the mushroom compared to the growing media.[55] Regulatory criteria are, however, set higher. For example, under REACH,[56] substances must have a bioconcentration factor (BCF) in aquatic species of at least 2000 to be classified as bioaccumulative and 5000 to be considered very bioaccumulative, corresponding to log BCFs of 3.3 and 3.7, respectively. On the basis of the data provided by Golovko et al.,[31] approximate BAFs were calculated also for oyster mushrooms (samples of mushrooms and substrate were not taken at the same day). Similar to what has been observed in the present study, the BAFs for oyster mushrooms were essentially equal for C7 to C12 (−1.3 to −1.4), and slightly higher for C6 (−0.96). A similar pattern was observed for PFSAs in oyster mushrooms; a higher BAF for PFBS (−0.72) and similar BAFs for PFHxS (−1.4) and PFOS (−1.5). Although the BAFs calculated based on data for the oyster mushrooms were higher than the BAFs calculated in the present study, the oyster mushroom log BAFs were also well below 0.

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Since there were a high number of nondetects for PFAS in the mushrooms, worst-case BAFs were calculated to assess a theoretical potential for uptake of PFAS in the mushrooms based on the LOQs (Table S16). The worst-case BAFs were all well below 1 for compounds in the spiked treatment and for about half of the compounds in the control treatment. The worst-case BAFs were particularly high for those compounds having a relatively higher mushroom LOQs (e.g., up to 27 for C14) and for those having a low substrate concentration (e.g., up to 14 for 11Cl). Nevertheless, compared to the BCF regulation limit of 2000, the potential for uptake of PFAS in mushrooms is clearly very low.

The spiking concentrations in the present experiment were in the same order of magnitude as concentrations reported in French urban wastes such as sewage sludge and municipal waste (average sum of 160 PFAS was 307 ng g^{-1} dm, and the median was 265 ng g^{-1} dm).[9] Overall, the limited uptake of PFAS into the edible parts of the fungi suggests that it is possible to use PFAS-containing waste material to produce mushrooms that are safe for human consumption. However, considering that PFAS are one of many organic contaminants which may occur in sewage sludge, additional mushroom uptake studies are urgently needed. Of particular importance are pharmaceuticals and personal care products, which occur widely in sewage sludge. Investigations into the uptake of these substances in mushrooms by our lab are ongoing and will be presented in a companion paper to the present study in the future.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.2c03790.

Additional information on target analytes, methods (incl. instrumental parameters for UPLC/MS/MS and quality control), PFAS concentrations in the substrate and mushrooms, mushroom yield, pH, BAFs, and statistical analysis (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Astrid Solvåg Nesse − *Faculty of Environmental Sciences and Natural Resource Management, Norwegian University of Life Sciences, 1433 Ås, Norway;* orcid.org/0000-0002-6525-6045; Phone: +47 67 23 18 99; Email: astrid.solvag.nesse@nmbu.no

Authors

Agnieszka Jasinska − *Lindum AS, 3036 Drammen, Norway; Department of Vegetable Crops, Faculty of Horticulture, Poznan University of Life Sciences, 60-637 Poznań, Poland*

Aasim Musa Ali − *Department of Contaminants and Biohazards, Institute of Marine Research, 5005 Bergen, Norway; Faculty of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, 1433 Ås, Norway*

Oskar Sandblom − *Department of Environmental Science, Stockholm University, 106 91 Stockholm, Sweden*

Trine A. Sogn − *Faculty of Environmental Sciences and Natural Resource Management, Norwegian University of Life Sciences, 1433 Ås, Norway*

Jonathan P. Benskin − *Department of Environmental Science, Stockholm University, 106 91 Stockholm, Sweden;* orcid.org/0000-0001-5940-637X

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jafc.2c03790

Author Contributions

The study design was developed by A.J., J.P.B., A.M.A., T.A.S., and A.S.N. J.P.B. and O.S. prepared the spiking solution and did the analytical work related to extraction and analysis of PFAS. A.J. conducted the experiment and did the sampling with the assistance of A.S.N. Analysis of substrate quality was performed by A.J. and A.S.N. The original draft was written by A.S.N. and J.P.B. with the input of all coauthors.

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Notes

The authors declare no competing financial interest.

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Supplementary Information

Uptake of ultrashort chain, emerging, and legacy per- and polyfluoroalkyl substances (PFAS) in edible mushrooms (*Agaricus spp.*) grown in a polluted substrate

Astrid Solvåg Nesse*[1], Agnieszka Jasinska[2,3], Aasim Musa Ali[4,5], Oskar Sandblom[6], Trine Aulstad Sogn[1], Jonathan P. Benskin[6]

[1] Faculty of Environmental Sciences and Natural Resource Management, Norwegian University of Life Sciences, 1433 Ås, Norway
[2] Lindum AS, 3036 Drammen, Norway
[3] Department of Vegetable Crops, Faculty of Horticulture, Poznan University of Life Sciences, 60-637 Poznań, Poland
[4] Department of Contaminants and Biohazards, Institute of Marine Research, 5005 Bergen, Norway
[5] Faculty of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, 1433 Ås, Norway
[6] Department of Environmental Science, Stockholm University, 106 91 Stockholm, Sweden
* astrid.solvag.nesse@nmbu.no, phone: 00 47 67 23 18 99

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Table S1. List of PFAS standards used for mushroom dosing experiment along with abbreviations and CAS-number

Full name		Acronym		CAS-number
Trifluoroacetic acid		TFA	C2	76-05-1
Perfluoropropanoic acid		PFPrA	C3	422-64-0
Perfluorobutanoic acid		PFBA	C4	375-22-4
Perfluoropentanoic acid		PFPeA	C5	2706-90-3
Perfluorohexanoic acid		PFHxA	C6	307-24-4
Perfluoroheptanoic acid		PFHpA	C7	375-85-9
Perfluorooctanoic acid		PFOA	C8	335-67-1
Perfluorononanoic acid		PFNA	C9	375-95-1
Perfluorodecanoic acid		PFDA	C10	335-76-2
Perfluoroundecanoic acid		PFUnDA	C11	2058-94-8
Perfluorododecanoic acid		PFDoDA	C12	307-55-1
Perfluorotridecanoic acid		PFTriDA	C13	72629-94-8
Perfluorotetradecanoic acid		PFTeDA	C14	376-06-7
Perfluorobutane sulfonic acid		PFBS		375-73-5
Perfluorohexane sulfonic acid		PFHxS		355-46-4
Perfluorooctane sulfonic acid		PFOS		1763-23-1
4.8-dioxa-3H-perfluorononaoic acid		ADONA		958445-44-8
2,3,3,3-tetrafluoro-2-(heptafluoropropoxy)propanoic acid		Gen-X		13252-13-6
F-53B	9-chlorohexadecafluoro-3-oxanone-1-sulfonic acid (major component)	9Cl-PF3ONS		73606-19-6
	11-chloroeicosafluoro-3-oxaundecane-1-sulfonic acid (minor component)	11Cl-PF3OUdS		83329-89-9

Table S2. Mushroom yield (g fresh weight), with standard deviation in parenthesis. Number of replicates was four for the control treatment, three for the spiked treatment at harvest 1 (H1), and two for the spiked treatment at harvest 2 (H2).

	Agaricus subrufescens		*Agaricus bisporus*	
	H1	H2	H1	H2
Control	114 (56)	68 (15)	154 (22)	42 (21)
Spiked	127 (22)	45 (7)	52 (33)	26 (27)

Table S3. pH of the substrate, with standard deviation in parenthesis. C = control, S = spiked

	Control			Spiked		
Digestate	7.9					
Day 3	9.0 (0.01)			9.0 (0.05)		
Day 6	8.8 (0.06)			8.9 (0.1)		
Day 9	9.2 (0.06)			9.1 (0.2)		
	Sub - C	Bisp - C	No - C	Sub - S	Bisp - S	No - S
Inoculation	9.0 (0.08)	8.8 (0.2)	9.1 (0.09)	9.0 (0.1)	8.9 (0.04)	9.0 (0.2)
Casing	6.2 (0.4)	6.7 (0.1)	8.0 (0.3)	6.4 (0.3)	6.9 (0.7)	7.7 (0.4)
Harvest 1	5.6 (0.06)	7.6 (0.7)	8.7 (0.09)	5.8 (0.3)	8.3 (0.6)	8.5 (0.1)
Harvest 2	5.9 (0.4)	8.4 (0.2)	8.2 (0.03)	7.1 (0.9)	8.4 (0.06)	8.1 (0.02)

Table S4. Instrumental parameters for PFPrA. PFBA. and PFPeA

Instrument	Dionex Ultimate 3000 liquid chromatograph coupled to a Q Exactive HF Orbitrap (Thermo Scientific)		
Column	Thermo Scientific Acclaim Trinity P1 Charged nanopolymer silica hybrid (3µm. 2.1 × 100mm)		
Mobile Phase	A: 20 mM ammonium acetate in SPE-polished HPLC grade water B: 20 mM ammonium acetate in methanol		
Injection volume	10 µL		
Column temperature	40 ˚C		
Flow rate	0.2 ml/min		
Gradient	**Time (min)**	**%A**	**%B**
	0	75	25
	0.25	75	25
	10	5	95
	12	5	95
	13	75	25
	16	75	25
Spray voltage	3.7 kV		
Capillary Temp	320 C		
Sheath gas	30		
Aux gas	10		
Aux gas heater	320 C		
S-lens RF level	50		
polarity	Negative		
Resolution	120 000		
AGC target	3e6		
Maximum IT	250 ms		
Scan range	100 – 1500 m/z		
resolution	15 000		
AGC target	2e5		
Maximum IT	30 ms		
Loop count	5		
Isolation window	0.4 m/z		
Collision energy	35		
Inclusion list			
Target	**Exact mass (m/z)**	**Internal standard**	**Exact mass (m/z)**
PFPrA	162.98239	$^{13}C_4$PFBA	216.99262
PFBA	212.97920	$^{13}C_4$PFBA	216.99262
PFPeA	262.97601	$^{13}C_5$PFPeA	267.99278

Table S5. Target compounds and internal standards, selected instrumental parameters for quantification by UPLC/MS/MS.

Instrument	Acquity UPLC + Xevo TQ-S (Waters Corp. Milford. MA)			
Column	Guard: BEH C18 (5 × 2.1 mm. 1.7 µm) Analytical: BEH C18 (50 × 2.1 mm. 1.7 µm)			
Mobile Phase	A: 90 % water and 10 % acetonitrile containing 2 mM ammonium acetate. B: 100 % acetonitrile containing 2 mM ammonium acetate.			
Injection volume	5 µL			
Column temperature	40 ˚C			
Gradient	**Time (min)**	**%A**	**%B**	**Flow Rate (ml/min)**
	0.0	90	10	0.40
	0.5	90	10	0.40
	5.0	20	80	0.40
	5.1	0	100	0.40
	6.6	0	100	0.40
	8.0	0	100	0.55
	10.0	90	10	0.40
Source temperature	150 ˚C			
Desolvation temperature	350 ˚C			
Cone gas flow rate	150 l/hr (nitrogen)			
Desolvation gas	650 l/hr (nitrogen)			
Nebulizer gas	7 bar			
Capillary voltage	3000 V			

Abbreviation[¤]	**Precursor Ion**	**Quantitative Product ion**	**Qualitative product ion**	**Internal standard**	**IS transition**
PFHxA	313	269	119	$^{13}C_2$-PFHxA	315>270
PFHpA	363	319	169	$^{13}C_4$-PFHpA	367>322
PFOA	413	169	369	$^{13}C_4$-PFOA	417>372
PFNA	463	419	219	$^{13}C_5$-PFNA	468>423
PFDA	513	469	269	$^{13}C_2$-PFDA	515>470
PFUnDA	563	519	269	$^{13}C_2$-PFUnDA	565>520
PFDoDA	613	569	169	$^{13}C_2$-PFDoDA	615>570
PFTrDA	663	619	169	$^{13}C_2$-PFDoDA	615>570
PFTeDA	713	669	169	$^{13}C_2$-PFDoDA	615>570
ADONA	377	251	85	$^{13}C_4$-PFOA	417>372
9Cl-PF3ONS	531	351	83	$^{13}C_4$-PFOS	503>80
11Cl-PF3OUdS	631	451	83	$^{13}C_4$-PFOS	503>80
PFBS	299	80	99	$^{18}O_2$-PFHxS	403>84
PFHxS	399	80	99	$^{18}O_2$-PFHxS	403>84
PFOS	499	80	99	$^{13}C_4$-PFOS	503>80
PFDS	599	80	99	$^{13}C_4$-PFOS	503>80
				$^{13}C_8$-PFOS (recovery standard)	507>80
				$^{13}C_8$-PFOA (recovery standard)	421>376

Table S6. Results of spike/recovery experiment performed in mushrooms (n=6)

		PFPrA	PFBA	PFPeA	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUnDA	PFDoDA	PFTriDA	PFTeDA	PFBS	PFHxS	PFOS	9Cl-PF3ONS	11Cl-PF3OUdS	ADONA
Method		**P1**	**P1**	**P1**	**C18**	**C18**	**C18**	**C18**	**C18**	**C18**	**C18**	**C18**	**C18**	**C18**	**C18**	**C18**	**C18**	**C18**	**C18**
Spiking amount (ng)		50	39	39	39	39	39	39	39	39	39	39	39	39	39	39	39	39	39
% recovery	1	106	103	121	113	107	107	106	111	115	117	112	111	92	103	109	118	92	89
	2	93	96	116	113	109	102	108	117	118	116	107	111	84	99	108	102	78	86
	3	143	97	112	106	107	106	98	112	121	112	113	111	94	98	107	108	92	101
	4	98	102	110	106	107	100	99	105	113	126	113	107	89	99	106	106	83	101
	5	111	102	125	104	108	106	107	112	118	123	101	95	89	101	102	108	84	89
	6	118	99	118	109	106	104	104	116	120	118	111	128	84	95	104	116	100	88
AVG		**112**	**100**	**117**	**108**	**107**	**104**	**104**	**112**	**118**	**119**	**110**	**110**	**89**	**99**	**106**	**110**	**88**	**92**
STDEV		18	3	5	4	1	3	4	4	3	5	5	10	4	3	3	6	8	7

Table S7. Results of spike/recovery experiment performed in fish muscle (n=6)

		PFPrA	PFBA	PFPeA	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUnDA	PFDoDA	PFTriDA	PFTeDA	PFBS	PFHxS	PFOS	9Cl-PF3ONS	11Cl-PF3OUdS	ADONA
	Method	**P1**	**P1**	**P1**	**C18**	**C18**	**C18**	**C18**	**C18**	**C18**	**C18**	**C18**	**C18**	**C18**	**C18**	**C18**	**C18**	**C18**	**C18**
	Spiking amount (ng)	40	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
% recovery	1	108	107	104	154	118	133	131	128	121	138	117	127	156	123	131	111	49	138
	2	108	110	117	136	113	118	127	133	107	128	101	119	183	123	137	131	67	117
	3	106	106	99	126	115	115	110	127	109	105	92	101	164	121	120	98	46	112
	4	100	114	95	127	108	121	116	121	158	105	98	109	159	116	126	95	45	112
	5	109	115	119	132	119	126	136	117	125	134	112	116	200	150	133	121	51	108
	6	95	102	91	133	107	113	117	115	136	131	85	103	145	86	128	105	47	113
	AVG	**104**	**109**	**104**	**135**	**113**	**121**	**123**	**124**	**126**	**124**	**101**	**112**	**168**	**120**	**129**	**110**	**51**	**117**
	STDEV	**5**	**5**	**12**	**10**	**5**	**7**	**10**	**7**	**19**	**15**	**12**	**10**	**20**	**20**	**6**	**14**	**8**	**11**

Table S8. Results of analysis of NIST SRM 2781 in sludge. Reference values for 9Cl-PF3ONS, 11Cl-PF3OUdS, ADONA, and PFPrA are unavailable.

	PFBA	PFPeA	PFHxA	PFHpA	PFOA (L+Br)	PFNA	PFDA	PFUnDA	PFDoDA	PFTriDA	PFTeDA	PFBS	PFHxS	PFOS (L+Br)[a]
Method	**P1**	**P1**	**C18**	**C18**	**C18**	**C18**	**C18**	**C18**	**C18**	**C18**	**C18**	**C18**	**C18**	**C18**
1	2.13	2.65	6.62	4.38	12.7	1.67	3.07	<LOQ	0.99	0.46	<LOQ	<LOQ	4.48	168
2	2.38	2.85	7.75	4.77	16.1	1.64	3.60	<LOQ	1.23	0.23	<LOQ	<LOQ	7.03	203
3	2.49	3.00	9.03	4.68	17.5	1.82	3.31	2.00	1.21	0.38	<LOQ	<LOQ	7.54	241
AVG	**2.33**	**2.84**	**7.80**	**4.61**	**15.4**	**1.71**	**3.33**	**<2.00**	**1.14**	**0.36**	**<0.04**	**<0.03**	**6.35**	**204**
STDEV	0.19	0.17	1.21	0.21	2.46	0.10	0.27	-	0.14	0.11	-	-	1.64	36
Range of values reported[b,c]	3.34-35.9	<3-9.56	9.97-17	4.83-9.6	18.7-31	1.7-6.59	2.67-6.05	<1.5-7.13	<1.5-4.81	<1.4-24	<0.63-1	<0.186-34.9	5-10	166-373

[a]sum of linear and branched isomers acquired from the m/z 499/80 transition. [b] Munoz et al. (2021). [c] Reiner et al. (2015)

Table S9. Limit of quantification (LOQ) and PFAS levels in the control substrate [ng g^{-1} dw] with standard deviation in parenthesis. At day 0, only the levels in the digestate were measured (not the complete compost mix). *Only one out of three replicates had levels above the LOQ. Mean is given as the level in that sample divided on the number of replicates. See Table S17 for decision rule on when to substitute observations below the LOQ. On Day 0, the levels of PFAS were measured in the digestate, not in the compost mixture.

						Inoculation			Harvest 1			Harvest 2		
	LOQ	**Day 0**	**Day 3**	**Day 6**	**Day 9**	**Sub**	**Bisp**	**No**	**Sub**	**Bisp**	**No**	**Sub**	**Bisp**	**No**
C3	2.40	<LOQ	4.0 (2.1)	1.5*	7.1 (1.1)	12 (4.7)	5.4 (3.1)	5.6 (2.8)	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
C4	1.06	<LOQ	<LOQ	<LOQ	<LOQ	0.47*	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
C5	0.25	<LOQ	<LOQ	<LOQ	0.07*	0.49 (0.31)	0.25 (0.09)	0.41 (0.17)	3.1 (1.7)	0.53 (0.56)	0.50 (0.24)	1.4 (0.43)	0.59 (0.11)	1.1 (0.25)
C6	0.04	<LOQ	0.22 (0.17)	0.72 (0.38)	0.68 (0.12)	1.6 (0.53)	0.81 (0.30)	1.1 (0.28)	1.4 (0.47)	1.6 (0.83)	1.0 (0.29)	1.3 (0.11)	1.6 (0.11)	2.3 (0.45)
C7	0.04	<LOQ	0.16 (0.14)	0.47 (0.35)	0.77 (0.06)	1.1 (0.51)	0.57 (0.36)	0.96 (0.32)	0.83 (0.29)	0.99 (0.83)	0.62 (0.27)	0.90 (0.11)	1.1 (0.14)	1.8 (0.46)
C8	0.04	0.29 (0.08)	0.47 (0.11)	0.67 (0.34)	0.84 (0.08)	1.1 (0.50)	0.72 (0.20)	0.84 (0.12)	1.33 (0.39)	1.4 (0.78)	0.57 (0.22)	1.3 (0.14)	1.3 (0.11)	1.6 (0.28)
C9	0.04	0.08 (0.04)	0.35 (0.14)	0.77 (0.46)	0.64 (0.28)	0.66 (0.40)	0.50 (0.20)	0.52 (0.16)	0.80 (0.30)	1.2 (0.75)	0.31 (0.20)	1.1 (0.20)	1.1 (0.20)	0.93 (0.18)
C10	0.03	0.17 (0.03)	0.48 (0.20)	0.87 (0.44)	0.80 (0.38)	0.74 (0.42)	0.61 (0.31)	0.54 (0.07)	0.94 (0.34)	1.4 (0.77)	0.45 (0.28)	1.3 (0.25)	1.4 (0.32)	1.0 (0.20)
C11	0.04	0.13 (0.05)	0.29 (0.08)	0.49 (0.59)	0.51 (0.18)	0.33 (0.19)	0.36 (0.19)	0.25 (0.09)	0.47 (0.14)	0.93 (0.44)	0.35 (0.19)	0.74 (0.26)	0.78 (0.22)	0.56 (0.15)
C12	0.03	0.06 (0.04)	0.42 (0.23)	0.89 (0.27)	0.51 (0.24)	0.39 (0.26)	0.42 (0.22)	0.28 (0.10)	0.44 (0.16)	1.3 (0.73)	0.35 (0.15)	0.67 (0.27)	0.78 (0.32)	0.56 (0.23)
C13	0.07	0.13 (0.07)	0.51 (0.28)	1.2 (0.24)	0.60 (0.19)	0.37 (0.25)	0.46 (0.19)	0.32 (0.19)	0.41 (0.16)	1.5 (0.95)	0.37 (0.14)	0.63 (0.25)	0.77 (0.38)	0.56 (0.17)
C14	0.03	0.03*	0.44 (0.24)	1.1 (0.15)	0.58 (0.12)	0.37 (0.26)	0.40 (0.27)	0.28 (0.04)	0.32 (0.14)	1.6 (1.64)	0.28 (0.11)	0.51 (0.20)	0.60 (0.29)	0.53 (0.21)
PFBS	0.03	<LOQ	<LOQ	0.21 (0.19)	0.05*	0.20 (0.13)	0.12 (0.12)	0.23 (0.07)	0.33 (0.09)	0.37 (0.19)	0.23 (0.05)	0.23 (0.04)	0.26 (0.08)	0.44 (0.18)
PFHxS	0.04	0.30*	0.55 (0.29)	0.52 (0.20)	0.54 (0.30)	0.87 (0.40)	0.59 (0.20)	0.75 (0.03)	0.76 (0.27)	0.90 (0.59)	0.12 (0.06)	0.70 (0.18)	0.59 (0.10)	0.44 (0.23)
PFOS	0.05	0.82 (0.47)	7.5 (5.7)	2.1 (2.7)	3.3 (2.6)	13 (7.6)	7.5 (5.7)	5.8 (3.0)	0.65 (0.19)	0.84 (0.35)	0.09 (0.07)	0.76 (0.20)	0.67 (0.15)	0.24 (0.11)
9Cl	0.03	<LOQ	0.05 (0.02)	0.11 (0.03)	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.08 (0.05)	<LOQ	0.08 (0.04)	0.07 (0.01)	0.01*
11Cl	0.04	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
ADONA	0.04	<LOQ	<LOQ	0.02*	<LOQ	0.06 (0.03)	0.04 (0.01)	<LOQ	0.05 (0.02)	0.05*	0.05 (0.02)	0.07 (0.02)	0.09 (0.04)	0.15 (0.05)

Table S10. Limit of quantification (LOQ) and PFAS levels in the spiked substrate [ng g^{-1} dw], with standard deviation in parenthesis.

					Inoculation			Harvest 1			Harvest 2		
	LOQ	**Day 3**	**Day 6**	**Day 9**	**Sub**	**Bisp**	**No**	**Sub**	**Bisp**	**No**	**Sub**	**Bisp**	**No**
C3	2.40	265 (95)	158 (88)	203 (49)	100 (46)	142 (16)	144 (57)	91 (75)	123 (94)	178 (93)	52 (43)	144 (72)	184 (114)
C4	1.06	96 (32)	64 (30)	107 (4.9)	72 (27)	106 (10)	79 (26)	77 (52)	77 (43)	102 (39)	49 (38)	78 (27)	186 (135)
C5	0.25	124 (31)	99 (41)	179 (11)	191 (92)	305 (15)	184 (62)	189 (56)	214 (94)	226 (53)	131 (82)	169 (42)	293 (67)
C6	0.04	161 (28)	146 (66)	286 (41)	332 (144)	536 (23)	341 (132)	282 (29)	348 (145)	386 (67)	216 (112)	263 (34)	366 (31)
C7	0.04	213 (31)	198 (90)	349 (51)	367 (119)	570 (14)	418 (162)	330 (33)	459 (199)	503 (94)	304 (140)	358 (46)	465 (59)
C8	0.04	242 (39)	210 (92)	334 (17)	307 (85)	455 (15)	361 (99)	401 (24)	563 (249)	605 (171)	378 (137)	448 (66)	598 (70)
C9	0.04	267 (41)	217 (87)	333 (17)	281 (71)	403 (9.1)	342 (64)	452 (26)	590 (268)	629 (174)	442 (149)	516 (78)	717 (97)
C10	0.03	315 (42)	254 (86)	363 (30)	308 (83)	424 (8.7)	378 (55)	496 (29)	598 (259)	625 (154)	465 (123)	561 (65)	636 (66)
C11	0.04	202 (32)	176 (61)	238 (23)	205 (53)	270 (8.8)	246 (36)	317 (15)	368 (163)	392 (119)	304 (82)	336 (42)	410 (50)
C12	0.03	246 (42)	205 (67)	277 (33)	229 (62)	307 (14)	286 (30)	320 (12)	329 (129)	403 (117)	297 (62)	331 (51)	394 (76)
C13	0.07	299 (39)	250 (83)	340 (57)	255 (65)	346 (21)	324 (24)	322 (7.0)	300 (120)	440 (113)	301 (63)	308 (65)	415 (69)
C14	0.03	277 (31)	235 (83)	323 (64)	220 (56)	301 (24)	314 (36)	257 (6.3)	206 (76)	394 (144)	230 (43)	225 (46)	328 (78)
PFBS	0.03	95 (22)	80 (23)	105 (10)	102 (34)	144 (4.1)	100 (16)	135 (28)	141 (59)	129 (31)	98 (41)	102 (15)	156 (54)
PFHxS	0.04	278 (48)	253 (103)	402 (17)	350 (116)	535 (18)	390 (134)	456 (34)	522 (250)	472 (127)	379 (82)	388 (77)	474 (21)
PFOS	0.05	232 (34)	194 (65)	271 (22)	233 (50)	330 (24)	273 (59)	400 (18)	429 (183)	411 (111)	368 (59)	390 (79)	435 (72)
9Cl	0.03	40 (5.9)	23 (8.1)	30 (2.2)	25 (5.7)	36 (7.0)	27 (5.2)	44 (2.0)	52 (21)	55 (25)	41 (10)	46 (12)	61 (3.4)
11Cl	0.04	4.7 (0.1)	4.0 (1.4)	5.1 (1.0)	4.0 (0.9)	6.5 (1.1)	4.1 (1.0)	6.8 (0.3)	6.9 (2.8)	10 (3.9)	6.5 (2.3)	6.3 (1.5)	11 (0.9)
ADONA	0.04	20 (3.6)	17 (7.8)	30 (3.3)	33 (8.5)	52 (0.9)	39 (10)	39 (3.6)	70 (33)	80 (23)	41 (19)	46 (6.7)	78 (4.8)

Table S11. Concentration of PFAS in the control substrate on ash basis [ng g^{-1} ash]. *Only one out of three replicates had levels above the limit of quantification (LOQ). Mean is given as the level in that sample divided on the number of replicates. See Table S17 for decision rule for when to substitute observations below the LOQ.

				Inoculation			Harvest 1			Harvest 2		
	Day 3	Day 6	Day 9	Sub	Bisp	No	Sub	Bisp	No	Sub	Bisp	No
C3	34 (20)	10*	40 (9.1)	65 (22)	31 (18)	33 (18)	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
C4	<LOQ	<LOQ	<LOQ	1.9*	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
C5	<LOQ	<LOQ	0.36*	2.7 (1.6)	1.4 (0.55)	2.5 (1.1)	12 (6.2)	1.8 (1.9)	1.6 (0.66)	5.6 (2.1)	2.0 (0.38)	3.6 (0.96)
C6	1.6 (1.1)	4.7 (2.4)	3.8 (0.14)	8.9 (2.9)	4.6 (1.7)	6.5 (1.9)	5.1 (1.6)	5.5 (2.7)	3.3 (0.76)	5.0 (0.37)	5.2 (0.36)	7.7 (1.7)
C7	1.3 (1.0)	3.1 (2.3)	4.3 (0.25)	5.9 (2.5)	3.2 (2.0)	5.7 (2.1)	3.1 (1.0)	3.5 (2.7)	2.1 (0.73)	3.5 (0.50)	3.5 (0.50)	6.2 (2.2)
C8	3.9 (0.55)	4.4 (2.3)	4.7 (0.58)	5.7 (2.5)	4.1 (1.1)	5.0 (0.90)	4.7 (1.4)	5.0 (2.5)	1.9 (0.59)	5.0 (0.72)	4.3 (0.41)	5.4 (1.2)
C9	2.7 (0.56)	5.0 (3.1)	3.5 (1.1)	3.5 (2.0)	2.8 (1.1)	3.1 (1.1)	3.0 (1.1)	4.4 (2.5)	1.0 (0.60)	4.2 (1.1)	3.7 (0.71)	3.2 (0.80)
C10	3.8 (0.81)	5.7 (2.9)	3.8 (1.6)	4.0 (2.1)	3.5 (1.7)	3.2 (0.57)	3.5 (1.2)	5.1 (2.5)	1.5 (0.80)	5.0 (1.3)	4.7 (1.1)	3.4 (1.0)
C11	2.3 (0.10)	3.2 (3.9)	2.8 (0.58)	1.8 (0.99)	2.1 (1.0)	1.5 (0.59)	1.8 (0.52)	3.3 (1.5)	1.1 (0.54)	3.0 (1.2)	2.6 (0.77)	1.9 (0.69)
C12	3.1 (1.3)	5.8 (1.8)	2.8 (0.92)	2.1 (1.3)	2.4 (1.2)	1.7 (0.68)	1.6 (0.58)	4.8 (2.9)	1.2 (0.42)	2.7 (1.3)	2.6 (1.1)	1.9 (0.98)
C13	3.8 (1.6)	7.8 (1.6)	3.3 (0.57)	2.0 (1.3)	2.6 (1.0)	1.9 (1.2)	1.5 (0.57)	5.3 (3.6)	1.2 (0.38)	2.5 (1.2)	2.6 (1.3)	1.9 (0.76)
C14	3.4 (1.4)	7.2 (1.0)	3.2 (0.71)	2.0 (1.4)	2.3 (1.5)	1.7 (0.29)	1.2 (0.51)	5.9 (6.1)	0.93 (0.30)	2.1 (0.95)	2.0 (1.0)	1.9 (0.89)
PFBS	<LOQ	1.3 (1.3)	0.27*	1.1 (0.72)	0.70 (0.68)	1.4 (0.47)	1.2 (0.34)	1.3 (0.63)	0.78 (0.11)	0.9 (0.14)	0.9 (0.28)	1.5 (0.73)
PFHxS	4.3 (2.0)	3.4 (1.4)	2.9 (1.2)	4.7 (1.9)	3.4 (1.1)	4.4 (0.30)	2.8 (0.96)	3.2 (1.9)	0.39 (0.18)	2.8 (0.94)	2.0 (0.34)	1.5 (0.94)
PFOS	65 (51)	14 (18)	18 (13)	74 (48)	43 (33)	34 (18)	2.4 (0.68)	3.0 (1.1)	0.30 (0.20)	3.1 (1.1)	2.2 (0.53)	0.85 (0.4)
9Cl	0.35 (0.11)	0.73 (0.20)	<LOQ	<LOQ	<LOQ	<LOQ	0.25 (0.11)	0.28 (0.19)	<LOQ	0.32 (0.17)	0.25 (0.044)	0.041*
11Cl	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
ADONA	<LOQ	0.11 (0.20)	<LOQ	0.30 (0.16)	0.23 (0.074)	<LOQ	0.18 (0.058)	0.13*	0.18 (0.068)	0.29 (0.051)	0.29 (0.13)	0.51 (0.19)

Table S12. Concentration of PFAS in the spiked substrate on ash basis [ng g^{-1} ash] with standard deviation in parenthesis.

				Inoculation			Harvest 1			Harvest 2		
	Day3	**Day 6**	**Day 9**	**Bisp**	**No**	**Sub**	**Bisp**	**No**	**Sub**	**Bisp**	**No**	**Sub**
C3	3304 (2778)	975 (379)	1454 (247)	873 (65)	1993 (1262)	1416 (1206)	867 (521)	661 (326)	314 (252)	448 (279)	1154 (1058)	189 (165)
C4	729 (223)	403 (125)	780 (102)	655 (49)	456 (168)	477 (214)	297 (174)	378 (132)	266 (175)	242 (123)	1186 (1117)	179 (146)
C5	942 (211)	626 (190)	1297 (101)	1884 (138)	1066 (409)	1262 (704)	831 (388)	844 (168)	656 (185)	522 (235)	1700 (1051)	472 (325)
C6	1222 (170)	930 (349)	2058 (157)	3315 (240)	1982 (853)	2193 (1113)	1349 (608)	1445 (196)	981 (93)	797 (287)	1991 (676)	777 (448)
C7	1616 (182)	1262 (519)	2510 (191)	3521 (197)	2432 (1047)	2420 (969)	1779 (826)	1882 (282)	1148 (98)	1087 (388)	2509 (753)	1090 (567)
C8	1835 (230)	1332 (491)	2414 (104)	2810 (192)	2097 (669)	2024 (710)	2185 (1030)	2259 (557)	1396 (69)	1360 (490)	3234 (1001)	1348 (566)
C9	2023 (225)	1399 (543)	2416 (301)	2490 (58)	1977 (463)	1849 (601)	2286 (1103)	2349 (565)	1574 (79)	1567 (570)	3862 (1126)	1576 (624)
C10	2395 (223)	1633 (533)	2638 (357)	2621 (100)	2185 (416)	2027 (690)	2317 (1071)	2334 (490)	1727 (108)	1697 (588)	3450 (1109)	1653 (532)
C11	1534 (181)	1139 (401)	1733 (268)	1667 (76)	1420 (274)	1346 (440)	1424 (670)	1462 (392)	1102 (57)	1014 (358)	2217 (676)	1080 (351)
C12	1868 (224)	1332 (485)	2025 (400)	1900 (142)	1654 (253)	1503 (494)	1276 (543)	1505 (381)	1115 (57)	995 (361)	2096 (491)	1055 (280)
C13	2272 (180)	1629 (619)	2487 (611)	2138 (171)	1873 (228)	1673 (528)	1161 (507)	1644 (363)	1120 (38)	921 (356)	2222 (584)	1069 (281)
C14	2104 (145)	1540 (646)	2366 (621)	1862 (161)	1812 (289)	1445 (447)	799 (328)	1469 (483)	894 (32)	676 (261)	1725 (326)	815 (184)
PFBS	718 (138)	514 (126)	765 (139)	890 (52)	579 (120)	671 (276)	544 (242)	482 (96)	469 (90)	309 (114)	928 (662)	350 (170)
PFHxS	2108 (256)	1616 (574)	2912 (193)	3302 (26)	2264 (876)	2308 (939)	2020 (1015)	1762 (409)	1585 (111)	1180 (458)	2601 (982)	1343 (358)
PFOS	1763 (170)	1251 (421)	1971 (311)	2041 (200)	1582 (416)	1527 (446)	1664 (760)	1536 (360)	1394 (81)	1195 (486)	2326 (611)	1303 (271)
9Cl	301 (33)	151 (56)	219 (35)	225 (49)	157 (37)	167 (49)	200 (88)	204 (86)	153 (8.1)	141 (63)	336 (123)	146 (44)
11Cl	36 (2.2)	26 (11)	37 (9.7)	40 (7.2)	24 (6.8)	27 (7.9)	27 (12)	36 (13)	24 (1.4)	19 (8.7)	58 (20)	23 (10)
ADONA	148 (24)	107 (41)	219 (7.5)	320 (7.3)	224 (70)	214 (72)	274 (137)	299 (76)	137 (10)	141 (52)	428 (155)	146 (77)

Table S13. Limit of quantification (LOQ) and mean PFAS concentration in the mushroom hats on fresh weight basis (ng g^{-1} fw), with standard deviation in parenthesis. When the average concentration of a PFAS is below the LOQ, it is because one or two of the replicates had a concentration below the LOQ which was replaced by LOQ/sqrt(2). * There was uptake in only one replicate, the concentration of that replicate divided by the number of replicates are given. See Table S17 for decision rule for when to substitute observations below the LOQ.

		Control				Spiked			
		Harvest 1		Harvest 2		Harvest 1		Harvest 2	
	LOQ	Sub	Bisp	Sub	Bisp	Sub	Bisp	Sub	Bisp
PFPrA	**1.3**	<1.3	<1.3	0.58*	<1.3	0.55*	1.0*	2.2 (0.93)	3.4 (2.1)
PFBA	**0.71**	<0.71	<0.71	<0.71	<0.71	<0.71	0.31*	<0.71	1.1 (0.50)
PFPeA	**0.05**	<0.05	<0.05	<0.05	<0.05	<0.05	0.06*	<0.05	0.43 (0.49)
PFHxA	**0.03**	<0.03	0.03 (0.01)	0.016*	<0.03	<0.03	0.14 (0.15)	0.13 (0.01)	0.24 (0.11)
PFHpA	**0.03**	<0.03	<0.03	0.011*	<0.03	<0.03	0.11 (0.11)	0.08 (0.00)	0.06 (0.05)
PFOA	**0.04**	0.05 (0.01)	0.05 (0.01)	0.05 (0.01)	<0.04	0.059 (0.01)	0.12 (0.08)	0.08 (0.00)	0.06 (0.01)
PFNA	**0.04**	<0.04	<0.04	<0.04	<0.04	<0.04	0.10 (0.08)	<0.04	0.05 (0.03)
PFDA	**0.03**	<0.03	<0.03	<0.03	<0.03	0.012*	0.09 (0.09)	0.06 (0.05)	0.07 (0.07)
PFUnDA	**0.05**	<0.05	<0.05	<0.05	<0.05	<0.05	0.08 (0.07)	0.06 (0.04)	0.07 (0.05)
PFDoDA	**0.17**	<0.17	<0.17	<0.17	<0.17	<0.17	<0.17	<0.17	<0.17
PFTriDA	**0.05**	<0.05	<0.05	<0.05	<0.05	<0.05	0.13 (0.13)	0.06 (0.04)	0.06 (0.04)
PFTeDA	**1.06**	<1.06	<1.06	<1.06	<1.06	<1.06	<1.06	<1.06	<1.06
PFBS	**0.02**	<0.02	<0.02	0.012*	<0.02	<0.02	0.10 (0.11)	0.08 (0.00)	0.15 (0.04)
PFHxS	**0.03**	<0.03	<0.03	0.013*	0.01*	0.011*	0.12 (0.11)	0.08 (0.07)	0.03 (0.01)
PFOS	**0.03**	<0.03	<0.03	<0.03	<0.03	<0.03	0.05 (0.04)	0.05 (0.04)	0.04 (0.03)
9Cl	**0.05**	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
11Cl	**0.06**	<0.06	<0.06	<0.06	<0.06	<0.06	<0.06	<0.06	<0.06
ADONA	**0.06**	<0.06	<0.06	<0.06	<0.06	<0.06	<0.06	<0.06	<0.06

Table S14. Mean PFAS concentration in the mushroom hats on a dry weight basis, with standard deviation given in parenthesis. * There was uptake in only one replicate, the concentration of that replicate divided by the number of replicates are given. See Table S17 for decision rule for when to substitute observations below the limit of quantification (LOQ).

	Control				Spiked			
	Harvest 1		Harvest 2		Harvest 1		Harvest 2	
	Sub	Bisp	Sub	Bisp	Sub	Bisp	Sub	Bisp
PFPrA	<LOQ	<LOQ	4.2*	<LOQ	4.2*	7.9*	14 (6.5)	28 (21)
PFBA	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	2.4*	<LOQ	9.0 (5.5)
PFPeA	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.46*	<LOQ	3.7 (4.5)
PFHxA	<LOQ	0.27 (0.11)	0.14*	<LOQ	<LOQ	1.2 (1.1)	0.78 (0.12)	2.0 (1.2)
PFHpA	<LOQ	<LOQ	0.10*	<LOQ	<LOQ	0.91 (0.79)	0.50 (0.03)	0.47 (0.47)
PFOA	0.33 (0.09)	0.40 (0.11)	0.35 (0.16)	<LOQ	0.45 (0.11)	1.0 (0.55)	0.48 (0.04)	0.50 (0.15)
PFNA	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.80 (0.62)	<LOQ	0.43 (0.33)
PFDA	<LOQ	<LOQ	<LOQ	<LOQ	0.09*	0.79 (0.64)	0.34 (0.29)	0.60 (0.64)
PFUnDA	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.71 (0.48)	0.38 (0.21)	0.59 (0.49)
PFDoDA	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
PFTriDA	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	1.1 (0.94)	0.38 (0.21)	0.52 (0.39)
PFTeDA	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
PFBS	<LOQ	<LOQ	0.10*	<LOQ	<LOQ	0.77 (0.80)	0.46 (0.03)	1.2 (0.53)
PFHxS	<LOQ	<LOQ	0.11*	0.09*	0.09*	1.1 (0.83)	0.50 (0.38)	0.23 (0.13)
PFOS	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.46 (0.28)	0.31 (0.25)	0.35 (0.30).
9Cl	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
11Cl	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
ADONA	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

Table S15. Log 10 Bioaccumulation factors, with standard deviation in parenthesis. *There was mushroom uptake in only one replicate. The BAF given in the table is the BAF of that replicate, not divided by the number of replicates (unlike what has been done for the concentration data). C12 (PFDoDA), C14 (PFTeDA), ADONA, and F-53B compounds are not included in the table as there was no detectable uptake of these compounds in any of the mushroom samples.

	Control				Spiked			
	Harvest 1		Harvest 2		Harvest 1		Harvest 2	
	Sub	Bisp	Sub	Bisp	Sub	Bisp	Sub	Bisp
C3					-1.14*	-0.98*	-0.33 (0.27)	-0.85 (0.56)
C4						-1.23*		-1.05 (0.36)
C5						-2.31*		-1.99 (0.75)
C6		-0.75 (0.35)	-0.36*			-2.64 (0.63)	-2.29 (0.07)	-2.20 (0.26)
C7			-0.42*			-2.83 (0.57)	-2.65 (0.01)	-3.05 (0.52)
C8	-0.58 (0.02)	-0.52 (0.28)	-0.61 (0.22)		-2.96 (0.14)	-2.75 (0.27)	-2.80 (0.02)	-2.99 (0.16)
C9						-2.94 (0.45)		-3.20 (0.41)
C10					-3.25*	-2.99 (0.52)	-3.16 (0.47)	-3.19 (0.61)
C11						-2.75 (0.34)	-2.87 (0.31)	-2.87 (0.37)
C13						-2.55 (0.47)	-2.89 (0.31)	-2.84 (0.24)
PFBS			0.22*			-2.39 (0.34)	-2.21 (0.06)	-1.98 (0.18)
PFHxS				-0.30*	-0.22*	-3.20*	-2.81 (0.59)	-2.90 (0.42)	-3.29 (0.31)
PFOS						-3.01 (0.39)	-3.12 (0.44)	-3.18 (0.44)

Table S16. "Worst-case" – bioaccumulation factors (not log-transformed). When there are empty cells, it means there was uptake in all the replicates of the treatment. For some compounds, a value is given both in Table S6 and in Table S7. This happens when there is uptake above the LOQ in some of the replicates but not all. Then the values above the LOQ, and possible imputations, are used to calculate the real BAFs in Table S6, while the LOQ-values are used to calculate the worst-case BAFs.

	Control				Spiked			
	Harvest 1		Harvest 2		Harvest 1		Harvest 2	
	Sub	Bisp	Sub	Bisp	Sub	Bisp	Sub	Bisp
PFPrA	3.8	4.8	2.9	5.1	0.14	0.13		
PFBA	4.7	6.0	4.8	6.3	0.09	0.10	0.16	
PFPeA	0.14	1.6	0.28	0.81	<0.01	<0.01	<0.01	
PFHxA	0.17	0.07	0.12	0.18	<0.01	<0.01		
PFHpA	0.29	0.40	0.18	0.27	<0.01	<0.01		<0.01
PFOA	0.10	0.08	0.04	0.29				
PFNA	0.41	0.38	0.28	0.35	<0.01	<0.01	<0.01	<0.01
PFDA	0.26	0.23	0.17	0.21	<0.01	<0.01	<0.01	<0.01
PFUnDA	0.82	0.62	0.51	0.66	<0.01	<0.01	<0.01	<0.01
PFDoDA	3.1	1.9	2.0	2.4	<0.01	<0.01	<0.01	<0.01
PFTriDA	0.99	0.58	0.65	0.77	<0.01	<0.01	<0.01	<0.01
PFTeDA	27	14	17	20	0.03	0.05	0.03	0.04
PFBS	0.46	0.58	0.46	0.85	<0.01			
PFHxS	0.31	0.41	0.25	0.39	<0.01	<0.01		<0.01
PFOS	0.35	0.36	0.30	0.45	<0.01	<0.01	<0.01	<0.01
9Cl	7.2	10	5.2	6.6	<0.01	0.01	<0.01	<0.01
11Cl	11	13	11	14	0.07	0.09	0.07	0.07
ADONA	9.4	11	5.8	8.8	0.01	0.01	0.01	<0.01

Spiking procedure

The PFAS spiking solution was poured into the liquid digestate and mixed with a hand-held cement mixer (Elektromix SY-HM-140; Yongkang Well-King Industry and Trade Co. Ltd., China), prior to mixing with straw, chalk, gypsum, and hot compost. The solution container was rinsed three times with 10 ml methanol which was poured into the digestate. The same procedure was done in the control treatment, where the corresponding amount of methanol and water was added. The control batch was always handled first to limit the risk of cross contamination.

Preparation of mushroom substrate

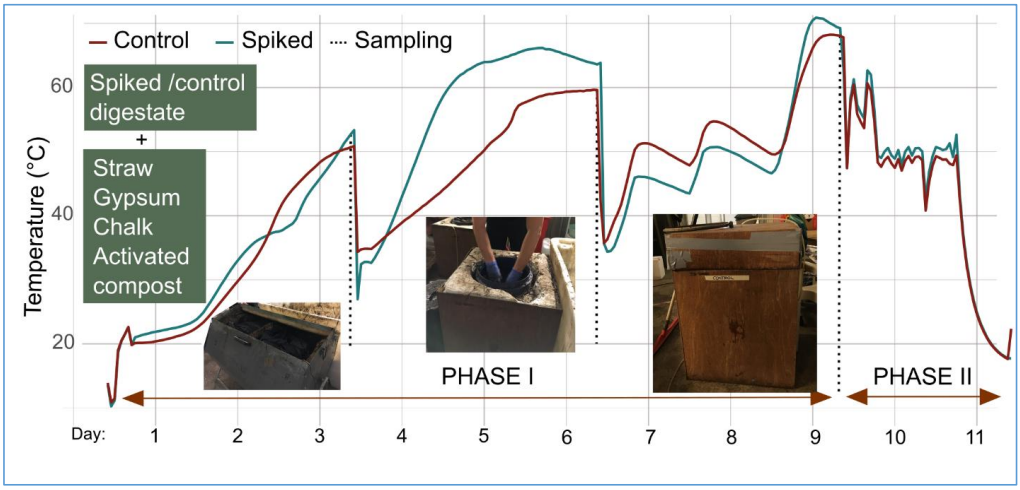

Figure S1. Preparation of the mushroom substrate. Phase I: Composting, Phase II: Pasteurisation. The blue and red lines show the temperature (°C) development in the spiked and control batches, respectively. The night between day 7 and 8 there was a power break which prolonged the composting process by one day. At day 11 (inoculation day), the substrate was inoculated with fungi and spiked with pharmaceuticals.

Phase I – Composting. The ingredients were mixed well before being composted in two separated composting drums (one for each batch – control and spiked, JK270; Joraform; Mjölby. Sweden) for three days until there was a volume loss due to heat generation. Thereafter the substrate was mixed with a hay fork and moved to two 60 L plastic containers (Peguform, Bötzingen, Germany) with a sealed lid and controlled air flow (to obtain similar conditions as in commercial bulk systems; 70-80°C and 6-9% O_2 v/v). The O_2 levels was measured three times a day with a portable gas analyser (GA5000, GeoTech, QED Environonmental Systems Ltd., Coventry, UK). The containers were built into a plywood box and polystyrene was used as isolation between the container and plywood box. A further description can be

found in Stoknes et al. (2013). The substrate was turned twice (by use of the hay fork) at an interval of 3 days, giving a total of 9 days for Phase I. The temperature development in the spiked and control batches are found in Figure S1 during Phase I and II along with pictures from the preparation process.

Phase II – Pasteurisation. The mushroom substrate was moved into a miniature Phase II tunnel, where temperature was slowly increased to 56-60°C over 24 hours and then maintained for 6 hours of pasteurisation. A rebuild 400 L freezer box was used as a Phase II tunnel. The freezer had stainless steel sheet dividers which allowed both batches to be treated simultaneously (see Stoknes et al. (2013) for details). During conditioning the temperature was decreased to 55°C for another 6 hours. Then the temperature was kept at 46-50°C until ammonia (NH_3 gas) had dissipated below 10 ppm. The NH_3 gas level was measured with Dräger accuro Pump with gas detection tubes of the type Ammonia 2/1 2-30 ppm (67 33 231; Dräger Inc, Huston, TX, USA). About 10 kg material was lost during composting, and at the end of Phase II there was 29.7 kg of control mushroom substrate, and 30.8 kg of spiked mushroom substrate.

Mushroom cultivation – additional information.

Casing material: The casing material was dark peat (from Holmen transport) mixed with $Ca(OH)_2$ to increase the pH of the casing material up to 7 – 7.5 and with gypsum for structure maintenance (120 g gypsum / 40 l peat). Casing is crucial for the development of fruiting bodies (i.e.. mushroom hats), as it holds enough water for basidiocarps to develop properly. The casing layer was sprinkled for the first few days with distilled water. The opened bags were moved to a cultivation chamber holding 25-30°C, where the air humidity was kept at 70-75%. The chamber received LED-light with colour temperature of 6000 K.

Harvesting. The mushroom fruiting bodies were picked/harvested from the individual growing bags as they obtained maturity. The maturity in mushrooms can be described as when the cap of the mushroom is starting to open, however the veil under the mushroom cap is still intact and attached to the cap edges. The gills are pink and the spores has not yet been released. All mushrooms in a single bag were harvested at once. Each bag had 1-3 harvests/flushes, of which the first was harvested 24 days after the casing was applied (83 days after inoculation) and the last was harvested 12 weeks after the casing day. For those units where the mushrooms had three flushes. the third was not analysed for PFAS.

Sampling procedure for the mushroom substrate

During phase I and II, small amounts of substrate were collected from a variety of spots in the batch and combined to a composite sample. The composite sample was cut into small pieces by a scissor and mixed well before the substrate was weighed into polypropylene sample tubes for the various analysis. When the casing was applied, the composite sample was made by taking three subsamples from each

bag: one from the top, one from the bottom, and one from the middle by making a small hole in the bag and taking out the substrate with an awl. Dry matter, ash, and pH were determined immediately. Samples for all other analysis were kept at -18°C until analysis.

Temperature logging

The temperature was monitored by loggers from Onset HOBO S-WCA-M003 (Bourne. MA. USA). During Phase I and II, three loggers were placed in each compost, one in the middle and two towards the sides. During mushroom cultivation there was one logger in the middle of each bag.

Substrate extraction procedure

Substrate samples were homogenized and then oven dried at 70˚C for 12 hrs. A 1 g (dry weight) sub-sample was then weighed into a polypropylene centrifuge tube and fortified with isotopically labelled internal standards (complete list provided in Table S4). Methanol (5 ml) was added, and the samples were shaken vigorously for 5 min, sonicated for 15 minutes, and then centrifuged (3000 rpm, 10 min). The resulting supernatant was transferred to a 13 ml PP tube, and after repeating the extraction once the supernatants were combined and concentrated under a stream of nitrogen to approximately 1 ml. The extract was transferred to a 1.7 ml Eppendorf centrifuge tube containing 25 mg ENVI-Carb and 50 µl glacial acetic acid, vortexed, and centrifuged (3000 rpm, 10 min). A portion (300 µl) of the extract was transferred to a microvial and fortified with 25 µl of a recovery standard solution (Table S4) and 300 µl of 4 mM NH_4OAc in water prior to instrumental analysis.

Mushroom extraction procedure

Mushroom samples were extracted by placing 1 g (wet weight) into a centrifuge tube together with 8 stainless steal beads (4.8 mm diameter) and 5 ml acetonitrile. The samples were then placed in a bead blender (SPEX SamplePrep 1600 MiniG) for 5 min at 1500 rpm, then centrifuged (3000 rpm, 10 min). The supernatant was removed to a 13 ml PP tube, and the extraction was repeated; thereafter the supernatants were combined and concentrated under a stream of nitrogen to approximately 1 ml. The extract was transferred to a 1.7 ml Eppendorf centrifuge tube containing 25 mg ENVI-Carb and 50 µl glacial acetic acid, vortexed, and centrifuged (3000 rpm, 10 min). A portion of the extract (300 µl) was transferred to a microvial and fortified with 25 µl of a recovery standard solution (Table S4) and 300 µl of 4 mM NH_4OAc in water prior to instrumental analysis.

Instrumental analysis

Mushroom and substrate extracts were analysed using two different methods. PFPrA, PFBA, and PFPeA were analysed with a Dionex Ultimate 3000 liquid chromatograph equipped with a Thermo Scientific Acclaim Trinity P1 column (3 µm, 2.1 × 100mm) and coupled to a Q Exactive HF Orbitrap (Thermo Scientific). The instrument was operated in negative electrospray ionization, full scan-data dependent MS2 mode, using a list of parent ions for PFPrA, PFBA, and PFPeA as an inclusion list. Finally, C6-C14 PFCAs, C4, C6, and C8 PFSAs, ADONA, 9Cl-PF3ONS, and 11Cl-PF3OUdS were determined using an Acquity UPLC equipped with a Waters BEH C18 guard column (5 × 2.1 mm, 1.7 µm) and a Waters BEH C18 analytical column (50 × 2.1 mm, 1.7 µm). The instrument was operated in negative electrospray ionization, multiple-reaction-monitoring mode. Quantification was carried out using an internal standard or isotope dilution approach, with a 5 to 8 point linear calibration curve with 1/x weighting, excluding the origin (see tables S2-S4 for PFAS and their corresponding internal standards). Correlation coefficients (r^2) were >0.99 for all targets with the exception of PFTrDA (>0.85), PFTeDA (>0.95), PFDS (>0.95), and 11Cl-PF3OUdS (>0.98), which were lower, possibly due to use of non-exactly matched isotopically labelled internal standards. Nevertheless, since accuracy of QC samples was reasonable for these targets, the curves were deemed sufficient for quantification of samples. Details of all instrumental methods, including LC gradients are provided in Tables S2, S3, and S4.

Quality control

To account for laboratory background contamination, procedural blanks (no matrix) were processed together with all samples. Among the remaining ultra-short chain PFAS analysed by LC-Orbitrap MS, blanks (n=2 for mushrooms and n=4 for substrates) displayed detectable levels of PFBA; consequently, LODs for both substrates and mushrooms were defined as the average concentration in the blank plus 3 time the standard deviation of the blanks. PFPrA and PFPeA were not observable in blanks and therefore LODs were based on the concentration of the lowest calibration point. Finally, for the remaining targets, LODs were based on the higher of either the lowest point on the calibration curve or the average concentration in the blanks plus 3 time the standard deviation of the blanks (n=3 for substrates and n=2 for mushrooms).

To assess accuracy and precision, replicate spike/recovery experiments were performed using mushrooms purchased from the grocery store (Agaricus bisporus; n=6 fortified with 39 – 180 ng of individual PFAS; n=3 unfortified, Table S5) as well as in-house pooled fish muscle (chosen due to low background PFAA contamination; n=6 fortified with 2.5 - 200 ng of individual PFAS; n=3 unfortified, Table S6). Percent recoveries, calculated by comparing the measured concentrations to expected concentrations in the spiked samples (after accounting for background levels) ranged from 88 – 119%

(standard deviation: 1 – 25%) in mushrooms. In fish muscle, recoveries were 101 – 129% (standard deviation 5 – 20%) for most PFAS, with the exception of PFBS which showed higher recoveries than expected (168 ± 20%, respectively) and 11Cl-PF3OUdS, which displayed lower recoveries than expected (51±8%), which was attributed to the use of non-exactly matched isotopically labelled internal standards for these substances. Overall, however, accuracy and precision were deemed suitable for the present work. In addition, samples of NIST 2781 (domestic sludge) were extracted and compared to measurements reported elsewhere (Table S7). In general, concentrations were either within the range of those reported previously or slightly lower. Most notably, concentrations of PFBA (determined using the Trinity P1 column) were considerably lower (2.33 ± 0.19 ng/g compared to 3.34 – 35.9 ng/g reported previously using C18 columns). Recently a PFBA interference, 3 oxo-dodecanoic acid, was identified in biological samples (Bangma et al.. 2021), and we speculate that this substance may also exist in SRM 278, but was removed using the Trinity P1 column, resulting in lower concentrations than reported elsewhere. Overall, these data indicate reasonable accuracy and precision of the method.

Substitution of values below the limit of quantification

Values below LOQ were substituted with LOQ/sqrt(2) as described elsewhere (Nyberg et al., 2018), in certain cases. About 80% of the observations of PFAS in the mushroom hats were below the LOQ. Replacing all <LOQ values by e.g., LOQ/sqrt(2) gives the following problems: (i) The mushroom uptake would seem higher for those compounds with higher LOQs, even when there was no detectable uptake in any of the mushroom hats. (ii) The analysis of PFAS in mushrooms were done on a wet weight basis before the concentrations was recalculated into dry weight basis. Even in cases where all replicates were <LOQ, the mushroom concentration would have a standard deviation due to the varying dry matter content of the mushrooms.

Therefore, it was decided to replace the <LOQ observations by the LOQ/square root of 2 only in certain cases, as seen in the following overview. The number of replicates for one treatment (combination of species and control/spiked) at one sampling time was between 2 and 4. The rules were applied for the mushroom substrate as well, where about 20% of the observations were <LOQ.

Table S17. Decision rule for imputation of observations below the limit of quantification (LOQ)

Number of replicates >LOQ	Replace <LOQ?	Mean given as
None	No	<LOQ
One (in cases with 3 or 4 replicates)	No	The concentration in the >LOQ sample divided by the number of replicates (labelled by a star)
At least 50% (1 when there are 2 replicates, 2 when there is 3 or 4)	Yes, by LOQ / sqrt(2)	The mean of all replicates, including imputations.

```
Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']
Formula: C8 ~ Treatment + Time + Species + (1 | Unit)
   Data: C8

REML criterion at convergence: 6.5

Scaled residuals:
    Min      1Q  Median      3Q     Max
-1.1261 -0.3600 -0.0645  0.4167  1.2407

Random effects:
 Groups   Name        Variance Std.Dev.
 Unit     (Intercept) 0.06816  0.2611
 Residual             0.01626  0.1275
Number of obs: 22, groups:  Unit, 14

Fixed effects:
               Estimate Std. Error       df t value Pr(>|t|)
(Intercept)     0.27047    0.12560 10.27406   2.153   0.0560 .
TreatmentSpiked 0.32465    0.15233 10.04458   2.131   0.0588 .
TimeH2          -0.03765    0.06290  6.55778  -0.598   0.5696
SpeciesBisp     0.22053    0.15195 10.40008   1.451   0.1762
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Correlation of Fixed Effects:
            (Intr) TrtmnS TimeH2
TretmntSpkd -0.502
TimeH2      -0.199 -0.037
SpeciesBisp -0.590 -0.029  0.117
```

Figure S2. Result of linear mixed model with treatment (spiked/control), harvest time, and mushroom species as fixed effects, experimental units as random effects, and concentration of PFOA in the mushrooms as the dependent variable.

```
Call:
lm(formula = LogBAF ~ CF2, data = pFCA_C3toC7_Spiked)

Residuals:
     Min       1Q   Median       3Q      Max
-1.00070 -0.34628  0.09527  0.29432  0.62029

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)  0.85763    0.29827   2.875  0.00833 **
CF2         -0.53839    0.05474  -9.836 6.79e-10 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 0.4262 on 24 degrees of freedom
  (24 observations deleted due to missingness)
Multiple R-squared:  0.8012,    Adjusted R-squared:  0.7929
F-statistic: 96.74 on 1 and 24 DF,  p-value: 6.787e-10
```

Figure S3. Result of linear regression on the log10 BAFs of the PFCAs C3 through C7. Non-detects are not included in the regression. CF2 equals the number of carbons in the compounds.

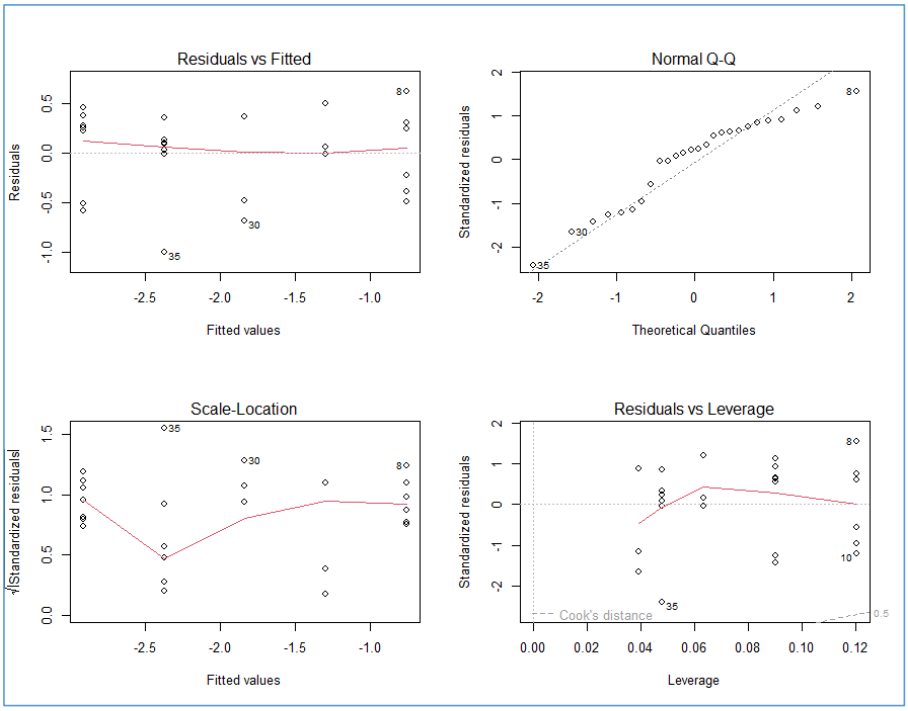

Figure S4. Diagnostic plot for the linear regression of the log BAF as a function of carbon chain length, for the PFCAs C3 to C7.

```
Call:
lm(formula = LogBAF ~ CF2, data = PFSA_Spiked)

Residuals:
    Min      1Q  Median      3Q     Max
-0.7328 -0.2880  0.1263  0.3127  0.5321

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept) -1.47734    0.32852  -4.497 0.000220 ***
CF2         -0.21733    0.05291  -4.107 0.000548 ***
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 0.396 on 20 degrees of freedom
  (8 observations deleted due to missingness)
Multiple R-squared:  0.4575,    Adjusted R-squared:  0.4304
F-statistic: 16.87 on 1 and 20 DF,  p-value: 0.0005476
```

Figure S5. Result of linear regression on the log10 BAFs of the PFSAs PFBS, PFHxS, and PFOS. Non-detects are not included in the regression. CF2 equals the number of carbons in the compounds.

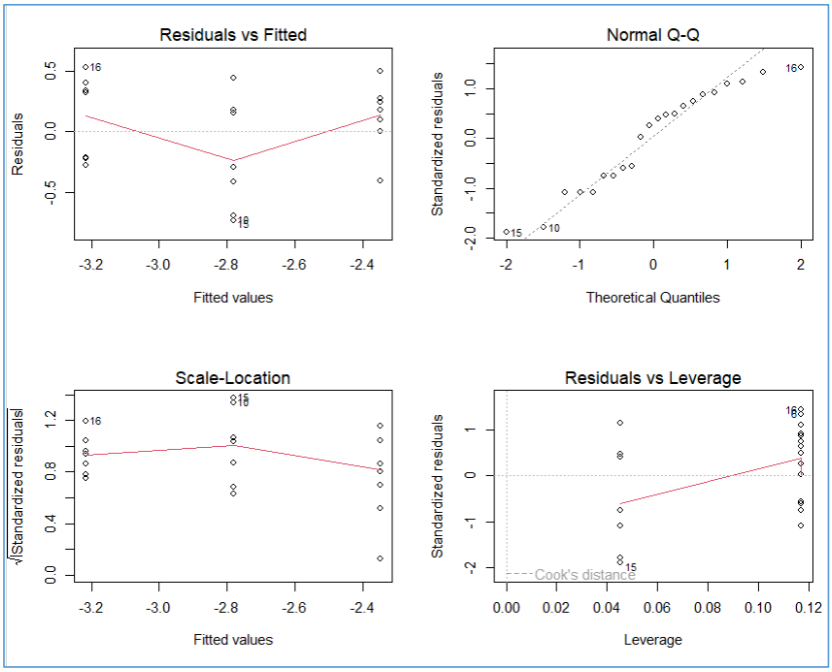

Figure S6. Diagnostic plots for linear regression of the log BAF as a function of carbon chain length, for the PFSAs PFBS, PFHxS, and PFOS.

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Paper IV

Low uptake of pharmaceuticals in edible mushrooms grown in contaminated biogas digestate

Astrid S. Nesse [a]*, Agnieszka Jasinska [b,c], Ketil Stoknes [b], Stine Göransson Aanrud [d], Kristin Ogner Risinggård [d], Roland Kallenborn [e], Trine A. Sogn [a], Aasim M. Ali [e,f]

[a] *Norwegian University of Life Sciences, Faculty of Environment and Natural Resources, Elizabeth Stephansensvei 31, 1433 Ås, Norway*

[b] *Lindum AS, Lerpeveien 155, 3036 Drammen, Norway*

[c] *Poznan University of Life Sciences, Department of Vegetable Crops, ul. J.H. Dabrowskiego 159, 60-594 Poznan, Poland*

[d] *Norwegian University of Life Sciences, Faculty of Veterinary Medicine, Elizabeth Stephansensvei 15, 1433 Ås, Norway*

[e] *Norwegian University of Life Sciences, Faculty of Chemistry and Biotechnology, Chr. M. Falsens vei 18, 1433 Aas, Norway*

[f] *Institute of Marine Research, Nordnesgaten 50, 5005 Bergen, Norway*

* Corresponding author.

Email: astrid.solvag.nesse@nmbu.no,

Phone: +47 672 31 899

Abstract

The uptake dynamics of two sulfonamide antibiotics, two fluoroquinolone antibiotics, and the anticonvulsant carbamazepine during the cultivation of two species of edible mushrooms (*Agaricus subrufescens* and *A. bisporus*) was investigated. None of the antibiotics were accumulated by the mushrooms, while carbamazepine and its transformation product carbamazepine-10,11-epoxide were taken up by *A. bisporus* fruiting body but only in small amounts (up to 0.76 and 1.85 μg kg^{-1} dw, respectively). The sulfonamides were quickly removed from the mushroom growth substrate, while the recalcitrant fluoroquinolones and carbamazepine were only partially removed. Removal efficiencies were generally higher for *A. subrufescens* than *A. bisporus*, but *A. subrufescens* was also grown at a slightly higher culture temperature. *A. subrufescens* also showed a lower uptake of contaminants. Comparison of maximum dietary intake with other common exposure sources showed that these mushrooms can safely be eaten although produced on a polluted substrate, with respect to the investigated compounds.

Keywords: *Agaricus, Antibiotics, Carbamazepine, LC-MS/MS, Biogas digestate, Remediation*

1. Introduction

Edible mushrooms are common commercially available food products globally produced in quanta of about 43 million tons a year, comparable in amount to e.g., lettuce and carrots (FAOSTAT, 2023). The common button mushroom (*Agaricus bisporus*) is grown mainly on agricultural residues such as hay and wheat straw with nitrogen supplements such as chicken manure or inorganic nitrogen (Suwannarach et al., 2022), but can also be grown in a substrate composed of biogas digestate and straw (Stoknes et al., 2013).

Biogas digestates are produced by anaerobic digestion (AD) of various organic wastes such as sewage sludge, food waste, fish silage, and manure. The digestate can contain organic contaminants, including antibiotics (Nesse et al., 2022), pharmaceuticals and personal care products (PPCPs) (Ali et al., 2019), per- and polyfluoroalkyl substances (PFAS), and legacy pollutants such as dioxins, polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) (Suominen et al., 2014). Chicken manure may also contain e.g., antibiotics and PFAS (García-Valcárcel and Tadeo, 2013; Ghirardini et al., 2020).

Despite the large production volume of mushrooms and the likely presence of pollutants in their growth substrate, only a few studies have investigated the uptake of organic pollutants into the fruiting bodies. Using the same experimental setup as reported here, we have recently investigated the uptake of PFAS in *A. bisporus* and *A. subrufescens*. Mushroom uptake was overall very low, and there was a distinct chain-length dependency with mushroom uptake decreasing with increasing PFAS alkyl chain (Nesse et al., 2023). Schildt et al. (2021) and Gbylik-Sikorska (2020) investigated the uptake of pharmaceuticals from spiked commercial *A. bisporus* kits. Golovko et al. (2021) assessed the uptake of selected PFAS, pharmaceuticals and one paraben in the white-rot oyster mushroom (*Pleurotus ostreatus*) grown in a substrate fertilized with biogas digestate, and in a standard substrate. Common for all these experiments was that most compounds were rapidly removed from the substrate and did not accumulate in the mushrooms, whereas some persistent compounds were taken up in low amounts.

In addition to uptake in the mushroom, the contaminant levels in the substrate are of interest and importance, as the spent mushroom substrate can be further used in horticultural growth media (Stoknes et al., 2019b) or as soil amendment (Leong et al., 2022), which could lead to plant uptake of the pollutants (Carter et al., 2014).

Fluoroquinolones and sulfonamides are two frequently used classes of antibiotics applied in human and veterinary treatment. In addition to their high stability towards metabolization, fluoroquinolones are also resistant towards hydrolysis as well as thermal and biological degradation (Al-Ahmad et al., 1999; Thiele-Bruhn, 2003), sorb to solids, and are not degraded during anaerobic digestion (AD) (Giger et al., 2003; Golet et al., 2003). Thus, 70 – 90% of norfloxacin and ciprofloxacin in raw sewage sludge ends in the digestate after AD (Golet et al., 2003; Lindberg et al., 2006). Sulfonamides are readily degraded (Hu et al., 2022; Ingerslev and Halling-Sørensen, 2000) but are nevertheless frequently found in high concentrations in manures (Ghirardini et al., 2020) and waste waters (Hu et al., 2022). Both compound groups can be found in digestate in concentrations in the µg – mg kg^{-1} range (Golet et al., 2003; Lindberg et al., 2006; McClellan and Halden, 2010) and are reported to accumulate in soils irrigated with wastewater along with the recalcitrant pharmaceutical carbamazepine (Dalkmann et al., 2012). Carbamazepine was found in all digestate samples in a Norwegian screening of biogas digestates, regardless of feedstock (Ali et al., 2019). In a national screening of 110 biosolids samples in the U.S.A, carbamazepine, norfloxacin, ciprofloxacin and caffeine were detected in all samples (McClellan and Halden, 2010).

Fungi are known for their ability to degrade complex organic molecules and have shown great potential for bioremediation purposes (Harms et al., 2011). The most studied fungi in this context are the wood-degrading white-rot fungi (Tortella et al., 2015), which (depending on e.g., species and growth conditions) can degrade both ciprofloxacin, norfloxacin and carbamazepine, along with a variety of other organic pollutants (Olicón-Hernández et al., 2017; Prieto et al., 2011). Although adapted to partly decomposed organic material (Morin et al., 2012)., *Agaricus spp.* are as well equipped with extracellular enzymes (Doddapaneni et al., 2013; Ullrich et al., 2005) as well as the intracellular enzyme system Cytochrome P_{450}

(CYP_{450}) (Doddapaneni et al., 2013), which is important for biochemical degradation of xenobiotics of e.g., carbamazepine (Golan-Rozen et al., 2015, 2011).

In this experiment, uptake of four antibiotics and carbamazepine in two edible mushrooms *Agaricus bisporus* and *Agaricus subrufescens* were studied, along with the removal of the compounds in the mushroom growth substrate. The mushroom substrate was based on biogas digestate and wheat straw, as described by Stoknes et al. (2013) with modifications by Jasinska et al. (2022). In addition to the five spiked compounds, the mushroom substrate was screened for 37 PPCPs found in biogas digestate (Ali et al., 2019). Mushroom uptake of detected non-added compounds was included in the study.

2. Materials and methods

A list of standards and reagents, dose preparation procedure, analytical methods for determination of compost quality and concentrations of PPCPs, and quality control can be found in the supplementary information. Target compounds which were detected in the mushroom substrate are listed in Table S1 and include five compounds which were added to the substrate (carbamazepine, norfloxacin, ciprofloxacin, sulfamethazine and sulfadiazine) in known concentrations, and four non-added compounds which were detected in the substrate (carbamazepine-10,11-epoxide, caffeine, tris (1-chloro-2-propyl) phosphate (TCPP), metoprolol, and *N*-*N*-Diethyl-3-methylbenzamide (DEET). The mushroom substrate was screened for an additional 33 compounds, which are listed in Table S2.

2.1 Experimental setup

2.1.1 Preparation of mushroom substrate. Control and spiked batches of mushroom substrate were prepared simultaneously. Each batch was prepared by mixing 33 kg biogas digestate of 4.3% dry matter (DM), 10.5 kg wheat straw, 300 g lime as calcium dihydroxide ($Ca(OH)_2$), 300 g lime as calcium sulphate ($CaSO_4$),and

1 kg activated garden compost for inoculation, to a total DM of 25%. The digestate was collected from a municipal plant with a feedstock of 73% household food waste and 27% cow manure. The batches were composted for 9 days and pasteurized for 2 days. Details on the substrate preparation and mushroom cultivation can be found in Nesse et al. (2023), while details on the equipment used can be found in Stoknes et al. (2013).

2.1.2 Inoculation with mushroom spawn and spiking of mushroom substrate. After pasteurization, each batch was split in 11 units of 2.8 kg (Spiked) and 2.7 kg (Control) each, which served as the experimental units. Four were inoculated with *A. subrufescens* (strain M7700), four with *A. bisporus* (strain M7243), and three were not inoculated and served as control. 90 grams granular spawn was used for each bag (Mycelia, Deinze, Belgium). Simultaneously, each spiked unit received 250 mL Milli-Q water containing 910 µg ciprofloxacin, 875 µg norfloxacin, 893 µg sulfadiazine, 923 µg sulfamethazine and 933 µg carbamazepine, to a final substrate concentration of 1166 – 1382 µg kg^{-1} dm or 6.8 – 9.4 mg kg^{-1} ash. The control units received 250 mL Milli-Q water.

2.1.3 Mushroom cultivation and sampling. The 22 units from the control and spiked treatment were kept at 25°C in sealed 7 L polypropylene bags with ventilation filters (PP50/SEU4/V40-51, SacO2 Microsac, Deinze, Belgium) for 60 days, when the spawn had overgrown the substrate. Then, the bags were opened, a casing layer (5-8 cm of dark peat, chalk and gypsum) was added, and the open bags were moved to a cultivation chamber holding 25-30°C and 70-75% relative humidity. After 9 days, the mycelia had overgrown the casing layer as well and the emergence of mushroom fruiting bodies were initiated by adjusting the growth conditions. *A. bisporus* bags were moved to a chamber holding 17-18°C, while the tropical *A. subrufescens* and the uninoculated control bags were kept at 20°C in the first growth chamber. The CO_2 concentration was kept below 1000 ppm in both chambers. The cultivation chambers received LED light with colour temperature 6000 K for 12 hours. From each bag, mushrooms were harvested twice as they gained maturity. The first harvest was 93 – 118 days after inoculation, and the second was 154 days after inoculation.

2.1.4 Mushroom substrate sampling. At inoculation and at harvest 2 the substrate was thoroughly mixed before composite samples were taken. During mushroom growth (i.e., at casing application and at harvest 1), three subsamples were taken, not to disturb the mycelia: from the top, the bottom, and the middle (by taking out the substrate with an awl through a small hole in the bag). All composite samples were cut into small pieces using a scissor, and pH, electrical conductivity, dry matter and ash were determined immediately. For the remaining analysis, frozen samples (-18°C) were used.

2.2 Sample characterization.

In the substrate, pH (30:100, substrate:distilled water), dry matter (DM, 120°C) and ash content (550°C) were determined immediately upon sampling. Dissolved organic carbon (DOC) were extracted with deionized water (1:5, substrate:water). The extract was centrifuged before the supernatant was filtered through a 0.45 µm nylon filter during centrifugation. The filtrate was analysed with a TOC analyser (Shimadzu TOC-V CPN. See the supplementary information for details).

For analysis of PPCPs, 2 g fresh (substrate) or 1 g freeze dried (mushrooms) sample were fortified with internal standards to an extract concentration of 250 ng mL^{-1} and extracted with EDTA-McIlvaine buffer and acetonitrile. The extracts were cleaned-up using Oasis HLB solid phase extraction columns and the PPCPs were eluted with 100% methanol. The elute was filtered through a 0.2 µm microcentrifuge filter before quantitative Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS). For analysis, two different methods were used. Method A were used to quantify carbamazepine, three of its degradation products, caffeine, metoprolol, DEET, and 31 other compounds (See Table S2). Method B were used to quantify the antibiotics norfloxacin, ciprofloxacin, sulfadiazine and sulfamethazine. Both methods applied dynamic multiple reaction monitoring (dMRM) mode and an Agilent 1200 series HPLC coupled to an Agilent 6490 triple quadrupole mass spectrometer with an Agilent Jet Stream electrospray ion source. The parameters of HPLC and ion source can be found in the supplementary information, along with details on the extraction of PPCPs and quality control.

158 **2.3 Data analysis.**

159 The Kruskal-Wallis H-test was used to statistically compare the amount left in the substrate between the
160 three treatments *A. subrufescens*, *A. bisporus* and the uninoculated control. Significant differences were
161 further tested with the Dunn`s test for multiple comparisons, using the Holm-Bonferroni method to adjust
162 the p-values. For summary statistics, values below the LOD were substituted by ½ LOD and values below
163 the LOQ were substituted by ½ LOQ. The substitution does not affect the statistical output since rank based
164 statistics were used. In tables, <LOD means all replicates were below the LOD, <LOQ means all replicates
165 were below the LOQ, which may include values below the LOD. Mean concentrations calculated in part
166 on censored data are labelled with a star.

167

168 **2.4 Calculation of dietary intake of PPCPs**

169 Maximum yearly intake of PPCPs through mushroom consumption was calculated according to equation
170 1, where the maximum detected mushroom concentration found in the present study was multiplied with
171 the average dry matter content of the mushrooms and the average global per capita consumption of
172 mushrooms (Royse et al., 2017).

173 $$Yearly\ intake\ (mg\ /\ year) = Mushroom\ conc.\left(\frac{mg}{kg\ dm}\right) * \frac{0.13\ kg\ dm}{kg\ fw} * 5\ kg\ fw/\ year \quad (1)$$

174

175 **2.5 Calculation of bioaccumulation factors**

176 Bioaccumulation factors were estimated by dividing the mushroom concentration of the individual PPCPs
177 by the respective concentration in the corresponding substrate (i.e., same experimental unit at the same
178 time). Concentrations on dry weight basis were used. When mushroom concentrations were below the LOD
179 or the LOQ, the LOD or LOQ limits were used as mushroom concentration, to estimate worst-case
180 bioaccumulation factors.

3. Results and discussion

3.1 Concentration of target compounds in the mushroom substrate with time

The concentration of the five spiked target compounds, three transformation products of carbamazepine, and 34 non-spiked PPCPs (Table S1 and S2) were quantified in the substrate at inoculation/spiking (day 0), casing application (day 60), and at harvest 1 (day 93 – 118) and 2 (day 154). Of these, the transformation product carbamazepine-10,11-epoxide (hereafter called carbamazepine epoxide), caffeine, *N,N*-Diethyl-3-methylbenzamide (hereafter called DEET), and metoprolol were detected, in addition to the five spiked compounds (Table 1).

Table 1. Mean concentration and standard deviation (µg kg^{-1} ash) of target compounds in the spiked substrates at inoculation (I), casing application (C), harvest 1 (H1), and harvest 2 (H2). Number of replicates are 3 for the mushroom treatments and 2 for the uninoculated control. LOD = limit of detection. LOQ = limit of quantification. * Values are partly calculated on substituted values (see methods section). NOR: norfloxacin, CIP: ciprofloxacin, SDZ: sulfadiazine, SMZ: sulfamethazine, CBZ: carbamazepine, CBZ-EP: carbamazepine-10,11-epoxide, CAF: caffeine, DEET: *N,N*-Diethyl-3-methylbenzamide, MTP: metoprolol.

	NOR	CIP	SDZ	SMZ	CBZ	CBZ-EP	CAF	DEET	MTP
A. subrufescens									
I	1750 ± 750	2470 ± 680	1870 ± 360	2030 ± 510	1650 ± 440	<LOD	218 ± 110	72 ± 52	30 ± 12
C	950 ± 490	703 ± 360	43 ± 3.7	58 ± 8.2	430 ± 270	216 ± 89	32 ± 48*	<LOQ	<LOD
H1	396 ± 210	39 ± 57*	39 ± 7.1	42 ± 12	112 ± 140	54 ± 28	20 ± 30*	<LOQ	<LOD
H2	1570 ± 2700*	268 ± 450*	17 ± 10*	34 ± 7.6	82 ± 130	24 ± 34	<LOD	<LOQ	<LOD
A. bisporus									
I	5440 ± 1200	5140 ± 1500	6410 ± 1600	5720 ± 1800	4510 ± 1600	<LOD	226 ± 9.2	115 ± 16	27 ± 7.4
C	3380 ± 3600	1810 ± 2200	67 ± 59	86 ± 30	1610 ± 1600	227 ± 140	128 ± 30	<LOQ	2.0 ± 3.3*
H1	3680 ± 2400	1870 ± 1000	46 ± 28	72 ± 22	2590 ± 2600	316 ± 110	102 ± 25	<LOQ	<LOD
H2	3200 ± 1800	734 ± 1300*	31 ± 21	63 ± 30	763 ± 520	279 ± 110	64 ± 36	<LOQ	<LOD
Control									
I	3270 ± 1200	3940 ± 540	5830 ± 100	5670 ± 270	3270 ± 44	<LOQ	233 ± 28	86 ± 61	32 ± 17
C	4330 ± 710	4000 ± 110	99 ± 31	126 ± 15	3730 ± 360	108 ± 120	85 ± 53	<LOQ	4.1 ± 5.7*
H1	2970 ± 220	3970 ± 330	52 ± 2.5	67 ± 10	4090 ± 1800	109 ± 100	20 ± 23*	<LOQ	<LOD
H2	7540 ± 4400	5120 ± 3200	82 ± 30	131 ± 60	6400 ± 5900	84 ± 4.9	96 ± 82	<LOQ	<LOD

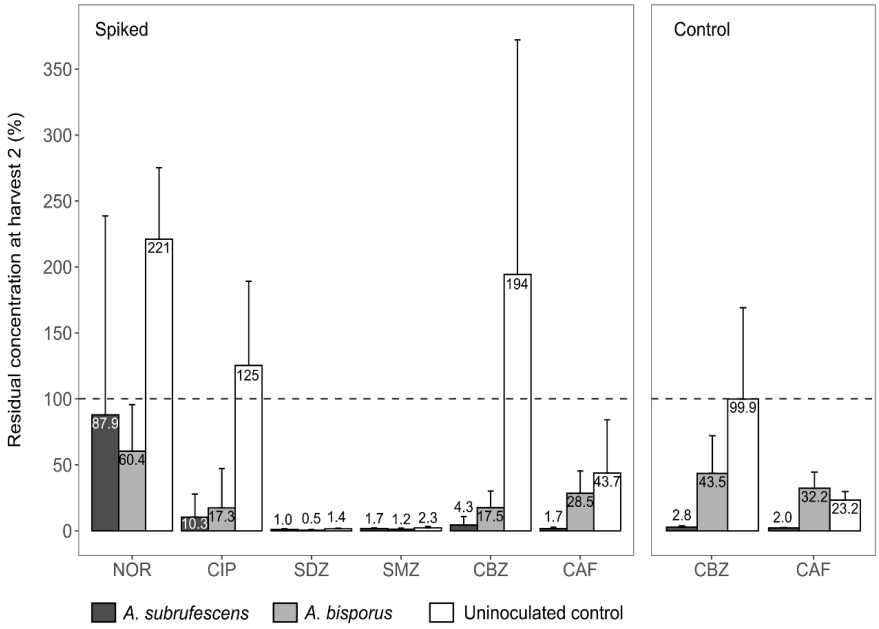


Figure 1. The amount of target compounds measured in the mushroom substrate at harvest 2, relative to the concentration at inoculation. The dashed line at 100% equals the amount present in the substrate prior to inoculation (calculated on ash basis). NOR: norfloxacin, CIP: ciprofloxacin, SDZ: sulfadiazine, SMZ: sulfamethazine, CBZ: carbamazepine, CAF: caffeine.

Substrate concentrations of target compounds are given on ash basis, since the continuous degradation of the substrate gives artificially low removal efficiencies on dry weight basis. The exception is the calculation of bioconcentration factors, since the mushroom concentrations are given on dry weight basis. Both dry weight and ash weight basis concentrations can be found in the SI, for both spiked and control substrate.

Both sulfonamides dissipated quickly and were removed by 97% to 99% already when casing were applied, regardless of treatment (Table 1). The recalcitrant fluoroquinolones and carbamazepine showed a slower removal or even increase and remained in the substrate to the end of the experiment (harvest 2, Figure 1). At the end of the experiment, the percentage removal of the compounds generally followed the order

213 *A. subrufescens* > *A. bisporus* > Control (except for norfloxacin), although this ranking was not statistically
214 significant (Kruskal-Wallis for the individual compounds, $\alpha = 0.05$, Figure 1). Removal is here used as a
215 collective term for all removal processes, including degradation (biotic or abiotic), residues entrapped or
216 sequestered in the mushroom substrate (which may be slowly released later), residues covalently bond to
217 the mushroom substrate (irreversibly bound), or by sorption to the mycelia (Gao et al., 2018; Schäffer et
218 al., 2018).

219 In the control substrate, all compounds but norfloxacin were quantified (Table S10), probably due to their
220 presence in the digestate prior to the experiments. Carbamazepine and caffeine were found at most sampling
221 events (up to 9.22 µg kg^{-1} and 188 µg kg^{-1} ash, respectively) and their concentration declined with time in
222 a similar manner as in the spiked substrate (Table S10, Figure 1). When the residual fraction at harvest 2
223 (as shown in Figure 1) were combined for the spiked and control substrate, the percentage removal of
224 carbamazepine and caffeine were significantly higher in the *A. subrufescens* substrate compared to the *A.*
225 *bisporus* substrate ($p = 0.035$ for carbamazepine, $p = 0.0044$ for caffeine, Dunn Test), and in the *A.*
226 *subrufescens* substrate compared to the uninoculated control ($p = 0.001$ for carbamazepine, $p = 0.008$ for
227 caffeine, Dunn Test). The sulfonamides were not detected in the control at inoculation but were later found
228 in the control *A. subrufescens* substrate in amounts up to 55% of the concentration in the spiked substrate.
229 Sulfonamides are sometimes found at higher concentrations in wastewater effluents than in the influents
230 (Göbel et al., 2007), usually attributed to cleavage of conjugated metabolites during active treatment to the
231 parent compounds (García-Galán et al., 2012). The removal efficiency of the spiked sulfonamides in *A.*
232 *subrufescens* substrate is thus probably underestimated. Otherwise, the target compounds were found
233 infrequently in the control substrate.

234 All target compounds except carbamazepine are highly hydrophilic, having a distribution coefficient (log
235 D) close to zero or even negative at pH 5.5 and 7 (Table S1). Fluoroquinolones are nevertheless strongly
236 sorbed, as they readily ionize and are present as zwitterions at the pH of the mushroom substrate (5.5 – 9,
237 Figure 2), and, hence, bind electrostatically to the substrate. Fluoroquinolones are strongly sorbed to organic

matter and partition mainly into the sewage sludge at wastewater treatment plants (Golet et al., 2003), whereas the sorption of sulfonamides and carbamazepine has been found to be of minor importance (Dwivedi et al., 2017; Göbel et al., 2005). Sorption to the mycelium is also an important removal process, which accounted for a large proportion of the non-extractable residues of especially ciprofloxacin, but also norfloxacin, in cultures with the white rot fungi *Phanerochaete chrysosporium* (Gao et al., 2018). Sorption is thus assumed to be a minor removal mechanism for carbamazepine and the sulfonamides in the present experiment, whereas for fluoroquinolones we assume removal by sorption to be higher.

The removal of carbamazepine was at least partly due to degradation, as seen by the formation of the transformation product carbamazepine epoxide. The concentration of carbamazepine epoxide was similar in *A. subrufescens* and *A. bisporus* substrate at casing application, before declining in *A. subrufescens* substrate along with declining carbamazepine concentration. In *A. bisporus* substrate the concentration of carbamazepine epoxide further increased (Table 1). Epoxidation is a common first step in the transformation of carbamazepine in fungi (Golan-Rozen et al., 2015; Kang et al., 2008), bacteria (Bessa et al., 2019) and plants (Riemenschneider et al., 2017), often followed by hydration to 10,11-dihydroxy-carbamazepine (not measured) (Golan-Rozen et al., 2015; Riemenschneider et al., 2017). Acridine, which is also formed from carbamazepine epoxide (via 9-acridine carboxaldehyde) (Bessa et al., 2019; Golan-Rozen et al., 2015; Riemenschneider et al., 2017), was not detected in any samples, indicating either that the acridine transformation pathway was not important or that the levels were too low to detect. A third transformation product, 3-hydroxy-carbamazepine was also not detected. 3-hydroxy-carbamazepine are formed by some fungi (Kang et al., 2008), but was not included in the proposed pathway for carbamazepine degradation by *P. ostreatus* (Golan-Rozen et al., 2015), the bacteria *Labrys portucalensis* (Bessa et al., 2019), and tomatoes (Riemenschneider et al., 2017).

Removal of carbamazepine by white-rot fungi has been extensively studied in culture media, and removal efficiencies range from negligible (Marco-Urrea et al., 2009) to almost complete (Golan-Rozen et al., 2015, 2011). Under optimal conditions, i.e., when a carbamazepine-degrading strain of a carbamazepine-

degrading fungi (*Pleurotus ostreatus* PC9) were grown in a media supporting enzymes involved in carbamazepine degradation (CYP450 and Manganese peroxidase), almost complete transformation can be achieved in a matter of days (Golan-Rozen et al., 2015, 2011). The unstable removal efficiencies of carbamazepine have been explained by the presence of the strong electron withdrawing amide group (–$CONH_2$), which appears to make the compound more resistant to fungal degradation (Yang et al., 2013).

Fluoroquinolones as well contain strong electron withdrawing groups (–F, =O and –COOH), but like many recalcitrant compounds, also fluoroquinolones can be efficiently degraded by white rot fungi. In culture media removal efficiencies have been found to reach up to 85 – 100% removal by *T. versicolor, Irpex lacteus* (Cvancarová et al., 2015; Prieto et al., 2011) and *Pycnoporus sanguineus* (Gao et al., 2018). White-rot fungi are however often poor competitors under non-sterile conditions (Asif et al., 2017; Harms et al., 2011), and *T. versicolor* removed only 35% of ciprofloxacin from non-sterile industrial wastewaters at low concentrations (Cruz-Morató et al., 2013). In the present experiment, norfloxacin were removed by 12-40%, and ciprofloxacin by 83 – 90%. Also *T. versicolor* was more efficient at removing ciprofloxacin than norfloxacin, while *P. sanguineous* removed both compounds at similar efficiencies (Gao et al., 2018; Prieto et al., 2011). Conversely, the white rot fungi *Ganoderma lucidum* degraded norfloxacin efficiently but not ciprofloxacin (Chakraborty and Abraham, 2017). The removal of ciprofloxacin was somewhat lower than found by Schildt et al., (2021) who found a removal in the substrate of *A. bisporus* of more than 80% after 14 days and 100% after 42 days.

Both sulfonamides dissipated quickly and were removed by 97 to 99% already at casing application (Table 1). Both sulfadiazine and sulfamethazine possess an amine (–NH_2) electron donating group in their structure, which appears to make the compounds more susceptible to fungal degradation (Yang et al., 2013). Thus, both sulfamethazine and the closely related sulfamethoxazole have been shown to be efficiently removed by white-rot fungi (Yang et al., 2013). As for carbamazepine and the fluoroquinolones, CYP450 enzymes seems to be involved in the degradation, as well as the laccase enzymes (García-Galán et al., 2011). The removal of sulfonamides was however equally high in the *No mushroom* treatment, showing

that the cultured fungi are not a prerequisite for removal. Removal efficiencies of sulfonamides during composting have been varying, from complete removal of sulfadiazine after three days of composting, to a half-life of up to 240 days for sulfamethazine (Mitchell et al., 2015). Sulfonamide antibiotics degrade in a similar manner and can be assessed as one group in environmental fate assessment (Ingerslev and Halling-Sørensen, 2000). Thus, it is unlikely that sulfonamide parent compounds will pose a threat towards the safe production of edible mushrooms in waste materials, despite their frequent use in veterinary medicine (European Medicines Agency, 2020). Considering the low sorption potential of the sulfonamides and their susceptibility to degradation, it is plausible that degradation was the main removal mechanism of these compounds. It is however important to note that many sulfonamide transformation products are more toxic than the parent compounds, and some may also be persistent (Puhlmann et al., 2022).

Of non-added compounds, only caffeine was detected throughout the experiment. As for most of the spiked pharmaceuticals, *A. subrufescens* was more efficient than *A. bisporus* at removing caffeine. The removal of caffeine was considerably higher than what was found by *Pleurotus ostreatus* cultivated in a mixture of 25% spent coffee grounds and 75% sawdust, where the decrease was 1.9 – 11 (Carrasco-Cabrera et al., 2019). However, when the substrate consisted of only spent coffee grounds, the removal was 86.9% (Carrasco-Cabrera et al., 2019).

Other relevant removal processes include hydrolysis and thermal degradation both more relevant for *A. subrufescens* substrate which were kept at a higher temperature and had a lower substrate pH (Figure 2, chemical hydrolysis of sulfonamides is especially relevant at low pH (Białk-Bielińska et al., 2012)). Most fluoroquinolones are chemically stable to both hydrolysis and high temperatures but are susceptible to photodegradation (Thiele-Bruhn, 2003). Photolysis is nevertheless assumed to be a minor removal mechanism, as it is unlikely that the light would penetrate substantially further into the mushroom substrate than into soil, where photolysis is restricted to the upper two mm (Hebert and Miller, 1990).

3.2 Uptake and distribution of pharmaceuticals and personal care products

Carbamazepine, carbamazepine epoxide, and DEET were the only compounds detected in the mushrooms, mainly in *A. bisporus* (Table 2). *A. bisporus* substrate also had a considerably higher concentration of carbamazepine and carbamazepine epoxide than the *A. subrufescens* substrate.

Table 2. Concentration of PPCPs in mushrooms grown in spiked substrate (µg kg^{-1} dw), bioaccumulation factors, maximum yearly intake of PPCPs through consumption of mushrooms, and a corresponding reference dose. For compounds not detected in the mushrooms, the "worst-case" bioaccumulation factors are calculated using the LOD or LOQ as mushroom concentration. These BAFs are indicated by < in the table. * Values are partly calculated on substituted values (see methods section). CBZ: carbamazepine, CBZ-EP: carbamazepine-10,11-epoxide, SDZ: sulfadiazine, SMZ: sulfamethazine, NOR: norfloxacin, CIP: ciprofloxacin, CAF: caffeine, DEET: *N,N*-Diethyl-3-methylbenzamide

	CBZ	CBZ-EP	SDZ	SMZ	NOR	CIP	CAF	DEET
Mushroom concentration (µg kg^{-1} dw)								
Sub – H1	< LOQ	1.02 [a]	< LOD	< LOD	< LOD	< LOD	< LOD	< LOQ
Sub – H2	< LOQ	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	0.08 [a]
Bisp – H1	0.76±0.92	1.85±1.57	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Bisp – H2	0.42±0.32	1.48±0.76	< LOD	< LOD	< LOD	< LOD	< LOD	0.15±0.06
Bioaccumulation factors								
Sub – H1	< 0.0027	< 0.0085	< 0.042	< 0.073	< 0.0068	< 0.40	< 0.26	< 0.45
Sub – H2	< 0.0071	< 0.012	< 0.11	< 0.090	< 0.026	< 0.22	< 0.70	< 0.41
Bisp – H1	0.0009	0.016	< 0.043	< 0.047	< 0.0008	< 0.0040	< 0.023	< 0.38
Bisp – H2	0.0021	0.013	< 0.071	< 0.051	< 0.0009	< 0.98	< 0.027	0.63
Comparison of exposure to pollutants through mushrooms and other sources								
Mushrooms (mg $year^{-1}$)	0.0049	0.0012	0.00031	0.00056	0.00046	0.0012		0.00010
Reference (mg)	200 – 1200 [b]		1000 [b]			500 – 1500 [b]	40 [c]	499 [d]

[a] At least one replicate was below the LOD or LOQ, see SI for substitution method. [b] Administered dose for adults per day (Norwegian pharmaceutical handbook, 2023). [c] Caffeine in one cup of black coffee. [d] Amount applied to one arm when using DEET-containing insect repellants (1 ml per arm is recommended, 50% DEET in formula, density of 0.998 g ml^{-1} (Apotek1, 2023))

Despite a low substrate concentration of carbamazepine epoxide relative to carbamazepine, the concentration in mushrooms were about twice as high for carbamazepine epoxide, resulting in 10-20 times

331 higher bioaccumulation factors (BAFs) for carbamazepine epoxide than for carbamazepine. The same
332 pattern was seen for tomatoes and wheat fertilized with carbamazepine containing biosolids (Mordechay et
333 al., 2018). Within-organism transformation from carbamazepine to the epoxide has been seen in other fungi
334 and plants, mainly by CYP_{450} enzymes transformation (Golan-Rozen et al., 2011). Further, carbamazepine
335 epoxide is more hydrophilic than carbamazepine (log K_{OW} of 0.95 and 2.25, respectively, Table S1), leading
336 to a higher bioavailability in the substrate. Nevertheless, translocation factors for both compounds were
337 well below 1 (maximum 0.016) and neither carbamazepine nor carbamazepine epoxide are bioaccumulative
338 in the present cultivation system.

339 The uptake of DEET in *A. bisporus* at harvest 2 was surprising, since the substrate concentration was below
340 the LOQ from casing and onwards. Consequently, the estimated minimum BAF was 0.63 (using the LOQ
341 as substrate concentration), indicating that DEET was readily taken up. In cereals and carrots, DEET has
342 been shown to be bioaccumulative with root concentration factors and leaf concentration factors in the
343 range 0.4 – 2.3 and 2.3 – 7.4, respectively (Eggen et al., 2013).

344 None of the antibiotics were detected in the mushrooms despite substrate concentrations similar to
345 (ciprofloxacin) or higher (norfloxacin) than carbamazepine. Similarly, Schildt et al. (2021) found that
346 ciprofloxacin concentration in *A. bisporus* grown in a substrate spiked with 1333 µg kg^{-1} dm was below 8.5
347 µg kg^{-1} at harvest 1 and not detected at harvest 2. The bioavailability of the fluoroquinolones in the substrate
348 can be assumed to be lower than that of carbamazepine due to strong sorption, which could explain the
349 differences in uptake. It is however important to note that the LOD for fluoroquinolones in the mushrooms
350 were in the same range as the detected concentrations of carbamazepine and carbamazepine epoxide. The
351 lacking uptake of sulfonamides is not surprising as these compounds were fast removed from the substrate.

352 Based on data from other similar experiments, the mushroom uptake of pharmaceuticals seems to be low
353 and highly dependent of substrate concentration. Schildt et al. (2021) and Golovko et al. (2021) cultivated
354 *A. bisporus* and *P. ostreatus*, respectively, in growth substrates spiked with PPCPs. For most PPCPs, the
355 substrate concentration declined rapidly, and the compounds were not taken up by the mushrooms.

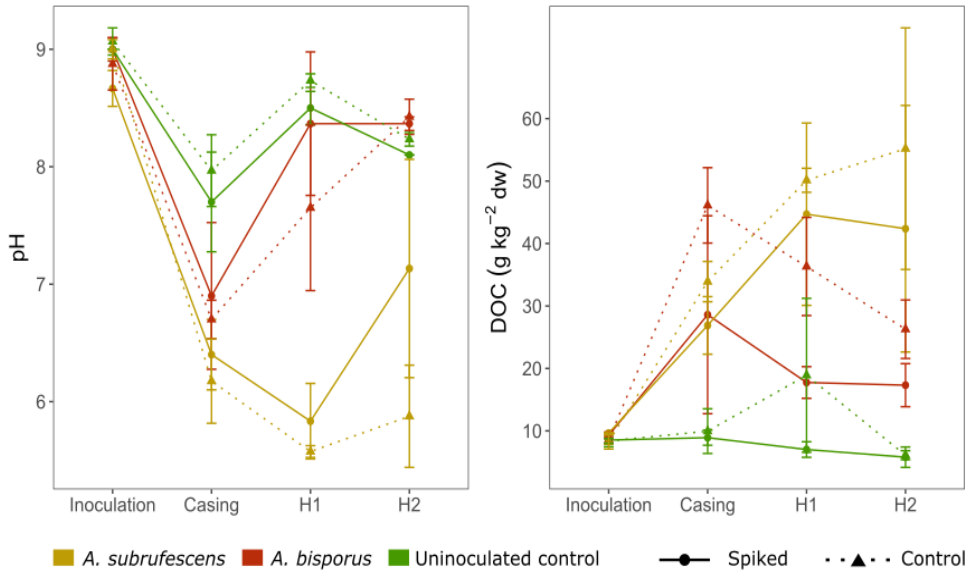
Praziquantel (Schildt et al., 2021) and clarithromycin (Golovko et al., 2021) on the other hand persisted in the substrates and were quantified in low amounts in the mushrooms. An approximate BAF of 0.11 for both compounds can be calculated based on data presented in the referred papers, indicating no accumulation potential. Similarly, Gbylik-Sikorska et al. (2020) investigated the uptake of doxycycline by *A. bisporus* and found consistently low BAFs, lower or equal to 0.012, for substrate concentrations up to 5 mg kg^{-1}. However, when *P. ostreatus* was cultivated on spent coffee grounds, the BAF for caffeine was approximately 1. Thus, although most compounds investigated so far do not accumulate in mushrooms, some compounds still might.

Worst-case BAFs were calculated for compounds not detected in the mushrooms, such as caffeine (indicated by a "less-than" sign (<) in Table 2). For caffeine, these range from 0.023 to 0.7 (Table 2). The higher worst-case BAF of 0.7 owes to the lack of caffeine in the substrate at Harvest 2 in *A. subrufescens* substrate, so that 0.7 equals the mushroom LOD divided on the substrate LOD. As seen from the worst-case BAFs in Table 2, none of the added or non-added compounds are bioaccumulative.

Maximum yearly intake of the target compounds through mushroom consumption was calculated based on the highest detected mushroom concentration for each compound. Again, the worst-case intake of compounds was calculated for those compounds which were not detected, by using their respective LODs. Maximum intake was compared with quanta of the compounds taking other exposure routes. For the pharmaceuticals, the reference intakes were the daily dose taken under treatment, for caffeine the amount in one cup of coffee was used, and for the insect repellent DEET the amount applied to one arm was used (Table 2). The maximum yearly intake of the target compounds were 5 to 7 orders of magnitude lower than the exposure through other sources.

3.3 Species dependent distribution

A. subrufescens was overall more efficient than *A. bisporus* at removing pollutants from the substrate (except for norfloxacin) and had a lower uptake of PPCPs. PFAS uptake was also lower in *A. subrufescens* than *A. bisporus* (Nesse et al., 2023). Since the tropical *A. subrufescens* was cultivated at a higher temperature than *A.* bisporus, the effect of species cannot be separated from the effect of temperature, or from the combined effect of both on substrate properties. Although the standard deviations are high, it can be seen from figure 2 that the pH and dissolved organic carbon (DOC) differed between both species and the uninoculated control. The pH declined and the DOC increased in the substrate of both fungi until the casing day after which they separate, coinciding with the relocation of *A. bisporus* to a lower temperature chamber (*A. subrufescens* and control: 20°C, *A. bisporus*: 17-18°C). From the inoculation and onwards, the DOC and pH follows a close to inverse relationship, which is not unexpected. Organic acids are formed during the degradation of organic matter, thus, the pH declines. In the uninoculated control there were only minor changes in DOC concentration with time, and the pH variations were also small compared to the changes seen in the mushroom treatment. Both pH and DOC affect the fate of organic contaminants in numerous ways, as for example the well-known effect of pH on compound speciation, as well as the role of DOC in complexation with the contaminants or competition for sorption sites. Thus, the observed differences in pH and DOC most likely affected the differences in removal efficiencies. This topic should be further investigated in a more systematic manner. From figure 2 it can also be seen that the pH was generally lower and the DOC higher in the control treatment compared to the spiked treatment, indicating that the fortification of the substrate affected the system.



399

400 **Figure 2.** pH and dissolved organic carbon (DOC) in the mushroom substrates at inoculation, casing
401 application, harvest 1 and harvest 2.

402

403 **3.4 Remediation potential and food security**

404 While the potential for mycoremediation of drugs is large, real-life applications are scarce (Harms et al.,
405 2011). White-rot fungi are adapted to wood with a pH of about 3-5 and are often outcompeted by
406 autochthonous microflora at the contaminated site which is often a very different habitat (Harms et al.,
407 2011; Marco-Urrea et al., 2015). Further, fungi degrade organic pollutants by co-metabolism, which means
408 that the presence of a readily degradable substrate is needed (Harms et al., 2011). The present system
409 overcomes both limitations, *Agaricus spp.* are grown under optimal conditions with rich access to organic
410 substrate. Considering the vast amounts of agricultural residuals which are stabilized and converted to food
411 by mushroom production yearly, this system is an example of real-life mycoremediation. The use of spent
412 mushroom substrate as soil amendment and in horticulture will constitute a lower risk with respect to
413 organic pollutants compared to the direct use of digestates.

Non-degradable compounds, such as metals or PFAS, will however be present in the spent mushroom substrate unless taken up. In the few experiments conducted, it was the small, charged molecules that were taken up in the largest amounts, i.e., ultra-short chain PFAS (Nesse et al., 2023) and cadmium (Stoknes et al., 2019a). Thus, care must be taken if these compounds are present in the substrate. However, Stoknes et al. (2019a) showed that the initial harvest (flush) of *A. subrufescens* removed 80% of the cadmium present in the substrate so that the remaining harvests did meet food safety standards. And even the ultra-short chain PFASs did not accumulate (Nesse et al., 2023). Uptake of pharmaceuticals has also been shown to be low, unless they are present at unrealistically high concentrations and persist in the substrate (i.e., praziquantel (Schildt et al., 2021), chloramphenicol (Golovko et al., 2021), and carbamazepine).

4. Conclusion and outlook

In the present study, mushroom fruiting bodies were shown to accumulate very low amounts of pharmaceuticals and personal care products. Most compounds were not detected at all, whereas carbamazepine and its transformation product carbamazepine-10,11-epoxide (CBZ-EP) were found in small amounts. The bioaccumulation factor of CBZ-EP was 10-20 times higher than for the parent compound, emphasizing the need to include transformation products in PPCP uptake studies. The sulfonamides were quickly removed from the mushroom growth substrate in all treatments, whereas the recalcitrant fluoroquinolones and carbamazepine showed a varying removal efficacy depending on treatment and compound. In the mushroom growth treatments, ciprofloxacin was removed from the growth substrate by 83 – 90% and carbamazepine by 57 – 97%. Considering that the current mushroom production system was tuned towards food production and not contaminant removal, these results must be considered promising. However, more studies are needed on the screening of potential transformation products and on the use of mushrooms for remediation purposes. The mushroom *Agaricus subrufescens* were generally shown to have a lower PPCP uptake than *A. bisporus*, and the PPCPs were also removed more effectively from its growth substrate in the *Agaricus subrufescens* treatment. All in all, the results demonstrate that

production of edible mushrooms may have the positive side effect of removing organic contaminants from the circular food-chain.

Supporting information

The supporting information includes information on target analytes, chemicals and solvents, sample preparation, analytical methods, method validation and quality control, concentration of analytes in substrate and mushrooms on dry weight and ash weight basis, and characterization of the mushroom growth substrate.

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Notes

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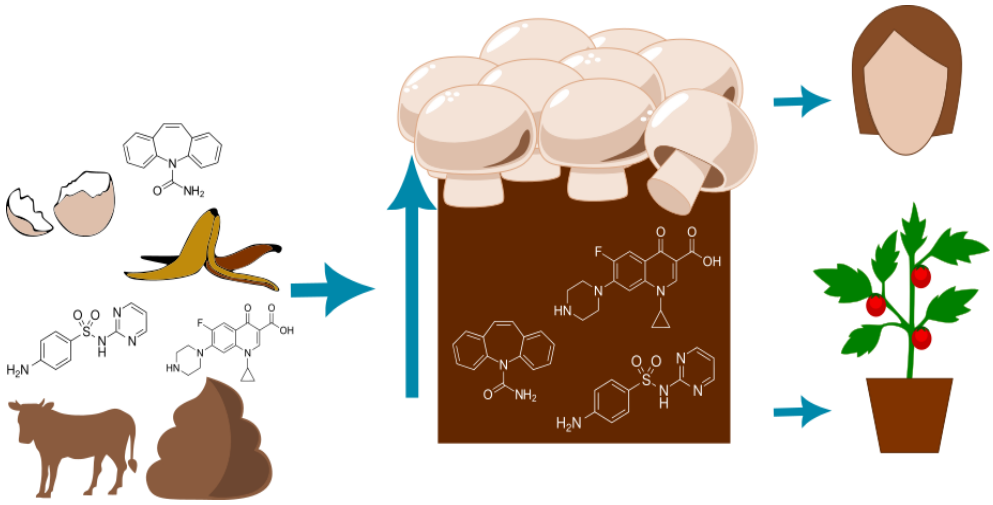
461 **TOC art**



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Supplementary information

Low uptake of pharmaceuticals in edible mushrooms grown in polluted biogas digestate.

Astrid S. Nesse[1]*, Agnieszka Jasinska[2,3], Ketil Stoknes[2], Stine Göransson Aanrud[6], Kristin Ogner Risinggård[6], Roland Kallenborn[4], Trine A. Sogn[1], Aasim M. Ali[4,5]

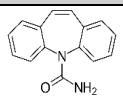
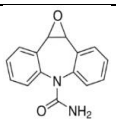
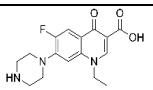
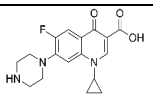
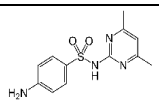
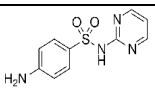
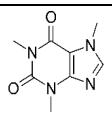
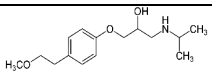
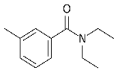
[1]*Norwegian University of Life Sciences, Faculty of Environment and Natural Resources, Elizabeth Stephansensvei 31, 1433 Ås, Norway*
[2]*Lindum AS, Lerpeveien 155, 3036 Drammen, Norway*
[3]*Poznan University of Life Sciences, Department of Vegetable Crops, ul. J.H. Dabrowskiego 159, 60-594 Poznan, Poland*
[4]*Norwegian University of Life Sciences, Faculty of Chemistry and Biotechnology, Chr. M. Falsens vei 18, 1433 Ås, Norway*
[5]*Institute of Marine Research, Nordnesgaten 50, 5005 Bergen, Norway*
[6]*Norwegian University of Life Sciences, Faculty of Veterinary Medicine, Elizabeth Stephansensvei 15, 1433 Ås, Norway*

*astrid.solvag.nesse@nmbu.no

Target analytes

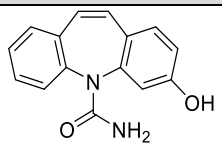
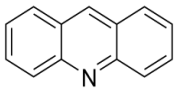
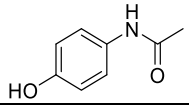
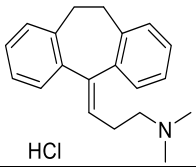
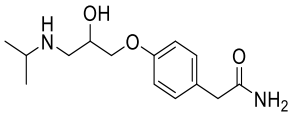
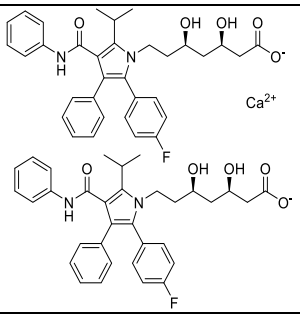
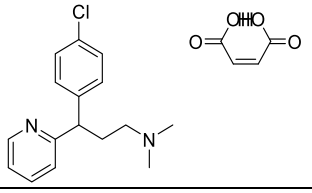
The complete list of target analytes screened in mushrooms and substrate is shown in Table S1 and Table S2. The selection of compounds was based on their high detection rates in Norwegian biogas digestate[1,2].

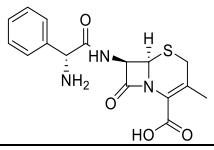
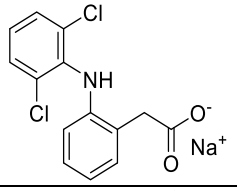
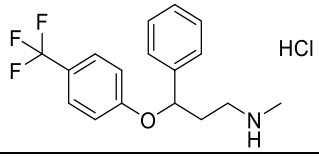
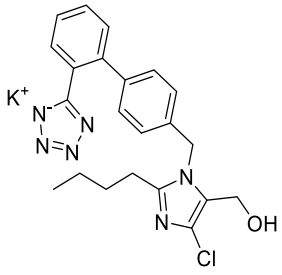
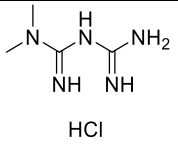
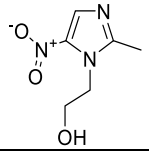
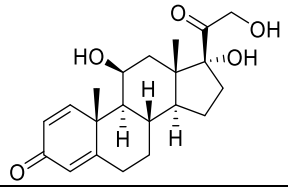
Table S1. List of substances quantified in the mushroom growth substrate, including physiochemical properties. All compounds in Table S1 were supplied by Sigma Aldrich, Oslo, Norway.

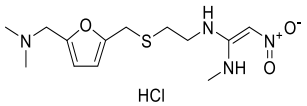
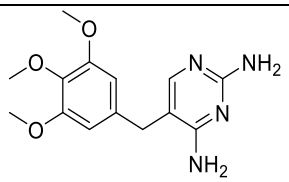
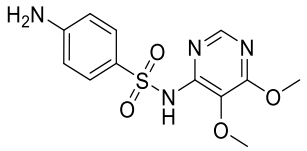
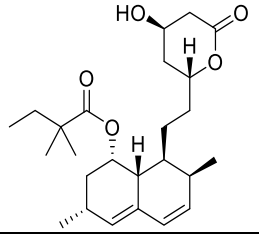
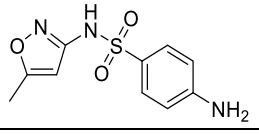
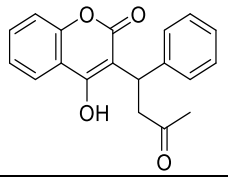
Compound (CAS-number)	Molecular formula	Structure	Description	Main species (pH 7)	pKa[1]	Log K_{OW}[2]	Log D (pH 5.5 / 7.4)[3]
Carbamazepine (298-46-4)	$C_{15}H_{12}N_2O$		Anti-convulsant	Neutral	13.9 [3]	2.25	2.28 / 2.28
Carbamazepine-10,11-epoxide (36507-30-9)	$C_{15}H_{12}N_2O_2$		CBZ transfor-mation product			0.95	
Norfloxacin (70458-96-7)	$C_{16}H_{18}FN_3O_3$		Fluoro-quinolone	Zwitterionic / Cationic	4.06, 6.20, 8.43, 10.56	– 0.31	-3.18 / -3.00
Ciprofloxacin (85721-33-1)	$C_{17}H_{18}FN_3O_3$		Fluoro-quinolone	Zwitterionic / Cationic	4.03, 6.23, 8.64, 10.58	0.00	-2.98 /-2.23
Sulfamethazine /Sulfadimidine (57-68-1)	$C_{12}H_{14}N_4O_2S$		Sulfon-amide	Neutral	2.24, 7.51	0.89[1]	
Sulfadiazine (68-35-9)	$C_{10}H_{10}N_4O_2S$		Sulfon-amide	Anionic / neutral	2.10, 6.36	– 0.34	-0.09 /-0.79
Caffeine (58-08-2)	$C_8H_{10}N_4O_2$		Psycho-stimulant	Neutral	14[4]	0.16	
Metoprolol (37350-58-6)	$C_{15}H_{25}NO_3$		β-blocker	Cationic	9.46	1.69	-1.20 /-0.25
N-*N*-Diethyl-3-methylbenzamide (DEET) (134-62-3)	$C_{12}H_{17}NO$		Insect repellent			2.26	2.24 /2.24

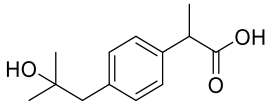
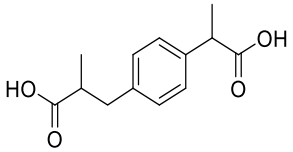
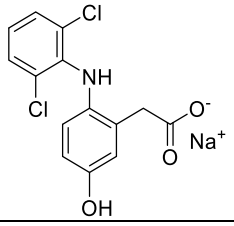
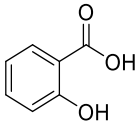
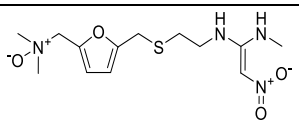
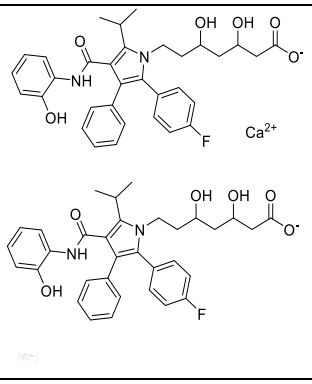
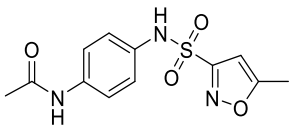
[1] Mean values from Babić et al.(2007) unless stated otherwise. [2] Predicted by EPISuite KOWWIN v1.67. [3] Predicted by ACD/Labs Percepta Platform, PhysChem Module. [4] Keerthanan 2021(Keerthanan et al., 2021)

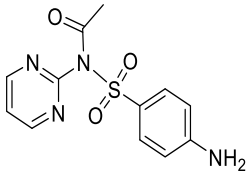
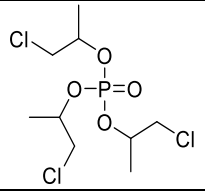
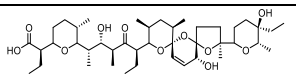
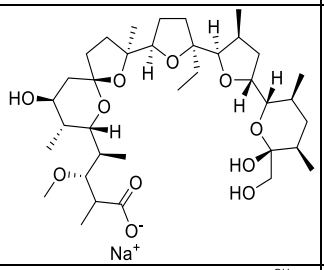
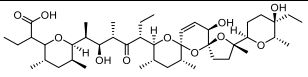
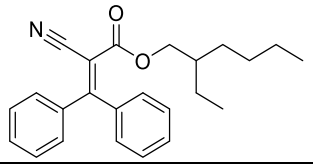
Table S2. List of substances which were included in the screening of the mushroom growth substrate, but which were not detected.

Compound (Abbreviation)	Molecular formula	Structure	CAS number	Description	Supplier
3-Hydroxy-carbamazepine (CBZ-3OH)	$C_{15}H_{12}N_2O_2$		68011-67-6	CBZ transformation product	Sigma Aldrich, Oslo, Norway
Acridine (ACR)	$C_{13}H_9N$		260-94-6	CBZ transformation product	Sigma Aldrich, Oslo, Norway
Acetaminophen (ACE)	$C_8H_9NO_2$		103-90-2	Nonsteroidal anti-inflammatory	Sigma Aldrich, Oslo, Norway
Amitriptyline hydrochloride (AMT)	$C_{20}H_{23}N \cdot HCl$		549-18-8	Antidepressant	Sigma Aldrich, Oslo, Norway
Atenolol (ATN)	$C_{14}H_{22}N_2O_3$		29122-68-7	β -blocker	Sigma Aldrich, Oslo, Norway
Atorvastatin calcium salt trihydrate (ATO)	$C_{66}H_{68}CaF_2N_4O_{10}$		134523-03-8	Antilipidemic	Toronto Research Chemicals, Toronto, Canada
(±)-Chlorphenirami ne maleate salt (CPA)	$C_{16}H_{19}ClN_2 \cdot C_4H_4O_4$		113-92-8	Antihistaminic	Sigma Aldrich, Oslo, Norway

Compound (Abbreviation)	Molecular formula	Structure	CAS number	Description	Supplier
Cephalexin (CPX)	$C_{16}H_{17}N_3O_4S$		15686-71-2	Antibiotic	Sigma Aldrich, Oslo, Norway
Diclofenac sodium salt (DCF)	$C_{14}H_{10}Cl_2NNaO_2$		15307-79-6	Nonsteroidal anti-inflammatory	Sigma Aldrich, Oslo, Norway
Fluoxetine hydrochloride (FLX)	$C_{17}H_{18}F_3NO \cdot HCl$		56296-78-7	Antidepressant	Sigma Aldrich, Oslo, Norway
Losartan potassium (LOS)	$C_{22}H_{22}ClKN_6O$		124750-99-8	Anti-hypertensive	Sigma Aldrich, Oslo, Norway
Metformin hydrochloride (MEF)	$NH_2C(=NH)NHC(=NH)N(CH_3)_2 \cdot HCl$		1115-70-4	Antidiabetic	Sigma Aldrich, Oslo, Norway
Metronidazole (MET)	$C_6H_9N_3O_3$		443-48-1	Antibiotic	Sigma Aldrich, Oslo, Norway
Prednisolone (PRE)	$C_{21}H_{28}O_5$		50-24-8	Corticosteroid	Sigma Aldrich, Oslo, Norway

Compound (Abbreviation)	Molecular formula	Structure	CAS number	Description	Supplier
Ranitidine hydrochloride (RAN)	$C_{13}H_{22}N_4O_3S \cdot HCl$		66357-59-3	Histamine H_1 and H_2 receptor antagonist	Sigma Aldrich, Oslo, Norway
Trimethoprim (TRI)	$C_{14}H_{18}N_4O_3$		738-70-5	Antibiotic	Sigma Aldrich, Oslo, Norway
Sulfadoxine (SUL)	$C_{12}H_{14}N_4O_4S$		2447-57-6	Antibiotic	Sigma Aldrich, Oslo, Norway
Simvastatin (SMV)	$C_{25}H_{38}O_5$		79902-63-9	Antilipidemic	Chiron AS, Trondheim, Norway
Sulfamethoxazole (SMX)	$C_{10}H_{11}N_3O_3S$		723-46-6	Antibiotic	Sigma Aldrich, Oslo, Norway
Warfarin (WAR)	$C_{19}H_{16}O_4$		81-81-2	Anticoagulant	Sigma Aldrich, Oslo, Norway

Compound (Abbreviation)	Molecular formula	Structure	CAS number	Description	Supplier
2-hydroxy-ibuprofen (IBP-OH)	$C_{13}H_{18}O_3$		51146-55-5	Metabolite of ibuprofen	Sigma Aldrich, Oslo, Norway
Carboxy ibuprofen (IBP-Car)	$C_{13}H_{16}O_4$		15935-54-3	Metabolite of ibuprofen	Sigma Aldrich, Oslo, Norway
5-hydroxy-diclofenac (5OH-DCF)	$C_{14}H_{11}Cl_2NO_3$		69002-84-2	Metabolite of diclofenac	Sigma Aldrich, Oslo, Norway
Salicylic acid (SA)	$C_7H_6O_3$		69-72-7	Anti-inflammatory	Sigma Aldrich, Oslo, Norway
Ranitidine N-oxide (RAN-O)	$C_{13}H_{22}N_4O_4S$		73857-20-2	Metabolite of ranitidine	Sigma Aldrich, Oslo, Norway
2-Hydroxy Atorvastatin Calcium Salt (2OH-ATO)	$C_{66}H_{68}CaF_2N_4O_{12}$		265989-46-6	Metabolite of atorvastatin	Toronto Research Chemicals, Toronto, Canada
N-Acetyl Sulfa-methoxazole (ACY-SMX)	$C_{12}H_{13}N_3O_4S$		21312-10-7	Metabolite of sulfameth-oxazole	Toronto Research Chemicals, Toronto, Canada

Compound (Abbreviation)	Molecular formula	Structure	CAS number	Description	Supplier
N-Acetyl Sulfadiazine (ACY-SAD)	$C_{12}H_{12}N_4O_3S$		127-74-2	Metabolite of Sulfadiazine	Toronto Research Chemicals, Toronto, Canada
Tris (chloroisopropyl) phosphate, mixture of isomers (TCPP)	$C_9H_{18}Cl_3O_4P$		13674-84-5	Flame retardant	Sigma Aldrich, Oslo, Norway
Salinomycin (SLM)	$C_{42}H_{70}O_{11}$		53003-10-4	Anticoccidial drug	Sigma Aldrich, Oslo, Norway
Monensin sodium salt (MON)	$C_{36}H_{61}NaO_{11}$		22373-78-0	Anticoccidial drug	Sigma Aldrich, Oslo, Norway
Narasin (NAR)	$C_{43}H_{72}O_{11}$		55134-13-9	Anticoccidial drug	Sigma Aldrich, Oslo, Norway
Octocrylene (OCR)	$C_{24}H_{27}NO_2$		6197-30-4	Sunscreen agents	Sigma Aldrich, Oslo, Norway

Chemicals and solvents

HPLC grade acetonitrile (CH_3CN) and methanol (MeOH) were purchased from VWR (West Chester, PA, USA). Reagent grade formic acid (CH_2O_2), ammonia solution (NH_4OH), ammonium acetate ($C_2H_7NO_2$), disodium ethylene diamine tetra acetate (Na_2EDTA), citric acid ($C_6H_8O_7$), sodium phosphate dibasic (Na_2HPO_4) and phosphoric acid (H_3PO_4) were purchased from Sigma-Aldrich (Oslo, Norway). Milli-Q system from Millipore (Bedford, MA, USA) was used for the mobile phase and preparation of the following solutions: McIlvaine buffer (pH = 4); SPE buffer (0.001% EDTA, 1% McIlvaine buffer, 2% CH_3CN, and 0.05% H_3PO_4 in Milli-Q).

Substrate Sample Preparation

The sample preparation method was adopted from Hu et al.[3] with modifications. An aliquot of 2 ± 0.05 g fresh sample was weighed into 50 mL polypropylene tubes. Samples were spiked with a mixture of internal standards ($^{2}H_{10}$-Carbamazepine, $^{13}C_3$-Caffeine, $^{2}H_{10}$-DEET, $^{2}H_7$-Metoprolol, $^{2}H_8$-Ciprofloxacin, $^{2}H_5$-Norfloxacin, $^{13}C_6$-Sulfadiazine, all at an end concentration of 0.25 µg/mL) followed by 3.5 mL 0.1 M EDTA-McIlvaine buffer. Subsequently, the mixture was vortex mixed for 10 s., followed by addition of 3.5 mL CH_3CN and vortex mixed for another 10 s before ultrasonication for 10 min at room temperature. The mixture was centrifuged for 15 min at 3500 rpm. The supernatant was transferred to a glass tube, and the extraction procedure was repeated once with 2 mL of each solvent without ultrasonication. The combined supernatants were dried to approximately 5 mL with controlled heating at 37 °C under a stream of compressed air (analytical quality) using a Reacti-Therm III evaporator (Thermo Fisher Scientific Inc, Rockford, USA). Each extract was then diluted with 2 mL water and shaken for 10 s before loading onto an Oasis HLB solid phase extraction (SPE) column (60 mg 3 cc; Waters, Milford, USA), which was preconditioned with 3 mL MeOH and 3 mL SPE buffer. The column was washed with 3 mL 5% MeOH in water and dried before the elution with 2.5 mL MeOH. The elute was evaporated to dryness using the Reacti-Therm III evaporator (Thermo Scientific, Waltham, MA, USA) at 37 °C, reconstituted with 1 mL 20% MeOH in water, vortexed and filtered through a 0.2 µm microcentrifuge filter (Spin-X, Costar, Corning Inc. NY, USA) before the samples were transferred to polypropylene vials for quantitative LC-MS/MS. Samples were analysed with two different instrumental methods both applying dynamic multiple reaction monitoring (dMRM) mode. Ciprofloxacin, norfloxacin, sulfadiazine and sulfamethazine were quantified with method B, while the remaining compounds were quantified with method A.

Mushroom Sample Preparation

For extraction of PPCPs from the mushrooms, 1 g freeze-dried mushrooms were used. The extraction procedure was identical to the substrate extraction procedure with one exception. Since the dry mushrooms absorbed most of the EDTA-McIlvaine buffer and acetonitrile added in the first extraction step, a double volume was used (i.e., 7 mL of each).

Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

Agilent 1200 series HPLC (Agilent Technologies, Waldbronn, Germany) was used for analytical separation coupled to an Agilent 6490 (Agilent Technologies, Santa Clara, CA, USA) triple quadrupole mass spectrometer with an Agilent Jet Stream electrospray ion source (AJS-ESI) which was used for detection. Table S3 and S3 show the parameters of HPLC and ion source, respectively. Table S5a and S4b shows the optimized multiple reaction monitoring (MRM) transitions for method A and B. Agilent MassHunter software (Version B.10.1 /Build 10.1.733.0, 2019) was used for instrument control, method validation and quantification.

Table S3 The optimized HPLC instrumental analysis parameters.

HPLC conditions				
Analytical column	Method A and B: Zorbax Eclipse plus C18 RRHD (2.1 x 100 mm, 1.8 μm) (Agilent, Palo Alto, USA)			
Guard column	Method A: Guard Cartridge (4 μm x 3.0 mm ID) (Zorbax, Agilent, Palo Alto, USA) Method B: Guard Cartridge Eclipse Plus C18 (2.1x5 mm, 1.8 μm) (Agilent, Palo Alto, USA)			
Column temperature (°C)	Method A: 35 Method B: 30			
Mobile phase	Method A: A: 0.1% formic acid in H_2O B: 100% CH_3CN Method B: A: 1 mM ammonium acetate and 0.1% formic acid in H_2O B: 0.1% formic acid in MeOH			
Injection volume (μL)	Method A: 10 Method B: 3			
Gradient profile	Method A			
	Time (min)	**Flow rate (mL/min)**	**Percentage A (%)**	**Percentage B (%)**
	0.0	0.3	90	10
	8.0	0.3	90	100
	15.0	0.3	10	100
	16	0.3	90	10
	20	0.3	90	10
	Method B			
	Time (min)	**Flow rate (mL/min)**	**Percentage A**	**Percentage B**
	0.0	0.3	80	20
	1.0	0.3	80	20
	6.0	0.3	60	40
	10	0.3	0	100
	12	0.3	0	100
	12.1	0.3	80	20
	14	0.3	80	20

Table S4 The optimized ion source

Ion source conditions	Method A	Method B
Gas temperature(°C)	200	180
Gas flow L min^{-1}	14	19
Nebulizer gas pressure (psi)	20	35
Sheath gas heater temperature (°C)	250	350
Sheath gas flow L min^{-1}	10	12
Capillary voltage (V)	3000	3000
Nozzle Voltage (V)	0	0
High Pressure RF (V)	150	150
Low Pressure RF (V)	70	70

Table S5.A MRM Parameters of the selected analytes and their internal standards for method A. The analysis was done in positive mode.

Compound	RT (min)	Prec Ion	Prod Ion1	Prod Ion 2	Frag (V)	CE (V) 1	CE (V) 2
Caffeine	2.46	195.1	138.1	110.1	380	18	24
Metoprolol	2.92	268.1	116.1	73.9	380	17	25
Carbamazepine-10,11-epoxide	3.38	253.1	209.9	180	380	12	12
Carbamazepine	3.78	237	193.9	178.9	380	39	18
DEET	4.16	192.1	119.1	91	380	17	25
$^{2}H_{10}$-Carbamazepine	3.78	247.1	204	202	380	21	39
$^{13}C_{3}$-Caffeine	2.46	198.1	140.1	112.1	380	18	25
$^{2}H_{10}$-DEET	4.16	202.2	119	91	380	17	25
$^{2}H_{7}$-Metoprolol	2.92	275.1	229.9	105	380	17	25
$^{2}H_{5}$-Atrazine	1.1	221.2	179	101	380	16	24

Table S5.B MRM Parameters of the selected analytes and their internal standards for method B. The analysis was done in positive mode.

Compound	RT (min)	Prec Ion	Prod Ion1	Prod Ion 2	Frag (V)	CE (V) 1	CE (V) 2	CAV (V) 1	CAV (V) 2
Ciprofloxacin	7.57	332.1	230.9	287.9	380	38	16	4	7
Norfloxacin	7.18	320	230.7	189	380	48	53	3	6
Sulfadiazin	2.78	251	91.8	156	380	23	11	7	2
Sulfamethazine	4.53	279.1	185.9	123.9	380	15	23	6	3
$^{2}H_{8}$-Ciprofloxacin	7.57	340.2	234.9	-	380	43	-	2	-
$^{2}H_{5}$-Norfloxacin	7.18	325	238	-	380	28	-	2	-
$^{13}C_{6}$-Sulfadiazine	2.78	257	98	-	380	27	-	2	-

Method validation and quality control

Method A:

Five samples of control mushroom substrate from inoculation and harvest 2 were combined and used for the preparation of matrix matched calibration curves (0.05 to 500 ng mL^{-1}) and spiked samples. The five samples were thoroughly mixed and divided into 15 centrifuge tubes (15 mL) of 2±0.05 g each. For mushroom matrix, store bought mushrooms were used.

To assess the method performance, accuracy and precision, spiked samples were used. Recovery and repeatability (described as the relative standard deviation (RSD)) were calculated using 6 replicates of sample spiked by the target analytes and their internal standards at a concentration of 200 µg kg^{-1} ww Spiked samples were prepared with the same extraction method. Percent recoveries ranged from 60.7 ± 7.5 – 100.9 ± 22.5%, (Table S6) indicating satisfactory accuracy and precision for the method used. To control the background contamination, procedural blank samples (n=4) of 2 mL MilliQ-water were prepared with the same method. In addition, carry over effect were controlled by injecting solvent blank every 10 samples. The estimation of limit of detection (LOD) and limit of quantification (LOQ) were calculated from the calibration curve (0.05 to 5 ng/mL) using the equation; $LOD = 3.3*\sigma/S$ and $LOQ = 10*\sigma/S$, where σ = the standard error of y-intercepts of regression lines and S = the of the slope of calibration curve. Limit of blank (LOB) was also calculated when compounds were detected in procedural blank samples, as the average concentration in the blank samples + 3 standard deviations. LOB values were lower than LOQ as depicted in Table S6.

Table S6 Method performance parameters of the method A

Compound	Mushroom (n=6) (Recovery±RSTD)%	Substrate (n=6) (Recovery±RSTD)%	LOB (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)
Carbamazepine	87.7±13.0	82.45±10.2	0.09	0.04	0.13
Carbamazepine-10,11-epoxide	82.3±24.5	60.7±7.5	0.04	0.07	0.20
Caffeine	64.1±38.0	100.3±7.3	0.59	0.66	1.99
DEET	116.4±19.5	88.9±7.8	0.10	0.06	0.17
Metoprolol	111.6±14.5	100.9±22.5	0.01	0.01	0.04

Method B:

For antibiotics, samples (n=6) were spiked at 250 ng/mL end concentration. In addition to solvent matched calibration, matrix matched calibration samples were spiked prior to sample preparation with end concentration at 250 ng/mL of ISTDs and concentration ranging from 0.5 to 500 ng/mL of native standards.

Recovery was calculated according to equation 1, where *signal (I)STD* refers to the actual signal area and *conc* refers to the spiked concentration of the sample in ng/mL. *Matrix* refers to the six replicates spiked to 250 ng/mL in matrix, *blank* refers to the blank matrix sample (n = 1). The *solvent* sample (n = 1) refers to the sample spiked to 200 ng/mL level in solvent.

$$\text{Recovery (\%)} = \sum_{i=1}^{n=6} \frac{\left(\frac{\text{signal STD (matrix)}_i}{\text{signal ISTD (matrix)}_i} - \frac{\text{signal (blank)}}{\text{signal ISTD (blank)}}\right) * \text{conc (solvent)}}{\frac{\text{signal STD (solvent)}}{\text{signal ISTD (solvent)}} * \text{conc (sample)}} * \frac{100}{n} \quad \text{(eq. 1)}$$

Repeatability is reported as the relative coefficient of variation (CV%) of the six matrix samples at 250 ng/mL according to equation 2 and 3, where *x* is the signal of the six matrix matched samples at 250 ng/mL and N is six. LOD and LOQ were calculated according to the description in Method A. Good recoveries and repeatability were observed for all target analytes as shown in Tables S6. No antibiotics were detected in the procedural samples used.

$$\sigma = \sqrt{\frac{1}{N-1}\sum_{i=1}^{N}(x_i - \bar{x})^2} \quad \text{(eq. 2)}$$

$$CV\% = \frac{\sigma}{\bar{x}} \cdot 100\% \quad \text{(eq. 3)}$$

Accuracy was calculated as the average difference between calculated concentration and expected concentration for each of the six 250 ng/mL samples and are reported in % of expected concentration (equation 4). The relative coefficient of variation (CV%) was calculated and are reported in the results as well (Table S7).

$$\text{Accuracy (\%)} = \sum_{i=1}^{n=6} \frac{\text{conc(calc)}_i}{\text{conc(exp)}_i} \cdot \frac{100}{n} \quad \text{(eq. 4)}$$

The calculations for recovery, repeatability, and accuracy were done for the quantifier transition for all target analytes. The calculations were done with samples spiked to 250 ng/mL for all compounds.

Table S7. Method performance parameters of method B, values from mushroom and substrate samples

Compound	Substrate (Recovery ±CV)%	Mushroom (Recovery ±CV)%	LOD (ng/mL)	LOQ (ng/mL)
Sulfadiazine	109.0±1.4	93.2±18.9	0.47	1.41
Sulfamethazine	55.2±45.5	104.3±21.1	0.86	2.6
Ciprofloxacin	117.87±20.5	95.6±6.6	1.8	5.6
Norfloxacin	105.7±7.6	78.3±12.1	0.7	2.2

Table S8. Mean concentrations and standard deviation in spiked substrate (μg kg^{-1} dw)

	NOR	CIP	SDZ	SMZ	CBZ	CBZEP	CAF	DEET	MTP
A. subrufescens									
Inoculation	253 ± 113	355 ± 102	270 ± 53	292 ± 76	237 ± 61	<LOD	31.6 ± 16	10.4 ± 8.0	4.29 ± 1.5
Casing	187 ± 92.6	138 ± 69	8.6 ± 0.84	11.6 ± 1.1	84.2 ± 53	42.3 ± 14	6.79 ± 10*	<LOQ	<LOD
H1	114 ± 60.9	11.1 ± 16.2*	11.3 ± 2.0	12.1 ± 3.5	32 ± 39	15.4 ± 7.9	5.79 ± 8.5*	<LOQ	<LOD
H2	418 ± 718	71.7 ± 121*	4.83 ± 2.6	9.62 ± 1.6	22 ± 34	6.58 ± 8.9	<LOD	<LOQ	<LOD
A. bisporus									
Inoculation	735 ± 185	692 ± 203	860 ± 209	769 ± 241	611 ± 230	<LOD	30.3 ± 1.1	15.4 ± 2.1	3.61 ± 1.0
Casing	597 ± 621	320 ± 379	11.7 ± 10	15.4 ± 4.8	284 ± 280	41.7 ± 27	23.0 ± 4.7	<LOQ	0.348 ± 0.58*
H1	950 ± 577	486 ± 249	12 ± 6.7	18.8 ± 5.0	662 ± 644	82.8 ± 27	27.0 ± 7.3	<LOQ	<LOD
H2	1070 ± 470*	224 ± 385*	9.95 ± 5.8*	20.7 ± 6.8	247 ± 140	93.7 ± 23	20.6 ± 7.7	<LOQ	<LOD
Uninoculated control									
Inoculation	462 ± 184	556 ± 94.9	819 ± 14	797 ± 66	460 ± 22.4	<LOQ	32.7 ± 2.8	11.9 ± 8.1	4.46 ± 2.3
Casing	759 ± 122	702 ± 22.3	17.4 ± 5.3	22 ± 2.6	654 ± 66	19.0 ± 20	14.9 ± 9.3	<LOQ	0.723 ± 1.0*
H1	792 ± 28	1060 ± 127	13.6 ± 0.16	17.7 ± 2.0	1080 ± 439	29.7 ± 28	5.48 ± 6.3*	<LOQ	<LOD
H2	1310 ± 248	879 ± 206	15 ± 0.97	23.4 ± 1.0	1020 ± 636	16.4 ± 6.0	15.6 ± 8.2	<LOQ	<LOD

* At least one of the replicates were below the LOD or LOQ

Table S9. Mean concentrations and standard deviation in the control substrate (µg kg^{-1} dw)

	NOR	CIP	SDZ	SMZ	CBZ	CBZEP	CAF	DEET	MTP
A. subrufescens									
Inoculation	<LOD	<LOQ	<LOD	<LOD	1.62 ± 0.91	<LOD	29.1 ± 6.03	20.2 ± 1.8	4.72 ± 1.89
Casing	<LOD	<LOD	4.69 ± 1.8*	3.37 ± 1.85*	0.66 ± 0.31	<LOD	5.05 ± 8.54*	<LOQ	<LOD
H1	<LOD	<LOD	4.01 ± 1.5*	4.48 ± 1.11	<LOD	<LOD	<LOD	<LOQ	<LOD
H2	<LOD	<LOD	<LOQ	2.36 ± 0.932*	<LOD	<LOQ	<LOD	<LOQ	<LOD
A. bisporus									
Inoculation	<LOD	3.37 ± 3.8*	<LOD	<LOD	1.60 ± 0.60	<LOQ	32.7 ± 6.2	16.3 ± 6.1	4.26 ± 1.1
Casing	<LOD	<LOD	2.45 ± 1.1*	<LOQ	0.95 ± 0.15	0.25 ±0.16*	14.3 ± 9.3	<LOQ	<LOD
H1	<LOD	<LOD	<LOQ	<LOQ	1.24 ± 0.89	0.33 ± 0.16*	16.5 ± 3.8	<LOQ	<LOD
H2	<LOD	<LOD	<LOD	<LOD	1.01 ± 0.36	0.38 ± 0.16*	17.2 ± 4.0	<LOQ	<LOD
Uninoculated control									
Inoculation	<LOD	<LOD	<LOD	<LOD	1.45 ± 0.032	<LOD	26.3 ± 2.7	20.1 ± 0.51	3.54 ± 0.22
Casing	<LOD	<LOD	<LOD	<LOD	1.65 ± 0.83	<LOQ	13.5 ± 3.6	<LOQ	2.08 ± 0.31
H1	<LOD	<LOD	<LOD	<LOD	1.99 ± 0.96	0.64 ± 0.23*	12.8 ± 1.0	<LOQ	<LOD
H2	<LOD	<LOD	<LOD	<LOD	2.38 ± 1.48	<LOQ	10.3 ± 1.8	<LOQ	<LOD

* At least one of the replicates were below the LOD or LOQ

Table S10. Mean concentrations and standard deviation in the control substrate (μg kg^{-1} ash)

	NOR	CIP	SDZ	SMZ	CBZ	CBZEP	CAF	DEET	MTP
A. subrufescens									
Inoculation	<LOD	<LOQ	<LOD	<LOD	8.67 ± 4.2	<LOD	158 ± 23	111 ± 16	25.3 ± 8
Casing	<LOD	<LOD	23.6 ± 8.7*	16.7 ± 8.2*	3.38 ± 1.7	<LOD	25.7 ± 44*	<LOQ	<LOD
H1	<LOD	<LOD	15.1 ± 5.8*	16.8 ± 4.5	<LOD	<LOD	<LOD	<LOQ	<LOD
H2	<LOD	<LOD	<LOQ	9.49 ± 4.3*	<LOD	<LOQ	<LOD	<LOQ	<LOD
A. bisporus									
Inoculation	<LOD	19.4 ± 22*	<LOD	<LOD	9.22 ± 3.6	<LOQ	188 ± 37	93.8 ± 36	24.5 ± 6.7
Casing	<LOD	<LOD	11.8 ± 4.9*	<LOQ	4.63 ± 0.83	1.22 +-0.77*	69.6 ± 45*	<LOQ	<LOD
H1	<LOD	<LOD	<LOQ	<LOQ	4.35 ± 2.9	1.17 ± 0.51*	59.4 ± 15	<LOQ	<LOD
H2	<LOD	<LOD	<LOD	<LOD	3.38 ± 1.2	1.26 ± 0.53*	57.5 ± 13	<LOQ	<LOD
Uninoculated control									
Inoculation	<LOD	<LOD	<LOD	<LOD	8.52 ± 0.32	<LOD	155 ± 18	119 ± 8.5	20.9 ± 2.1
Casing	<LOD	<LOD	<LOD	<LOD	8.18 ± 4.7	<LOQ	64.6 ± 19	<LOQ	9.84 ± 1
H1	<LOD	<LOD	<LOD	<LOD	6.64 ± 2.9	2.14 ± 0.9	43.2 ± 4	<LOQ	<LOD
H2	<LOD	<LOD	<LOD	<LOD	8.36 ± 5.6	<LOQ	35.2 ± 6.6	<LOQ	<LOD

* At least one of the replicates were below the LOD or LOQ

Table S11. Mean concentration and standard deviation of PPCPs in mushrooms (μg kg^{-1} dw)

			CBZ	CBZ-EP	CAF	DEET	SDZ	SMZ	NOR	CIP
Spiked	*A. subrufescens*	H1	<LOQ	1.02*	<LOD	<LOQ	<LOD	<LOD	<LOD	<LOD
		H2	<LOQ	<LOD	<LOD	0.08*	<LOD	<LOD	<LOD	<LOD
	A. bisporus	H1	0.76±0.92	1.85±1.57	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
		H2	0.42±0.32	1.48±0.76	<LOD	0.15±0.06	<LOD	<LOD	<LOD	<LOD
Control	*A. subrufescens*	H1	<LOD	<LOD	<LOD	0.15*	<LOD	<LOD	<LOD	<LOD
		H2	<LOD	<LOD	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOD
	A. bisporus	H1	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
		H2	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD

* At least one of the replicates were below the LOD or LOQ

Mushroom substrate characterization.

The substrate was mixed with distilled water at 20°C in a 30:100 ratio (v/v) for pH and 60:300 ratio (v/v) for EC determination. After 30 minutes of sedimentation, pH and EC were measured with a Milwaukee 802 pH/EC/TDS meter. At harvest 1 and 2 it was not possible to sample enough material for pH and EC analysis and the pH was therefore measured directly in the substrate with a "stick-in" pH meter from BIOGRÓD (product code G071505). Dry matter was measured using minimum 10 grams substrate at 120°C in duplicate. Dried substrate was further burned at 550°C to determine loss of ignition (i.e., ash). For determination of dissolved organic carbon (DOC), 5.0 g substrate were mixed with 25 mL deionized water, agitated at a horizontal shaker for one hour at 100 rpm, and centrifugated at 4000 rpm for 15 minutes. The supernatant was filtered through Whatman blue ribbon filters, and the filtrate were again filtrated through a 0.45 µm nylon filter (Thermo Scientific F2519-4) during centrifugation at 4000 rpm for 5 minutes. The filtrate was analyzed at Shimadzu TOC-V CPN.

Table S12. Substrate quality parameters, spiked substrate. DM: Dry matter. VS: Volatile solids.

	pH	DM (%)	Ash (%)	VS (%)
A. subrufescens				
Inoculation	8.7 ± 0.2	23.8 ± 1.0	14.4 ± 0.7	85.6 ± 0.7
Casing	6.4 ± 0.3	20.6 ± 1.6	20.0 ± 1.5	80.0 ± 1.5
H1	5.8 ± 0.3	19.5 ± 1.7	28.7 ± 0.5	71.3 ± 0.5
H2	7.1 ± 0.9	19.8 ± 4.2	28.4 ± 1.7	71.6 ± 1.7
A. bisporus				
Inoculation	9.0 ± 0.1	24.4 ± 0.5	13.5 ± 0.9	86.5 ± 0.9
Casing	6.9 ± 0.6	21.5 ± 0.7	18.0 ± 1.0	82.0 ± 1.0
H1	8.4 ± 0.6	16.6 ± 2.3	26.2 ± 1.5	73.8 ± 1.5
H2	8.4 ± 0.1	19.1 ± 1.8	35.5 ± 10	64.5 ± 10
Uninoculated control				
Inoculation	9.0 ± 0.0	23.7 ± 0.1	14.0 ± 0.5	85.9 ± 0.5
Casing	7.7 ± 0.4	21.4 ± 0.6	17.6 ± 0.1	82.4 ± 0.1
H1	8.5 ± 0.1	16.4 ± 0.1	26.7 ± 1.0	73.3 ± 1.0
H2	8.1 ± 0.0	15.6 ± 4.0	19.8 ± 8.3	80.2 ± 8.3

Table S13. Substrate quality parameters, control substrate. DM: Dry matter. VS: Volatile solids.

	pH	DM (%)	Ash (%)	VS (%)
A. subrufescens				
Inoculation	9.0 ± 0.1	23.4 ± 2.4	18.3 ± 1.9	81.7 ± 1.9
Casing	6.2 ± 0.4	21.4 ± 0.6	19.7 ± 1.3	80.3 ± 1.3
H1	5.6 ± 0.1	22.4 ± 1.9	26.7 ± 0.9	73.3 ± 0.9
H2	5.9 ± 0.4	20.5 ± 1.0	25.6 ± 3.9	74.4 ± 3.9
A. bisporus				
Inoculation	8.9 ± 0.2	24.2 ± 1.4	17.4 ± 0.4	82.6 ± 0.4
Casing	6.7 ± 0.2	21.8 ± 1.5	20.7 ± 0.6	79.3 ± 0.6
H1	7.7 ± 0.7	20.0 ± 1.5	27.8 ± 1.5	72.2 ± 1.5
H2	8.4 ± 0.2	21.7 ± 2.9	30.0 ± 0.3	70.0 ± 0.3
Uninoculated control				
Inoculation	9.1 ± 0.1	23.5 ± 2.0	17.0 ± 0.9	83.0 ± 0.9
Casing	8.0 ± 0.3	21.4 ± 1.7	21.1 ± 2.3	78.9 ± 2.3
H1	8.7 ± 0.1	17.2 ± 0.9	29.6 ± 2.2	70.4 ± 2.2
H2	8.2 ± 0.1	18.5 ± 1.1	29.5 ± 2.5	70.5 ± 2.5

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Norwegian University
of Life Sciences

Postboks 5003
NO-1432 Ås, Norway
+47 67 23 00 00
www.nmbu.no

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