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Doctoral thesis

*Valorization of poultry manure through anaerobic
co-digestion with locally available organic waste –
an integrated approach for bioenergy production
and nutrient recovery in a circular economy framework*

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ABSTRACT IN ENGLISH

This doctoral thesis presents a comprehensive study on the valorization of poultry manure through anaerobic co-digestion with locally available biowaste. Particular attention was devoted to improving process stability, increasing methane production efficiency, and evaluating the fertilizing value and safety of the resulting digestate. The research was driven by the need to develop sustainable strategies for managing poultry manure - one of the most abundant and nitrogen-rich agricultural residues, while simultaneously seeking technologies that enable energy and nutrient recovery in line with the principles of the circular economy.

The theoretical background, based on a series of review publications, identified anaerobic digestion as a promising technology for the stabilization of poultry manure and its conversion into renewable energy and nutrient-rich products. However, its practical application is constrained by the low carbon-to-nitrogen ratio and the risk of ammonia inhibition. To overcome these limitations, co-digestion with other locally available organic wastes, such as sewage sludge and grease trap residues was proposed. The management of these waste streams also poses significant environmental and technological challenges, while their complementary physicochemical properties make them suitable for joint processing. Combining these substrates helped balance feedstock composition, improve buffering capacity, and enhance process stability.

Experimental research confirmed that the co-digestion of poultry manure with sewage sludge is technically feasible and leads to a significant increase in methane yield compared with the digestion of sewage sludge alone. The optimal share of poultry manure in the feedstock mixture ranged from 20% to 40% of volatile solids, ensuring process balance, reducing ammonia accumulation, and creating a clear synergistic effect between substrates. The obtained digestate was characterized by a stable chemical composition, neutral to slightly alkaline pH, and a high content of nutrients, particularly nitrogen, phosphorus, and calcium - indicating its fertilizing potential and suitability as a soil-improving material.

Further research focused on the possible reuse of digestate and the intensification of biogas production from poultry manure-based mixtures. The studies demonstrated that digestate can be successfully used in the cultivation of *Agaricus bisporus* as an additive to the casing layer, provided that its proportion does not exceed a few percent of the casing mass. Moreover, chemical and thermo-alkaline pretreatments, particularly saponification with sodium hydroxide - significantly improved substrate solubilization, accelerated hydrolysis, and increased methane yield by up to 63% compared with untreated mixtures.

The results confirmed the validity of the research hypotheses. It was shown that co-digestion of poultry manure with selected organic wastes enhances process

efficiency and stability; that the resulting digestate exhibits valuable fertilizing potential but requires further assessment regarding its safe environmental application; and that pretreatment constitutes an effective tool for the intensification of methane production.

The dissertation demonstrates that integrating poultry manure co-digestion with appropriately selected biowastes, supported by targeted pretreatment methods and sustainable digestate management, provides an efficient and environmentally sound strategy for converting agricultural and municipal residues into renewable energy and value-added products. These outcomes align with the goals of the circular economy and support the development of energy self-sufficient wastewater treatment systems and waste-to-energy installations.

ABSTRACT IN POLISH

Rozprawa doktorska przedstawia kompleksowe badania nad możliwością zagospodarowania pomiotu kurzego w procesie ko-fermentacji beztlenowej z lokalnie dostępnymi bioodpadami. Szczególną uwagę poświęcono poprawie stabilności procesu, zwiększeniu wydajności produkcji metanu oraz ocenie wartości nawozowej i bezpieczeństwa stosowania uzyskanego pofermentu. Podjęte badania wynikają z potrzeby opracowania zrównoważonych metod postępowania z pomiotem kurzym - jednym z najbardziej rozpowszechnionych i zasobnych w azot odpadów pochodzenia rolniczego, przy jednoczesnym poszukiwaniu technologii umożliwiających odzysk energii i składników pokarmowych w zgodzie z zasadami gospodarki o obiegu zamkniętym.

Część teoretyczna, oparta na cyklu publikacji przeglądowych, wskazała fermentację beztlenową jako obiecującą technologię stabilizacji pomiotu kurzego i przekształcania go w energię odnawialną oraz produkty bogate w składniki odżywcze. Zwrócono jednak uwagę, że praktyczne zastosowanie tej technologii jest ograniczane przez niekorzystny stosunek C/N i ryzyko inhibicji amoniakiem. W celu przezwyciężenia tych ograniczeń zaproponowano ko-fermentację z innymi lokalnie dostępnymi odpadami organicznymi, takimi jak osady ściekowe czy tłuszcze z separatorów, których zagospodarowanie również stanowi istotne wyzwanie środowiskowe i technologiczne. Połączenie tych substratów, o komplementarnych właściwościach fizykochemicznych, pozwalało na zrównoważenie składu wsadu, poprawę pojemności buforowej i zwiększenie stabilności procesu.

Badania eksperymentalne potwierdziły, że ko-fermentacja pomiotu kurzego z osadem ściekowym jest technologicznie możliwa i prowadzi do istotnego wzrostu wydajności metanowej w porównaniu z fermentacją osadu prowadzonego samodzielnie. Optymalny udział pomiotu w mieszaninie wsadu mieścił się w zakresie 20–40% suchej masy organicznej, co zapewniało równowagę procesową, ograniczało akumulację amoniaku i umożliwiało wystąpienie efektu synergii między substratami. Uzyskany poferment charakteryzował się stabilnym składem chemicznym, obojętnym lub lekko zasadowym odczynem oraz wysoką zawartością składników pokarmowych, zwłaszcza azotu, fosforu i wapnia, co wskazuje na jego potencjał nawozowy oraz możliwość wykorzystania jako środka poprawiającego właściwości gleb.

Kolejne etapy badań obejmowały analizę możliwości wykorzystania pofermentu oraz intensyfikacji produkcji biogazu z mieszanin zawierających pomiot kurzy. Wykazano, że poferment może być z powodzeniem stosowany w uprawie *Agaricus bisporus* jako dodatek do warstwy okrywowej, o ile jego udział nie przekracza kilku procent masy okrywy. Z kolei zastosowanie chemicznej i termoalkalicznej obróbki wstępnej, w szczególności zmydlania za pomocą wodorotlenku sodu – znacząco poprawiło rozpuszczalność substancji organicznych, przyspieszyło hydrolizę i zwiększyło

wydajność produkcji metanu nawet o 63% w porównaniu z mieszaninami niepoddanymi modyfikacji.

Uzyskane wyniki potwierdziły zasadność postawionych hipotez badawczych. Wykazano, że ko-fermentacja pomiotu kurzego z wybranymi odpadami organicznymi zwiększa efektywność i stabilność procesu, uzyskany poferment posiada wartościowy potencjał nawozowy, przy czym wymaga dalszej oceny pod kątem bezpieczeństwa jego stosowania w środowisku, a zastosowanie obróbki wstępnej stanowi skuteczne narzędzie intensyfikacji produkcji biogazu.

Rozprawa dowodzi, że integracja ko-fermentacji pomiotu kurzego z odpowiednio dobranymi bioodpadami, wsparta ukierunkowanymi metodami obróbki wstępnej oraz zrównoważonym zagospodarowaniem pofermentu, stanowi efektywną i przyjazną środowisku strategię przekształcania odpadów rolniczych i komunalnych w energię odnawialną i produkty o wartości dodanej. Wyniki te wpisują się w cele rozwoju gospodarki o obiegu zamkniętym i wspierają dążenia do zwiększenia samowystarczalności energetycznej systemów oczyszczania ścieków oraz instalacji typu waste-to-energy.

ABSTRACT IN DUTCH

Dit doctoraatsonderzoek presenteert een uitgebreide studie over de valorisatie van pluimveemest via anaërobe co-vergisting met lokaal beschikbaar biologisch afval. Bijzondere aandacht werd besteed aan het verbeteren van de processtabiliteit, het verhogen van de methaanproductie-efficiëntie en het evalueren van de bemestingswaarde en de veiligheid van het verkregen digestaat. Het onderzoek werd gedreven door de noodzaak om duurzame strategieën te ontwikkelen voor het beheer van pluimveemest – één van de meest overvloedige en stikstofrijke landbouwresiduen – en tegelijkertijd technologieën te verkennen die energie- en nutriëntenherwinning mogelijk maken in overeenstemming met de principes van de circulaire economie.

De theoretische achtergrond, gebaseerd op een reeks overzichtspublicaties, identificeerde anaërobe vergisting als een veelbelovende technologie voor de stabilisatie van pluimveemest en de omzetting ervan in hernieuwbare energie en nutriëntenrijke producten. De praktische toepassing ervan wordt echter beperkt door de lage koolstof-stikstofverhouding en het risico op ammoniakinhibitie. Om deze beperkingen te overwinnen, werd co-vergisting met andere lokaal beschikbare organische afvalstromen, zoals zuiveringsslib en vetvangerresiduen, voorgesteld. Het beheer van deze afvalstromen vormt eveneens een belangrijke ecologische en technologische uitdaging, terwijl hun complementaire fysisch-chemische eigenschappen ze geschikt maken voor gezamenlijke verwerking. De combinatie van deze substraten hielp om de samenstelling van het substraat in evenwicht te brengen, de bufferende capaciteit te verbeteren en de processtabiliteit te verhogen.

Experimenteel onderzoek bevestigde dat co-vergisting van pluimveemest met zuiveringsslib technisch haalbaar is en leidt tot een aanzienlijke toename van de methaanopbrengst in vergelijking met de afzonderlijke vergisting van slib. Het optimale aandeel pluimveemest in het mengsel bedroeg 20–40% van de vluchtige vaste stoffen, wat processtabiliteit garandeerde, ammoniakaccumulatie verminderde en een duidelijk synergetisch effect tussen de substraten veroorzaakte. Het verkregen digestaat werd gekarakteriseerd door een stabiele chemische samenstelling, een neutrale tot licht alkalische pH en een hoog gehalte aan nutriënten – vooral stikstof, fosfor en calcium – wat wijst op zijn potentieel als meststof en bodemverbeterend middel.

Vervolgonderzoek richtte zich op de mogelijke hergebruiktoepassingen van het digestaat en de intensivering van de biogasproductie uit mengsels op basis van pluimveemest. De studies toonden aan dat digestaat met succes kan worden gebruikt bij de teelt van *Agaricus bisporus* als toevoeging aan de dekaarde, op voorwaarde dat het aandeel niet meer bedraagt dan enkele procenten van de massa van de dekaarde. Bovendien verbeterden chemische en thermo-alkalische voorbehandelingen – met

name verzeping met natriumhydroxide – de oplosbaarheid van organische stoffen aanzienlijk, versnelden de hydrolyse en verhoogden de methaanopbrengst tot 63% in vergelijking met onbehandelde mengsels.

De resultaten bevestigden de geldigheid van de onderzoekshypothesen. Aangetoond werd dat co-vergisting van pluimveemest met geselecteerde organische afvalstromen de efficiëntie en stabiliteit van het proces verhoogt; dat het verkregen digestaat een waardevol bemestingspotentieel heeft, maar verdere beoordeling vereist met betrekking tot de milieuveiligheid van het gebruik ervan; en dat voorbehandeling een doeltreffend instrument vormt voor de intensivering van de methaanproductie.

De thesis toont aan dat de integratie van co-vergisting van pluimveemest met zorgvuldig geselecteerde bioreststromen, ondersteund door gerichte voorbehandelingsmethoden en duurzaam digestaatbeheer, een efficiënte en milieuvriendelijke strategie biedt voor de omzetting van landbouw- en stedelijke residuen in hernieuwbare energie en producten met toegevoegde waarde. Deze resultaten sluiten aan bij de doelstellingen van de circulaire economie en ondersteunen de ontwikkeling van energiezelfvoorzienende waterzuiveringsinstallaties en waste-to-energy systemen.

LIST OF IMPORTANT ABBREVIATIONS AND SYMBOLS

A – Alkalinity

AD – Anaerobic digestion

AcoD/cAD – Anaerobic co-digestion

ARGs – Antibiotic resistance genes

BAT – Best available techniques

BE – Biological efficiency

BMP – Biochemical methane potential

C/N – Carbon-to-nitrogen ratio

CE – Circular economy

CHP – Combined heat and power systems

COD – Chemical oxygen demand

CPI – Co-digestion performance index

DCWG – Supercritical water gasification

DM – Dry mass

EC – Electrical conductivity

EDCs – Endocrine-disrupting chemicals

EPA – Environmental Protection Agency

EPS – Extracellular polymeric substances

ER – Potential ecological risk factor

ERA – Environmental risk assessment

ERF – Environmental risk factor

ETP – Electron transport phosphorylation

FTIR – Fourier-transform infrared spectroscopy

FAN – Free ammonia nitrogen

FM – Fresh matter

GC-MS – Gas Chromatography–Mass Spectrometry

GER – Global ecological risk

GHG/GHGs – Greenhouse gas(es)

GRI – Global Risk Index

GTS – Grease trap sludge

HRT – Hydraulic retention time

HTC – Hydrothermal carbonization

HTG – Hydrothermal gasification

HTL – Hydrothermal liquefaction

HTP – Thermal hydrolysis process

HTV – Hydrothermal vaporization

ICP–OES – Inductively coupled plasma optical emission spectrometry

ICF – Individual contamination factor

IER – Individual ecological risk

IQR – Interquartile range

ISR – Inoculum-to-substrate ratio

LB–EPS – Loosely bound extracellular polymeric substances

LCA – Life cycle assessment

LC–MS – Liquid Chromatography–Mass Spectrometry

MG – Modified Gompertz model

MSW – Municipal solid waste

NPOC – Non-purgeable organic carbon

OLR – Organic loading rate

OSW – Organic solid waste

OWtE – Organic waste-to-energy

PCA – Principal component analysis

PCR – Polymerase chain reaction

PE – Population equivalent

PFAS – Per- and polyfluoroalkyl substances

PNEC – Predicted no-effect concentration

PM – Poultry manure / digestate obtained from anaerobic co-digestion of sewage sludge with poultry manure

PPCPs – Pharmaceuticals and personal care products

QSAR - Quantitative structure–activity relationship

RA – Risk assessment

RAC – Risk assessment code

R_m – Maximum methane production rate

RA – Risk Assessment

RI – Risk index

RRfW – Resource recovery from waste

S–EPS – Soluble extracellular polymeric substances

SEM – Scanning electron microscopy

SS / Ss – Sewage sludge / digestate obtained from anaerobic digestion of sewage sludge

TB–EPS – Tightly bound extracellular polymeric substances

TC – Total carbon

TEM – Transmission electron microscopy

TF – Transfer function model

TKN – Total Kjeldahl nitrogen

TN – Total nitrogen

TOC – Total organic carbon

TP – Total phosphorus

TS – Total solids

VFAs – Volatile fatty acids

VS – Volatile solids

WWTP – Wastewater treatment plant

WtE – Waste-to-energy

UPLC – Ultra-performance liquid chromatography

1. INTRODUCTION

1.1. Background

The 21st century presents unprecedented challenges in the field of environmental protection, requiring difficult decisions that will determine the future of societies and ecosystems worldwide. Over recent decades, the European Union has undertaken significant efforts to establish uniform and enforceable legal frameworks in climate policy and the transition towards a sustainable economy. The selection of effective strategies for managing the Earth's resources and mitigating harmful emissions demands an interdisciplinary perspective that integrates scientific, social, and economic dimensions.

Understanding the historical background of the climate crisis is essential to shaping contemporary approaches to sustainability and social responsibility. The rapid expansion of the oil industry and fossil fuel use after World War II triggered growing environmental concerns, intensified scientific interest and stimulated early warnings about global climate risks. A milestone in this development was Charles Keeling's 1960 publication in *Tellus*, reporting rising atmospheric CO₂ concentrations at the South Pole, occurring "almost as fast as could be expected from the (global) combustion of fossil fuels" (Franta, 2018). Technological advances in the 1950s, particularly gas analysers, enabled systematic geophysical measurements and contributed to the International Geophysical Year (1957–1958). Although the main focus of this initiative was the physicochemical properties of the Earth, it also produced the now-iconic Keeling Curve – the first continuous, direct record of global CO₂ growth (Franta, 2018; Keeling et al., 2011). Despite the significance of this work, Keeling's pioneering research was long under-cited, perceived by many as "routine science" (Marx et al., 2017). Yet, concerns about industrial CO₂ emissions had already been voiced in the late 19th century, echoing John Tyndall's 1859 discovery that "carbonic acid" (now CO₂) absorbs thermal radiation (Lewandowsky, 2021).

The question therefore arises: why did it take so long for a decisive discursive breakthrough in climate governance? The first formal international initiative to address these issues is generally regarded as the 1972 Stockholm Conference (European Parliament, 2025). While the conference attracted unprecedented attention to environmental concerns, it fell short of expectations. Boycotts by numerous countries – influenced by geopolitical tensions and economic priorities – limited its impact (Lewandowsky, 2021; Linnér & Selin, 2013). In parallel, disinformation campaigns conducted by oil and gas corporations hindered the formation of public awareness and delayed environmental action. Research has shown that, by the 1970s, these companies already possessed internal studies confirming the risks of fossil fuel combustion and had developed tools to estimate its impact on global warming with considerable accuracy (Bonneuil et al., 2021; Supran et al., 2023). Their opposition

to eco-tax proposals further weakened Europe's position during the 1992 Earth Summit in Rio de Janeiro. Only in the years surrounding the 1997 Kyoto Conference did a shift occur, as the pursuit of scientific consensus and the prospect of a global climate agreement prompted major oil companies to abandon strategies of outright denial. Partial acceptance of the Intergovernmental Panel on Climate Change (IPCC) findings and the adoption of "green" narratives marked the beginning of a new phase in the interaction between science, politics, and industry (Bonneuil et al., 2021; Gertrudix et al., 2024).

The Kyoto Protocol, ratified in 2005, was expected to deliver substantial progress as an international agreement supplementing the United Nations Framework Convention on Climate Change (UNFCCC), developed at the Earth Summit in Rio de Janeiro. The Protocol was founded on the principle of common but differentiated responsibilities, taking into account the economic performance of the countries committed to reducing greenhouse gas emissions compared to the base year (1990 for most countries). Developing countries expressed clear concerns about environmentally related trade protectionism and demanded international financial support for climate protection and development (Linnér & Selin, 2013). Therefore, some nations could resort to flexibility mechanisms, while developed countries, recognised as the main emitters, bore the greatest burden of financing GHG reductions and providing technologies and projects to the former (Grunewald & Martinez-Zarzoso, 2016). These economic disparities, combined with differing expectations and demands, intensified tensions, disputes, and the difficulty of reaching consensus. The most significant opponent of the Protocol was the United States, whose objections were rooted in the lenient treatment of China and India, which were almost entirely excluded from the agreement's requirements. Despite initially accepting the agreement, Canada withdrew its commitments in 2011 (Linnér & Selin, 2013). The first commitment period of the Kyoto Protocol covered the years 2008–2012, and analyses indicate that the parties formally adhered to its provisions. However, the largest GHG reductions were recorded in former Eastern Bloc countries, largely due to the economic restructuring that followed the dissolution of the Soviet Union in the early 1990s (Shishlov et al., 2016). Ultimately, although 36 developed countries covered by the Protocol reduced their emissions, global GHG emissions increased by 32% between 1990 and 2010 (Hohne, 2012). Therefore, the problem of climate change mitigation not only remained unresolved but also escalated, prompting a continuous number of meetings at the international and local levels. In 2012, an agreement was reached in Doha to extend the Kyoto Protocol commitments until 2020, although the United States has yet to approve it, and countries such as Russia and Japan have decided to withdraw it. However, the meeting had a broader context, serving as a bridge for future initiatives and laying the groundwork for post-2020 climate goals (Savaresi, 2016; Stoddard et al., 2025).

Meanwhile, the European Union authorities undertook their own legislative work focused on more ambitious plans for reducing greenhouse gas emissions. In 2009, a climate package was introduced, which included the so-called "three 20 targets": a 20% reduction in greenhouse gas emissions in the EU compared to the 1990 legibility mechanism agreement, a 20% improvement in the EU's energy efficiency, and 20% of the EU's energy from renewable energy sources. However, standardizing legislation across the European Union, which encompasses many countries with diverse economic levels, is also challenging. Some member states have expressed concerns about rising energy costs resulting from the package's implementation. This is particularly true for countries economically dependent on coal, such as Poland, which, without appropriate support, could lose energy security and independence after adopting EU climate commitments (Skjærseth, 2018; Skoczkowski et al., 2018). Therefore EU authorities had to adopt a strategy to bridge the gap between climate policy and energy security concerns, emphasizing mutual benefits. Building consensus and mitigating resistance from coal-dependent states primarily involved taking into account the existing experience of implementing ambitious climate goals in individual countries and a promising long-term perspective in the negotiations (Skjærseth, 2018).

International cooperation has a significant impact on shaping public awareness and building trust that other countries are taking action to protect the environment. A lack of coordination in setting global climate policy priorities can contribute to increased economic incentives and increased emissions among countries not covered by commitments (van Asselt & Newell, 2022). In this context, UN conferences can serve as an instrument for establishing rules and standards of conduct, exchanging views, sharing best practices, and ultimately creating a space for dialogue on issues that often do not receive the attention they deserve. Although the Climate Summits did not demonstrate significant direct achievements, their ability to influence public opinion and, consequently, political agendas is significant (Linnér & Selin, 2013). Further international meetings were therefore inevitable, but the experience of the Kyoto commitments, which were disproportionate to the scale of the challenge, led to a shift away from the categorization of countries and the annex-based structure (Stoddard et al., 2025). Years of difficult negotiations culminated in the signing of the Paris Agreement in 2016, which sets a long-term goal of keeping global surface temperature increase well below 2°C, with a preferred limit of 1.5°C above pre-industrial levels (Mercure et al., 2021). Unlike the Kyoto Treaty, this agreement emphasizes consensus-building and allows for voluntary, nationally determined targets. This means partially lifting the barrier between developed and developing countries, encouraging all parties to the agreement to reduce GHG emissions. The treaty emphasizes the principle of transparency, obliging parties to regularly (every five years) communicate their nationally determined contribution to global climate change response. Furthermore, each subsequent contribution will be higher than the current

one, expressing the highest possible level of ambition (Mercure et al., 2021; Savaresi, 2016; United Nations Framework Convention on Climate Change (UNFCCC), 2016).

The Paris Agreement is widely regarded as a milestone and the first comprehensive global agreement, based on a declaration and review (Andresen et al., 2021; Busch & Judick, 2021). However, due to the lack of agreed-upon uniform rules of conduct and the voluntary nature of commitments, consensus-based decision-making has been criticized. Stoddard et al., (2025) conducted a comprehensive analysis of the actions of world leaders in mitigating climate change over the past three decades. Despite countless meetings and a multitude of international efforts, global CO₂ emissions have continued to rise, with 2020 recording a 60% increase compared to 1990. It is impossible to pinpoint a single cause for the failure to combat global warming; it turns out that the problem is complex and has many components. However, comparative data on cumulative GHG emissions and income inequality across countries since the end of the 20th century, in the face of a galloping free-market economy, are noteworthy. Progressive economic growth and fuelled consumption place money as the sole measure of value, overriding the least developed regions and future generations who will be most affected by climate change. This explains, among other things, the US withdrawal from the Paris Agreement, the presence of sceptical counterproposals to the scientific consensus on climate, and the reluctance to abandon the carbon paradigm (Andresen et al., 2021; Busch & Judick, 2021; Shabbir & Agha Shah, 2025). Despite scientific rationale, the Paris Agreement also fails to address the need to decarbonize and leave most fossil fuels in the ground, as revealed by lobbying from stakeholders in high-emission industries (Stoddard et al., 2025).

Despite the lack of a change in the global emissions curve and direct mitigation effects on climate, the Paris Agreement strengthened the position of a long-term global goal and heralded a shift in the dynamics of the science-policy relationship. In Paris, the United Nations Framework Convention on Climate Change requested the IPCC to engage scientists and develop a Special Report on Global Warming of 1.5°C. The report was published in 2018, although the scientific community was initially reluctant to engage in the project due to scepticism about the 1.5°C scenario and the need to adapt to political demands (Livingston & Rummukainen, 2020). Thanks to scientific mobilization, the report defined various greenhouse gas emission scenarios, indicating what conditions should be achieved to limit temperature increases. Global net carbon dioxide emissions would need to fall by about 45% by 2030, compared to 2010 levels, while net-zero emissions would need to be achieved around 2050. The report clearly emphasizes the urgency of action and emphasizes that climate change is intensifying, and its negative impacts are already being felt. The IPCC clearly identifies the burning of fossil fuels as the primary cause of the increase in greenhouse gas concentrations in the atmosphere. It also emphasizes the need to reduce emissions of other greenhouse gases, such as methane, whose main sources come from the energy sector, agriculture, and waste (V. Masson-Delmotte et al., 2018).

Following clear scientific messages and the imperative need for urgent action, Europe launched a bold political response based on the intensive implementation of climate transformation and building a global leadership position in the fight against climate change. As recently as 2018, the European Parliament and the Council of the EU introduced new or amended existing legislation on the emissions trading system, effort sharing (a shared target of 40% CO₂ reduction compared to 1990), land use change and forestry, increasing energy efficiency, promoting renewable energy sources, the energy performance of buildings, and managing the Energy Union and climate action (Burger, 2025). In 2019, the European Green Deal was launched, a draft of a comprehensive strategy to achieve climate neutrality in Europe by 2050 and decouple economic growth from the use of natural resources. As part of the above, the European Commission has identified 10 program priorities, which include reviewing existing legislation in the context of climate and environmental protection, as well as introducing regulations on the circular economy, building renovation, biodiversity, agriculture, and innovation (EUROPEAN COMMISSION. Brussels, 11.3.2020 COM(2020) 98 final). The European Union has set highly ambitious climate policy goals, encountering numerous challenges during their implementation, ranging from varying capacity of member states to adopt commitments to unexpected global events. The COVID-19 pandemic has exposed the weakness of economies and forced them to develop strategies to ensure resilience and maintain the supply chain of raw materials (Veugelers et al., 2024). Energy security concerns were exacerbated by the escalation of Russia's invasion of Ukraine and the need to increase the mobilization of defence funds at the expense of investments in green technologies (Krawczyńska et al., 2024). Particularly in the Visegrad Group countries, where public acceptance of the clean energy transition is weak, populist politicians and vested interest groups used the crisis as an opportunity to revive their commitment to fossil fuels (Durdovic et al., 2025). Nevertheless, the limited transport of energy resources from Russia forced Central European countries to accelerate their energy transition. Despite the crisis related to the pandemic and the conflict in Ukraine, the development of renewable energy sources and an increase in employment in this sector are observed, particularly using biomass. The main advantage of renewable energy installations is their local nature, which does not require the development of centralized technical infrastructure (Igłński et al., 2022; Krawczyńska et al., 2024).

The European Green Deal has so far produced, among other outcomes, the European Climate Law (Regulation (EU) 2021/1119), which sets a new, more ambitious target for 2030 – namely, to reduce greenhouse gas emissions to at least 55% compared to 1990 levels. Furthermore, the Energy Efficiency Directive (Regulation (EU) 2023/1791) was amended, establishing an 11.7% reduction in final energy consumption by 2030 relative to 2020. The updated Renewable Energy Directive ((EU) 2023/2413) raises the overall binding target for the share of renewable energy sources in the EU energy mix to 42.5%. The “Fit for 55” package was also introduced, aligning EU policies with the Union's

commitment to reduce net GHG emissions by 2030, addressing emissions from maritime and aviation sectors, expanding the emissions trading scheme, and proposing a carbon border tax (Burger, 2025).

The current geopolitical situation has underscored for decision-makers the importance of securing domestic fuel and energy resources. Energy independence translates into both economic and political autonomy and, in the case of renewable energy development, also supports the transition to clean energy technologies (Igliński et al., 2022). In response to Russia's invasion of Ukraine, the European Commission implemented the REPowerEU plan to gradually phase out imports of Russian fossil fuels, highlighting, among other measures, the role of biomethane as a low-emission substitute for natural gas (Sesini et al., 2024). Sustainable biogas and biomethane production was also designated as a strategic technology in the Net Zero Industry Act, a regulation introduced in March 2023 in response to the US Inflation Reduction Act (Veugelers et al., 2024). The integration and decarbonisation of the gas sector as part of the Fit for 55 package is currently high on the EU political agenda, emphasising the development of hydrogen, biomethane, and syngas production through power-to-gas, anaerobic digestion, and gasification processes, as well as CO₂ capture technology (CCS). While the power-to-gas, CCS, and gasification sectors are still in the early stages of development, anaerobic digestion is currently the most mature and well-understood technology in terms of industrial application (Brémond et al., 2021).

Anaerobic digestion involves the microbial conversion of organic matter into biogas – a mixture of methane, carbon dioxide, and trace amounts of other gases – under anaerobic conditions. This process is one of the most widely applied technologies in Europe for the treatment and valorisation of organic solid waste. Beyond energy recovery, anaerobic digestion contributes to sustainable waste management and is directly aligned with the Circular Economy Action Plan – another key element of the European Green Deal (Honcharuk et al., 2024). The plan specifically addresses the need to make sustainable products the norm in the EU and to reduce waste generation (European Commission, 2020). Anaerobic digestion can treat or co-treat (co-digestion) substrates such as municipal waste, agro-industrial residues, manure, or sewage sludge (Mata-Alvarez et al., 2014a). In addition to biogas, the process yields digestate with high fertiliser potential, which can be used directly on crops or further processed for nutrient recovery (Karki et al., 2021). The agricultural sector accounts for approximately 17% of greenhouse gas emissions, so recovering and recycling nutrients in the form of bio-based fertilisers from digestate offers a promising pathway to address agronomic challenges and enhance sustainable food production. Moreover, organic fertilisers are becoming increasingly competitive with mineral ones, whose production is associated with the generation of hazardous fossil-based waste and heavy reliance on non-renewable natural gas (Álvarez Salas et al., 2024).

The European biogas sector has expanded significantly over the past two decades, initially relying on energy crops, high feed-in tariffs, and electricity production through cogeneration units (Brémond et al., 2021). At present, however, the focus is shifting towards using waste as feedstock and producing biomethane – an upgraded form of biogas or gasified solid biomass that can be injected into the natural gas grid (Sesini et al., 2024). Apart from CH₄, all other gases contained in raw biogas, such as N₂, H₂S, and NH₃, are undesirable for further energy use. Biogas upgrading technologies are continuously being researched and developed, including physical, chemical, and biological processes (Angelidaki et al., 2018). In practice, the separated CO₂ is usually released into the atmosphere; although this does not contribute to a net increase in climate change due to the short carbon cycle of the feedstocks used, it nonetheless represents an untapped resource. Syngas can be produced from captured CO₂ using hydrogen derived, for example, from surplus renewable electricity (Cordova et al., 2023). The calorific value of pure methane is 35.7 MJ/m³, while that of raw biogas typically ranges from 16 to 23 MJ/m³, depending on its composition (Piechota & Igliński, 2021). The realistic biomethane potential estimated for 2050, along with methane potentials from other feedstocks reported in the *Gas for Climate* study (Terlouw et al., 2019) could reach 64.2 billion m³ in the optimised gas scenario.

Political incentives and continued subsidies remain crucial for the further development of biogas and biomethane plants. However, both existing and future installations must take into account local opportunities to create additional income streams and territorial synergies (Brémond et al., 2021). Concerns remain regarding the year-round availability of feedstock for anaerobic digestion and the operational risks associated with certain wastes. Materials such as manure or sewage sludge may contain substances that inhibit the digestion process or pose hygiene and sanitation challenges. These issues can, however, be mitigated through pre-treatment or co-digestion, optimising feedstock composition and operating parameters (Karki et al., 2021). Unlike other renewable technologies, biogas plants are not dependent on weather conditions, which represents a significant advantage for investment and encourages further research into deployment options. Processing diverse organic waste streams through digestion, combined with the possibility of substrate modification, minimises the use of natural resources and reduces greenhouse gas emissions (Łukomska et al., 2025). Moreover, the decentralised nature of biogas plants allows for more even distribution across a territory, reducing transmission losses. By contrast, large centralised power plants, such as coal-fired facilities, often suffer significant energy losses when transmitting electricity over long distances. They are also vulnerable targets for hostile forces, terrorism, or cyberattacks, which in the event of an incident could destabilise the national energy system (Igliński et al., 2022). Biogas production facilities can be constructed in various configurations, both in urban and rural areas. In Poland, owing to its strong agricultural base, the country possesses one of the largest waste biomass potentials in Europe. Collaboration with local biogas

plants enables farmers to implement good agricultural practices more effectively, for instance by using digestate as fertiliser instead of raw manure or mineral fertilisers, which are often a source of nitrate pollution (Igliński et al., 2022; Szymańska & Lewandowska, 2015). Biogas production facilities are also commonly co-located with landfills and wastewater treatment plants, which are of strategic importance given EU policies targeting their energy self-sufficiency and reduced pollutant emissions (European Parliament and of the Council, 2018, 2024).

Aligned with EU goals for climate change mitigation, resource protection, and the transition towards a sustainable economy, the biogas sector is expected to grow further in the coming years – particularly in Poland, where the energy transition is gaining momentum (Ciula et al., 2023). The EU-27 reported a doubling of primary biogas production between 2010 and 2022, with another doubling considered feasible by 2030 (Kusz et al., 2024). According to Poland's Energy Policy until 2040 (PEP2040), biogas plants will become a key element of national energy security, serving both as renewable energy generation facilities and as waste treatment units. Moreover, they can help reduce the need for large-scale electricity storage infrastructure, particularly given the high cost of rare metals required for battery production (Mamica et al., 2022). Although the biogas sector in Poland is still in its early stages compared to other EU countries, the concept of transforming organic waste into value-added products such as fertilisers, feed, food, and energy is steadily gaining attention across research, policy, investment, and society (Neczaj et al., 2021; Szymańska & Lewandowska, 2015).

1.2. Motivation and novelty of the research

Although energy recovery through anaerobic digestion is a relatively well-understood and widely used process, the sector's potential is still underutilized in Poland. According to the Biomethane Data Sheet for Poland published by Eurostat in 2023 (based on 2021 data), current biogas production in the country could increase 8-9 times over existing reserves (European Commission, 2023). It is estimated that the amount of sustainably produced biomethane in Poland could reach 3.3 billion m³ (including 3.1 billion m³ from methane fermentation) by 2030, placing the country among the top five biomethane markets in the EU-27. Ignoring this potential would miss an opportunity to increase the revenues of Polish agriculture, the agri-food, municipal, and energy sectors, as well as increase greenhouse gas emissions. One of the key messages for Poland outlined in the above report is to focus the biogas sector on short supply chains, which aim to reduce the transport costs of both raw materials and digestate, as well as proximity to the end user or gas pipeline. Using locally available biowaste for biogas production appears to be the most cost-effective and sustainable path to unlocking the industry's potential. The availability of a wide range of materials that can be processed by anaerobic digestion creates the need for further

research to optimize the process in terms of its stability and increasing its efficiency, or to counteract potential problems related to its inhibition.

One of the bio-wastes whose management still poses a challenge in Poland is poultry manure. Poland is one of the leaders in the poultry industry in Europe, and it is estimated that over 4 million tons of manure are generated annually (EUROSTAT, 2019). So far, the most common agricultural use in the world, as well as in Poland, has many disadvantages, as excessive dosing of these substances into the soil can lead to a threat to both crops and human health, and the effects of such activities may include, for example, eutrophication of reservoirs, phytotoxic effects on crops, air pollution and greenhouse gas emissions (it is estimated that ammonia emissions from manure spread on the field can be several dozen times higher than during combustion) (Neshat et al., 2017; Tańczuk et al., 2019). However, manure can be processed for energy purposes, for example, through anaerobic digestion, pyrolysis, gasification, incineration, or co-incineration. Of the technologies mentioned above, anaerobic digestion currently seems to be the most advantageous. Unlike other methods, it can be used for raw materials with a hydration content above 60%, and therefore does not require complex pre-treatment. An additional advantage is the production of digestate, which, after appropriate stabilization, can be used as a fertilizer, soil conditioner, or pyrolysis substrate (Bayrakdar et al., 2017). Despite its high content of biodegradable organic matter and extensive buffering capacity, poultry manure is considered difficult to digest. Limitations in processing this waste stem from its characteristics, specifically its low carbon-to-nitrogen ratio (ranging from 5 to 10), as well as the risk of ammonia accumulation during the process, as excessive concentrations of this intermediate product of uric acid and protein decomposition can lead to process inhibition.

Potential ways to improve the efficiency of the anaerobic digestion process of poultry manure may be its co-digestion with other organic waste, the addition of an adsorbent, e.g. in the form of biochar, or pre-conditioning of the feedstock or one of its components before introducing it into the digestion reactors (Li et al., 2014; Neshat et al., 2017; Sakar et al., 2009). Introducing a properly selected co-substrate into leads to improved nutrient balance, improved C/N ratio of the feedstock, increased biodegradable fractions, increased macro- and micronutrient content, and greater dilution of toxic compounds. Preferred co-substrates for poultry manure are waste with high carbon content, such as kitchen waste, the organic fraction of municipal waste, or agricultural waste with high lignocellulose content. In the literature, poultry manure is most often co-digested with only one co-substrate. Publications in which a multi-component mixture was subjected to anaerobic stabilization are rare (Li et al., 2014; Mata-Alvarez et al., 2014b), therefore, in this study, anaerobic co-digestion of poultry manure with several types of waste was conducted. Preconditioning the co-digestion mixture or just one of its components seems particularly interesting in the context of improving the feedstock hydrolysis rate, which is the bottleneck stage

of the process. Furthermore, the limited number of publications in this area, and the economic aspect of the proposed solutions are most often omitted, supports the need for research (Grosser & Neczaj, 2017).

The research I undertook touches upon aspects of both engineering and environmental protection (waste management) as well as energy. The novelty of this work is the processing of poultry manure in hybrid systems, combining several unit processes. The research was conducted in batch and semi-continuous modes. The first of the presented operating modes allowed for determining the biodegradability of the waste mixtures used, determining the unit biogas/methane production coefficient, and estimating their kinetic parameters. Implementing the process in a semi-continuous system allowed for determining the stability and efficiency of the proposed technological solutions, which is useful in the context of the potential for industrial-scale implementation. One of the components of the co-digestion mixture used was sewage sludge, which allowed for determining the potential for implementing the proposed solution in wastewater treatment plants.

Biomasa Magazine, the first nationwide publication fully dedicated to the renewable energy industry and organizer of Poland's largest conference for biogas industry representatives, points out that the biogas market struggles primarily with: 1) a lack of public understanding of who biogas infrastructure serves and what purpose; 2) neglected communication – emphasizing the role of biogas plants alongside other renewable energy sources; 3) insufficient investor knowledge of planning and implementing the investment process for such projects; 4) a shortage of properly trained personnel (Lisiecka, 2024). Observing the dynamics of changes in the natural environment and climate, its impact on political sentiment and social disorientation, often leading to the polarization of public opinion, I see the importance of systematizing available knowledge and promoting sustainable solutions. As a supporter of the implementation and transfer of scientific findings into practice, I undertook this research with the hope of highlighting the potential of solutions that are both environmentally friendly and realistically feasible in the context of locally available resources.

2. AIM AND SCOPE OF THE RESEARCH

2.1. Research aim

This doctoral thesis aims to develop an integrated approach to managing poultry manure through anaerobic co-digestion with sewage sludge and other locally available organic waste.

The research focuses on:

- increasing the efficiency and stability of the anaerobic digestion process,

- enhancing energy recovery in the form of biogas,
- assessing the quality and management potential of digestate,
- embedding the proposed solutions within the framework of the circular economy and EU climate policy.

2.2. Research scope

The scope of this thesis includes:

- characterisation of substrates: analysis of the physicochemical properties of poultry manure, sewage sludge, and other co-substrates (fruit waste, grease waste),
- BMP tests (batch tests): determination of the biodegradability of mixtures and optimisation of the inoculum-to-substrate ratio,
- semi-continuous (CSTR) test: analysis of the stability of the anaerobic co-digestion process under conditions close to industrial scale, including high manure content,
- feed conditioning: evaluation of the impact of preliminary substrate preparation (various conditioning methods) on process performance,
- analysis of digestate quality: assessment of fertilizer value and environmental safety of digestate, including testing its potential as a substrate for mushroom cultivation,
- energy and environmental aspects: calculation of energy balance and GHG emissions for the tested variants.

2.3. Practical aim of the research

The applied purpose of this research is to develop a technology that:

- increases the energy self-sufficiency of wastewater treatment plants,
- ensures the safe and cost-effective management of poultry manure and other local biowaste,
- enables the production of valuable digestate suitable for use as fertilizer or growing medium,
- reduces negative environmental impacts, including ammonia emissions, odour, and GHG emissions.

2.4. Research hypotheses

Based on the research objectives and scope, the following hypotheses have been formulated:

H1: Anaerobic co-digestion of poultry manure with sewage sludge and other organic waste significantly increases biogas production efficiency compared to mono-digestion of sewage sludge or poultry manure.

H2: Preliminary conditioning (pre-treatment) of the feedstock mitigate ammonia inhibition and improve process stability and efficiency.

H3: The digestate obtained from anaerobic co-digestion meets the quality requirements for use in agriculture and horticulture, and in particular can serve as an alternative substrate for mushroom cultivation.

3. LIST OF PUBLICATIONS

The scientific output related to this doctoral thesis comprises 7 thematically related papers published or prepared for publication between 2019 and 2025. The total impact factor in their respective years of publication amounted to 26.1, while the total number of points awarded by the Ministry of Science and Higher Education (MNiSW) at the time of publication was 720.

The following list presents publications and manuscripts associated with this doctoral thesis, together with their respective identifiers.:

1. **Anna Jasińska**, Anna Grosser, Erik Meers. (2023). *Possibilities and limitations of anaerobic co-digestion of animal manure – A critical review*. *Energies*. 16(9), 3885. – Review article, impact factor: **3.2**, scientific journal rank by MNiSW: **140** points (**D.1.**)
2. Małgorzata Kacprzak, Krystyna Malińska, Anna Grosser, Jolanta Sobik-Szołtysek, Katarzyna Wystalska, Danuta Drózdź, **Anna Jasińska**, Erik Meers. (2023). *Cycles of carbon, nitrogen and phosphorus in poultry manure management technologies – environmental aspects*. *Critical Reviews in Environmental Science and Technology*, 53(8), 914-938. – Review article, impact factor: **11.4**, scientific journal rank by MNiSW: **200** points (**D.2.**)
3. Anna Grobelak, Klaudia Całus-Makowska, **Anna Jasińska**, Marek Klimasz, Aleksandra Wypart-Pawul, Dominika Augustajtys, Estera Baor, Daria Sławczyk, Aneta Kowalska. (2024). *Environmental Impacts and Contaminants Management in Sewage Sludge-to-Energy and Fertilizer Technologies: Current Trends and Future Directions*. *Energies*. 17(19):4983. – Review article, impact factor: **3.2**, scientific journal rank by MNiSW: **140** points (**D.3.**)
4. **Anna Jasińska**, Anna Grosser, Erik Meers, Ana Robles Aguilar. (2024). *Energy recovery from poultry manure in the process of semi-continuous anaerobic co-digestion with sewage sludge*. *Energy Reports*, 12, 3969-3981. – Original research article, impact factor: **5.1**, scientific journal rank by MNiSW: **100** points (**D.4.**)
5. **Anna Jasińska**, Erik Meers, Anna Grosser. (2025). *Sustainable digestate management as a supplement to the casing layer in mushroom cultivation*. – Original research article, manuscript prepared for publication (**D.5.**)
6. **Anna Jasińska**, Anna Grosser, Erik Meers, Dagmara Piłyp. (2024). *Stimulating Methane Production from Poultry Manure Digest with Sewage Sludge and Organic Waste by Thermal Pretreatment and Adding Iron or Sodium Hydroxide*. *Energies*,

17(11), 2679. – Original research article, impact factor: **3.2**, scientific journal rank by MNiSW: **140** points (**D.6.**)

7. **Anna Jasińska**, Anna Grosser, Erik Meers, Dagmara Piłyp. (2025). *Evaluation of saponification and chemical pretreatments for poultry manure co-digestion: methane production, EPS fraction analysis and kinetic study*. – Original research article, manuscript prepared for publication (**D.7.**)

4. Overview of publications

To provide a clear overview of the research structure and logical progression of the studies included in this doctoral thesis, Figure 4.1 illustrates the interrelation between individual publications (D.1–D.7). The figure summarizes the conceptual background, experimental stages, and practical implications of the conducted research.

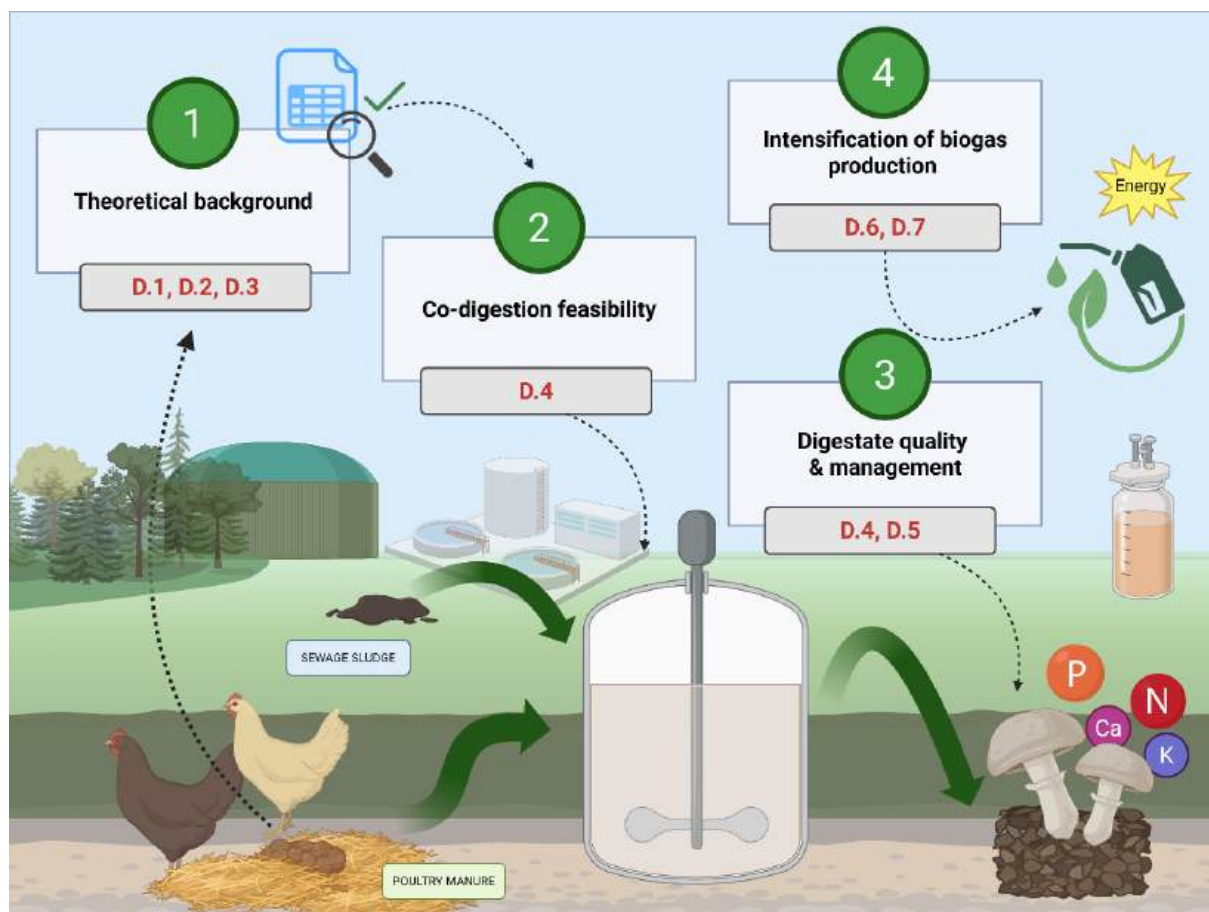


Figure 1. Graphical overview of the doctoral research structure and the corresponding publications (D.1–D.7) included in the thesis.

4.1. Introduction to the research topic

An important point of reference for the present doctoral thesis is a set of three review articles that provide an extensive overview of the state of knowledge in the field of anaerobic digestion and nutrient management, which I authored or co-authored.

The first *“Possibilities and Limitations of Anaerobic Co-Digestion of Animal Manure – A Critical Review”* (D.1.) presents a comprehensive analysis of the literature on anaerobic digestion of manure, with particular emphasis on poultry manure and the role of co-digestion in overcoming process limitations. In this publication special attention was devoted to identifying suitable co-substrates that could balance the unfavourable characteristics of poultry manure, especially its low C/N ratio and the risk of ammonia inhibition. This paper provides the main conceptual foundation for my doctoral research, directly linking the theoretical framework with the experimental part of the thesis.

The other two papers: *“Cycles of Carbon, Nitrogen and Phosphorus in Poultry Manure Management Technologies – Environmental Aspects”* (D.2.) and *“Environmental Impacts and Contaminants Management in Sewage Sludge-to-Energy and Fertilizer Technologies: Current Trends and Future Directions”* (D.3.) complement and expand upon the issues outlined in D.1. The second article focuses specifically on poultry manure, which constitutes the main substrate of my research, analysing the cycles of carbon, nitrogen, and phosphorus in different management technologies. It highlights anaerobic digestion as a technology that enables not only renewable energy recovery but also the recycling of nutrients back into the soil. The third article brings in the perspective of sewage sludge, which in my experimental work was used as a co-substrate in anaerobic co-digestion process with poultry manure. It addresses current environmental challenges related to contaminants such as heavy metals, pharmaceuticals, and microplastics, while also emphasizing the potential of integrating anaerobic digestion with complementary treatment methods to improve the safety and value of digestate application.

Taken together, the three review papers (D.1.–D.3.) portray anaerobic digestion as a process that faces certain challenges related to substrate characteristics, yet holds great potential for both energy recovery and nutrient recycling. Poultry manure, as the primary substrate, is characterized by high organic matter content and considerable methane potential, but its effective use requires balanced feedstock composition. Co-digestion with sewage sludge and other locally available organic residues emerges as a promising strategy to enhance process stability, increase biogas yield, and obtain digestate with a more favourable nutrient profile. When properly applied in agriculture, digestate contributes to the recycling of nitrogen and phosphorus, reduces the risk of eutrophication, and decreases the reliance on synthetic fertilizers. The collective findings of these three reviews therefore provide a solid theoretical background for this doctoral thesis, outlining the research directions in the anaerobic co-digestion of poultry manure with locally available organic wastes as a solution aligned with the principles of the circular economy.

4.2. Assessment of the possibility of anaerobic co-digestion of poultry manure with sewage sludge

The conclusions drawn from my earlier literature analysis highlighted that poultry manure and sewage sludge represent two of the most abundant organic waste streams generated in the agri-food and municipal sectors. Their management poses both environmental and operational challenges due to high organic and nutrient loads, as well as the need for sustainable utilization pathways. At the same time, these waste streams offer considerable potential for renewable energy recovery through anaerobic digestion, particularly in the context of the growing demand for energy self-sufficiency in wastewater treatment plants and the implementation of circular economy principles. These factors provided the rationale for undertaking experimental research on the co-digestion of poultry manure and sewage sludge, with the aim of improving process stability and increasing biogas production efficiency.

This part of the research is presented in the article *“Assessment of the possibility of anaerobic co-digestion of poultry manure with sewage sludge”* (D.4). The experiments were designed to gradually increase the proportion of poultry manure in mixtures with sewage sludge, while comprehensively assessing process performance and biogas quality under mesophilic, semi-continuous conditions.

The results demonstrated that co-digestion of poultry manure with sewage sludge was feasible up to a maximum of 60% VS contribution of manure in the feedstock mixture. However, the most stable process performance and the highest biogas yields were achieved at intermediate manure shares. The optimal outcomes were observed within the range of 20–40% VS, with the maximum methane production recorded at approximately 42% poultry manure. At these proportions, the complementary characteristics of both substrates created a clear synergistic effect (synergy index > 1): poultry manure provided readily biodegradable organic matter and high methane potential, whereas sewage sludge ensured adequate moisture, buffering capacity, and a more balanced nutrient composition. This synergy resulted in an improved C/N ratio, reduced ammonia inhibition, and a significantly enhanced specific methane yield. At higher manure shares (50–60% VS), the process could still be maintained, but efficiency declined and the risk of destabilization increased due to ammonia accumulation and elevated concentrations of volatile fatty acids.

The experimental design included detailed monitoring of process parameters such as biogas and methane production kinetics, specific methane yield, volatile fatty acids, alkalinity, pH, and ammonia levels. This comprehensive evaluation allowed for the identification of process conditions that ensure optimal and stable performance of the anaerobic co-digestion system.

The findings presented in D.4 demonstrate that co-digestion of these substrates is a promising technological pathway that can simultaneously address waste

management challenges and support renewable energy recovery. By ensuring higher and more stable biogas production compared to mono-digestion, this approach contributes to improving the energy balance of wastewater treatment plants. Moreover, it emphasizes the role of valorising locally available organic waste streams and integrating energy recovery with nutrient recycling in accordance with the principles of the circular economy.

4.3. Quality of digestate and opportunities for its utilization

In the context of anaerobic digestion, an equally important aspect as biogas production is the management and potential use of the solid–liquid residue known as digestate. This material, when appropriately stabilized, can serve as a secondary raw product and a source of nutrients that may be reintroduced into environmental or agricultural systems. In the study “*Assessment of the possibility of anaerobic co-digestion of poultry manure with sewage sludge*” (D.4), the characteristics of digestate obtained from both mono-digestion of sewage sludge and its co-digestion with poultry manure were analysed in detail to determine the impact of substrate composition on nutrient content and stability of the final product.

The results demonstrated that the inclusion of poultry manure as a co-substrate significantly affected the properties of the digestate. Compared with digestate from sewage sludge alone, the co-digestion product contained higher concentrations of nitrogen, phosphorus, calcium, and potassium, as well as increased alkalinity and improved buffering capacity. The digestate exhibited a neutral to slightly alkaline pH (7.7–8.3) and a moderate degree of mineralization, which indicates a stable and nutrient-rich material with potential fertilizing properties. The concentration of ammonium nitrogen increased with the proportion of poultry manure in the feedstock, reaching approximately 2750 mg/L at 60% PM, while volatile solids removal and methane yield improved simultaneously. Although the contents of trace elements such as Zn, Cu, Ni, Cr, Pb, and Cd remained low and within ranges typically regarded as safe for agricultural use, Regulation (EU) 2019/1009 of the European Parliament and of the Council of 5 June 2019 (European Parliament and the Council of the European Union, 2019) currently excludes sewage sludge–derived materials from the list of feedstocks permitted in EU fertilizing products. Nevertheless, the results of D.4 suggest that, provided the presence of other pollutants from sewage sludge is effectively reduced, such digestates could potentially meet the quality criteria for inclusion under this regulation.

The possibility of practical utilization of digestate was further examined in the study “*Sustainable digestate management as a supplement to the casing layer in mushroom cultivation*” (D.5), which focused on assessing the potential of digestates from sewage sludge mono-digestion (Ss) and from co-digestion with poultry manure (PM) as components of the casing layer used in *Agaricus bisporus* cultivation. The research aimed to verify whether digestate could partially replace peat in the casing layer,

offering an alternative route for its reuse. Both types of digestate were characterized by low total solids (1.4–2.6%), slightly alkaline pH (≈ 7.3), and moderate electrical conductivity (8–12 mS/cm). They contained high levels of calcium (53–59 g/kg), phosphorus (37–40 g/kg), and potassium (15–19 g/kg), while trace element concentrations (Fe, Zn, Mn) remained within acceptable limits. The incorporation of digestate into the peat-based casing mixture increased its nutrient content and electrical conductivity in a dose-dependent manner. Low inclusion levels (3–5% of the casing mass) did not negatively affect mushroom yield or quality, while higher doses (10%) reduced biological efficiency and cap size. The results of **D.5** therefore demonstrated that digestate, when applied in limited amounts, can improve the physicochemical properties of the casing layer and contribute to nutrient recycling without causing adverse effects within the tested scope.

The combined findings of **D.4** and **D.5** show that digestate can be effectively incorporated into nutrient recovery strategies within the circular economy framework. Co-digestion of sewage sludge with poultry manure allows for stabilization of nitrogen-rich organic residues and generation of a nutrient-dense digestate suitable for reuse. At the same time, its controlled application in mushroom cultivation provides a new direction for valorising this by-product. Such an integrated approach supports waste minimization, nutrient circularity, and sustainable management of secondary raw materials. Overall, the results confirm that digestate derived from sewage sludge and poultry manure co-digestion is chemically stable and rich in nutrients, and that its utilization either as a fertilizer or as a component of horticultural substrates represents a promising pathway for bioresource recovery, although further research is required to fully assess potential biological and chemical contaminants.

4.4. Intensification of biogas production from poultry manure

The results obtained in the previous study (**D.4**) demonstrated that the co-digestion of poultry manure with sewage sludge enables stable process performance and satisfactory methane yields. However, methane productivity in such mixtures remains limited due to the low biodegradability of certain organic fractions and the high nitrogen content characteristic of poultry manure. Therefore, subsequent research focused on intensifying biogas production through the application of physical and chemical pretreatment methods aimed at improving substrate solubilization and enhancing the kinetics of anaerobic digestion. The findings of this research are presented in the publications: *“Stimulating Methane Production from Poultry Manure Digest with Sewage Sludge and Organic Waste by Thermal Pretreatment and Adding Iron or Sodium Hydroxide”* (**D.6**) and *“Evaluation of saponification and chemical pretreatments for poultry manure co-digestion”* (**D.7**), which together provide a comprehensive evaluation of how different conditioning methods affect the efficiency of co-digestion systems based on poultry manure, sewage sludge, and grease trap sludge.

In the study described in **D.6**, the experimental work was conducted in four stages: selection of an additional organic co-substrate, determination of the optimal inoculum-to-substrate ratio, optimization of pretreatment parameters, and evaluation of methane production efficiency through biochemical methane potential (BMP) tests. Among the co-substrates tested, grease trap sludge—characterized by a high lipid content—proved to be the most effective, increasing methane yield from 320 to 340 mL CH₄/g VS. Of the pretreatment strategies applied (thermal, thermochemical with iron addition, and thermochemical with sodium hydroxide), the highest methane yield and the greatest degree of organic matter liquefaction were obtained for the thermochemical treatment with NaOH at 4.5 g/L. This method increased methane yield up to 501 mL CH₄/g VS, representing a 47% improvement compared to the untreated mixture. The enhanced efficiency was associated with an increase in non-purgeable organic carbon (NPOC) and the transformation of extracellular polymeric substances (EPS), which facilitated hydrolysis and improved substrate availability. The study also showed that appropriate NaOH dosing promoted solubilization without excessive ammonia accumulation, whereas higher reagent concentrations led to inhibition risks. These results demonstrate that controlled alkaline pretreatment can effectively intensify methane production from complex organic waste mixtures (**D.6**).

The follow-up research presented in **D.7** evaluated more advanced conditioning approaches—chemical (alkaline) and thermo-alkaline (saponification) – applied to mixtures of poultry manure, sewage sludge, and grease trap sludge (30:40:30 VS). Both methods used sodium hydroxide at doses ranging from 0.9 to 5.4 g/L, with saponification performed at 70 °C to enhance solubilization efficiency. Detailed analyses of physicochemical indicators and EPS fractions (S-EPS, LB-EPS, TB-EPS) showed that saponification caused a much stronger restructuring of the polymer matrix than alkaline pretreatment alone. The content of soluble EPS (S-EPS) increased by 95–120%, while the tightly bound fraction (TB-EPS) decreased by 25–45%. These changes were accompanied by increased concentrations of NPOC and volatile fatty acids (VFAs), confirming intensified hydrolysis and greater bioavailability of organic matter for methanogenic microorganisms.

As a result, methane yield under saponification conditions increased linearly with NaOH dose ($Y_m = 14.9 \times \text{dose} + 476.98$; $R^2 = 0.95$), reaching a maximum of 556 mL CH₄/g VS at 4.5 g NaOH/L—approximately 29% higher than for alkaline pretreatment and 63% higher than in the control sample. The process was also characterized by a shorter lag phase and a higher co-digestion performance index (CPI = 1.29), indicating a synergistic enhancement beyond the additive effects of individual substrates. Two-way ANOVA and correlation analyses confirmed a significant influence of both the method and NaOH dose on methane yield and EPS transformation ($p < 0.001$). Strong positive correlations between soluble EPS, NPOC, and VFAs ($r \approx 0.8$) demonstrated that thermo-alkaline conditions accelerated solubilization and improved methanogenic activity (**D.7**).

The findings obtained in D.6 and D.7 confirm that the use of appropriately selected pretreatment methods can effectively intensify biogas production from poultry manure-based mixtures. The most favourable results were achieved for thermo-alkaline saponification, which ensured a high degree of solubilization, effective destabilization of EPS structures, and improved methanogenic kinetics. This approach represents a promising direction for enhancing the energy recovery potential of anaerobic digestion processes treating nitrogen-rich and low-biodegradability wastes, thereby contributing to the development of energy self-sufficient wastewater treatment plants and supporting the implementation of circular economy principles.

5. CONCLUSIONS

This doctoral thesis addressed the valorization of poultry manure through anaerobic digestion and co-digestion with locally available organic residues, aiming to improve energy recovery and promote nutrient recycling within a circular bioeconomy framework. The research encompassed a comprehensive pathway—from the analysis of current challenges and technological gaps in organic waste management to the experimental verification of anaerobic digestion performance, digestate characteristics, and process intensification strategies.

The introductory part of the thesis defined the environmental and technological rationale for utilizing poultry manure and sewage sludge as major organic waste streams, underlining their high generation volumes, nutrient loads, and the need for sustainable treatment methods. The theoretical framework highlighted the potential of anaerobic digestion as a process capable of stabilizing these wastes, generating renewable energy, and producing nutrient-rich by-products suitable for reuse in agriculture and related sectors.

The experimental studies confirmed that the co-digestion of poultry manure with sewage sludge ensures stable process performance and increased methane yields compared with mono-digestion. The digestate produced under these conditions exhibited favorable physicochemical properties, including a balanced nutrient composition and stable organic matter, supporting its potential for controlled agricultural application or partial substitution of peat in horticultural uses. Further investigations demonstrated that targeted pretreatment methods—particularly thermo-alkaline conditioning—can significantly enhance substrate biodegradability and intensify methane generation, offering a practical route to higher energy recovery. Collectively, the research results provide a coherent understanding of how substrate selection, mixture composition, and pretreatment strategies influence both biogas production and digestate quality.

5.1. Verification of research hypotheses

The outcomes of the experimental work allowed for the verification of all research hypotheses formulated in the doctoral thesis:

Hypothesis H1, stating that anaerobic co-digestion of poultry manure with sewage sludge improves process stability and biogas yield compared with mono-digestion, was **confirmed**. The synergistic effect between substrates resulted in enhanced methane production, stable operation, and optimal performance within specific mixture ratios.

Hypothesis H2, assuming that the digestate obtained from the co-digestion process constitutes a valuable by-product with potential for agricultural or industrial utilization, was **partially confirmed**. The digestate demonstrated strong fertilizing potential and nutrient content suitable for reuse; however, full environmental safety cannot yet be guaranteed due to the possible presence of untested contaminants such as organic micropollutants or pathogenic organisms.

Hypothesis H3, proposing that the efficiency of biogas production from poultry manure-based mixtures can be enhanced through physical or chemical pretreatment, was **confirmed**. The application of thermo-alkaline methods significantly increased methane yield and improved substrate solubilization, proving the effectiveness of such strategies for process intensification.

The verification of these hypotheses confirmed the overarching assumption that the integration of anaerobic co-digestion with appropriate pretreatment and digestate management represents a viable and sustainable technological pathway for managing nitrogen-rich organic residues.

5.2. Scientific and practical significance

The findings of this thesis contribute to both scientific understanding and practical implementation of anaerobic digestion technologies. Scientifically, the research provides a detailed insight into the interactions between substrates of different origins and compositions, revealing how co-digestion and pretreatment affect the biochemical and physicochemical mechanisms governing methane formation. The analyses of extracellular polymeric substances (EPS) and organic carbon solubilization offer new perspectives on the mechanisms of improved biodegradability in complex organic matrices.

From a practical standpoint, the results deliver applicable knowledge for optimizing biogas plant operations and designing integrated waste-to-energy systems. They demonstrate that the combined treatment of poultry manure and sewage sludge can simultaneously enhance renewable energy recovery, reduce waste volumes, and contribute to nutrient recycling. Moreover, the demonstration of digestate reuse in horticultural applications introduces innovative possibilities for circular resource

management and supports the reduction of peat consumption. These outcomes are directly aligned with the objectives of the EU Green Deal and Circular Economy Action Plan, emphasizing sustainable waste management and energy self-sufficiency in wastewater treatment facilities.

5.3. Future research directions

Future research should continue to address the environmental safety of digestate, with particular emphasis on emerging contaminants such as pharmaceuticals, hormones, and microplastics, as well as the potential presence of pathogens. This topic is gaining importance due to evolving environmental regulations, growing societal awareness, and the rapidly expanding body of scientific evidence on trace pollutants in organic residues. Continuous monitoring and adaptation to new legal and analytical standards will be essential to ensure that digestate reuse remains safe and consistent with the principles of sustainable bioresource management.

In parallel, further studies should focus on optimizing biogas production technologies, particularly through scaling up the most effective pretreatment methods to pilot and full-scale installations. Such research will be crucial to assess their energy balance, cost-efficiency, and long-term stability under real operating conditions. The continued development of the biogas sector should also involve building strong cooperation networks among researchers, policymakers, farmers, and industry representatives. Strengthening this collaboration will facilitate the exchange of knowledge, accelerate the implementation of innovative technological solutions, and enhance the role of anaerobic digestion in achieving energy self-sufficiency and advancing the circular bioeconomy.

In conclusion, this doctoral research confirms that anaerobic co-digestion of poultry manure with sewage sludge, supported by targeted pretreatment and sustainable digestate utilization, constitutes an environmentally sound and technically feasible approach for transforming organic waste into renewable energy and valuable secondary products. The obtained results strengthen the scientific foundations for the development of energy self-sufficient wastewater treatment systems and contribute to advancing circular bioeconomy solutions for future sustainable waste management.

6. OTHER RESEARCH ACHIEVEMENTS

6.1. Publications

1. Anna Grosser, Ewa Neczaj, Anna Jasińska, Piotr Celary. (2020). *The influence of grease trap sludge sterilization on the performance of anaerobic co-digestion of sewage sludge*. Renewable Energy, 161, 988-997.

2. Anna Jasińska. (2020). *Usuwanie amoniaku jako etap obróbki wstępnej obornika w procesie fermentacji metanowej (Ammonia removal as a pretreatment stage for anaerobic digestion process of animal manure)* in: *Współczesne problemy ochrony środowiska i energetyki 2020 (Contemporary problems of power engineering and environmental protection 2020)*, edited by: Magdalena Bogacka, Krzysztof Pikoń. Department of Technology and Equipment for Waste Management, Silesian University of Technology, Gliwice, pp. 33-44.
3. Małgorzata Kacprzak, Iwona Kupich, Anna Jasińska, Krzysztof Fijałkowski. (2022). *Bio-Based Waste Substrates for Degraded Soil Improvement – Advantages and Challenges in European Context*. *Energies*, 15 (1).
4. Anna Jasińska, Małgorzata Kacprzak. (2022). *Assessment of the effectiveness of bioremediation of degraded areas with the use of stabilized sewage sludge and other biodegradable waste* in: *Environmental safety of bio-waste in the circular economy*, edited by: Ewa Neczaj, Anna Grosser. Politechnika Częstochowska (Częstochowa University of Technology), Częstochowa, pp. 234 – 251.
5. Anna Grosser, Anna Jasińska, German Smetana. (2022), *Technologie uszlachetniania biogazu do biometanu – przegląd literaturowy (Technologies for upgrading biogas to biomethane – a review)* in: *Inżynieria Środowiska i Biotechnologia – Wyzwania i Nowe Technologie (Environmental Engineering and Biotechnology – Challenges and New Technologies)*, edited by: Agata Rosińska, Beata Karwowska, Magdalena Madela, Politechnika Częstochowska, Częstochowa.

6.2. Conferences and speeches

1. 15th Conference on Sustainable Development of Energy, Water and Environment Systems (SDEWES) – Cologne, Germany 1-5.09.2020.
2. Environmental Protection and Energy Conference 2020 – Gliwice, Poland 11.12.2020.
3. V Kongres Biogazu „Biogazowa transformacja Polski” (3rd Biogas Congress “Poland's biogas transformation”) – Poland, 7.12.2020.
4. III Forum „Sieciowanie Partnerów SIR” (3rd Forum on Networking SIR Partners) – Wrocław, Poland 12-16.06.2021.
5. VI Kongres Biogazu „Biogaz i biometan - w kierunku neutralności klimatycznej” (6th Biogas Congress “Biogas and biomethane – towards climate neutrality”) – Ossa, Poland 13-14.12.2021.
6. European Sustainable Nutrient Initiative – Belgium 29.03.2022.
7. Energia-Klimat-Środowisko, 25 lat Wydziału Infrastruktury i Środowiska (Energy Climate Environment, ECE 2022, 25 years of the Faculty of Infrastructure and Environment) – Częstochowa, Poland 14-16.09.2022.
8. Webinar: Nutri2Cycle Lighthouse Network: Demonstrating nutrient and carbon recycling in EU agriculture in practice – within the Nutri2Cycle project, 19-28.04.2022.

9. Webinar: Best practices and barriers in nutrient efficient agriculture in Hungary, Croatia and Poland – within the Nutri2Cycle project, 03.02.2023.

6.3. Projects

1. Nutri2Cycle - Nurturing the Circular Economy: Transition towards a more carbon and nutrient efficient agriculture in Europe - European Union's Horizon 2020 research and innovation programme under grant agreement No. 773682.
2. The EnviSafeBioC PROJECT funded by the NAWA National Agency for Academic Exchange
3. Innovative technologies in environmental biotechnology, grant agreement No. BS/PB-400–301/24 of the Czestochowa University of Technology.
4. Implementation of innovations in the field of energy and fertilizer processing of sewage sludge and biodegradable waste, reducing the negative impact on the environment in a coastal tourist region, Project no. NORW.19.01.01-22-0003/20.

6.4. Internships and study visits

1. Study visits to foreign universities: Yildiz Technical University in Istanbul, Pamukkale University, Nigde Omer Halisdemir University, Gebze Technical University – 19-28.09.2021.
2. “FertiCycle and Nutri2Cycle joint 1st Virtual Winter-school - Waste Processing and Up-cycling Concepts” – 03.02.-31.03.2021.
3. Internship at Ghent University – 01.03.2022-15.05.2022; 15.08.2022-15.12.2022.
4. “3rd Nutri2Cycle summer school” – Barcelona, Spain, 07-09.06.2023.

6.5. Awards

1. Award in category “Most promising solution” in students competition: “Future and Nexus of Energy and Environment” supported by Visegrad Fund, project No. 21930003 Exchange of Smart Solutions between V4 and EU in Energy and Environment – 06.06.2022.

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8. APPENDIX

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D.1

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Possibilities and limitations of anaerobic co-digestion of animal manure – A critical review



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Review

Possibilities and Limitations of Anaerobic Co-Digestion of Animal Manure—A Critical Review

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Abstract: Anaerobic digestion is a well-known and long-used biological method for stabilizing organic materials. Among the benefits of this process in waste management are the reduction of greenhouse gases, the production of alternative energy, and the acquisition of valuable digestate that can be used in the form of biogas, thereby closing the cycle of elements in nature. For some materials, such as manure, which is heterogeneous in terms of morphology and chemical composition, digestion of a single substrate may not be very efficient. Therefore, more and more studies on the co-digestion process are appearing in the literature. This solution allows higher biogas production and the possibility of processing several wastes simultaneously. The prospect of the future effective application of anaerobic co-digestion depends on regulations, work regime, and access to raw materials. Therefore, there is a need to systematize the available knowledge and results, as well as to identify the possibilities and limitations of the discussed process, which is undertaken in this paper.

Keywords: anaerobic digestion; co-digestion; biogas; animal manure; renewable energy; methane



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1. Introduction

Rational management of the Earth's resources is becoming more and more important in widely understood environmental protection. Scientists are undoubtedly facing the challenge of reducing global warming, which has the consequence of ozone depletion and ecosystem decline. Recent reports by Greenpeace Southeast Asia and the Centre for Research on Energy and Clean Air (CREA) indicate that air pollution from the burning of fossil fuels (coal, oil, and natural gas) is responsible for approximately 4.5 million deaths each year worldwide, and the global economic loss from fossil fuel air pollution is estimated to be \$2.9 trillion per year or about 3.3 percent of global GDP. In the US alone, fossil fuel combustion emissions are associated with approximately 230,000 deaths and \$600 billion in economic losses annually [1]. There is increasing focus on the integration of energy, water, and environmental systems, including aspects related to the development of transport, industry, and agriculture. Therefore, it is important to take a holistic approach to the presented problem, and above all to adopt a policy aimed at the efficient use of alternative energy and rational waste management [2,3].

Reducing greenhouse gas emissions and developing a reliable strategy for saving fossil fuels is of particular interest to policymakers. In the area of climate policy, the European Union aims to achieve the so-called net-zero emission by 2050, which is the main postulate presented in the European Green Deal project. Over the 21st century, the proposed thresholds for reducing the emissions of gases responsible for global warming and the use of renewable energy sources are increasing [4]. Figure 1 shows the direction of the EU authorities' action in the field of climate and energy policy [5].

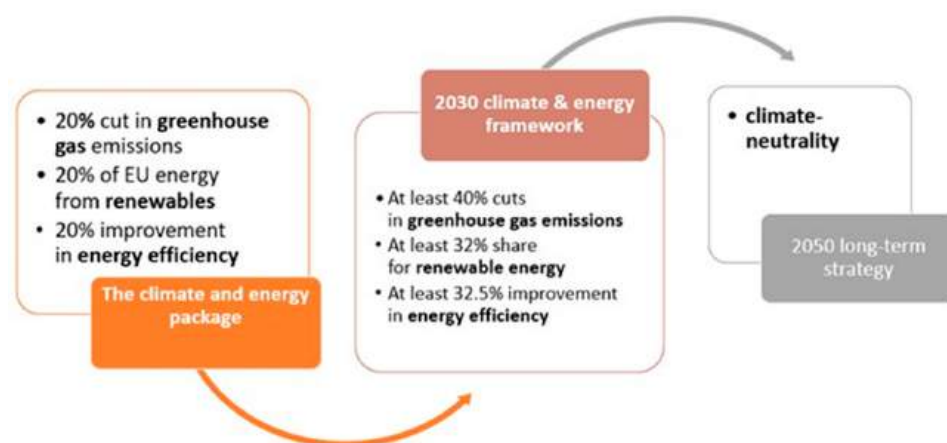


Figure 1. The most important demands of the 21st century regarding climate policy proposed by the European Parliament [5].

In the face of progressive climate change and the need for rapid reduction of conventional energy sources, the search for appropriate waste treatment methods is extremely important. One of the major producers of waste is the agri-food sector, which generates, among other forms of waste, animal excrement. Animal husbandry is one of the main sources of atmospheric pollution and greenhouse gas (GHG) emissions globally. Methane (CH_4), nitrous oxide (N_2O), carbon dioxide (CO_2), ammonia (NH_3), hydrogen sulfide (H_2S), methyl mercaptan (CH_3SH), di- and trimethyl sulfide, volatile organic compounds, endotoxins, and poisons can be emitted from livestock manure, causing serious environmental pollution and health problems [6]. GHGs discharged from animal production account for 18% of total global emissions [7]. Globally, cattle farming is leading the way (Figure 2, Tables 1 and S1), but due to the expected growth of the global human population, the increase in global total meat production is estimated to increase from 330 million tons (2017 data) to 465 by 2050 [8]. As a result, intensive livestock farms will increase, leading to an increase in the production of different types of manure, which, if untreated, can lead to serious environmental problems [9]. The slurry is a heterogeneous mixture of feces, urine, food residues, a small amount of litter, and technological waters (from animal washing, flushing, positions, and canals). Factors affecting manure properties include species and age of animals, type of feed used, and the content of litter and food residues, as well as ambient temperature (Table 2) [10]. Improper manure management can lead to ground and surface water pollution, deterioration of the biological structure of the soil, release of animal pathogens, attracting of pests, and generating odors [11,12]. In Poland, which ranks among the leaders in poultry farming in the world, this industry is developing extremely rapidly, resulting in increased manure production [13]. Environmental problems related to poultry production have been a concern in the last century. Farm animals are usually enclosed for most of their lives in large clusters in small rooms, which leads to large volumes of feces accumulating in concentrated areas [14]. Farmers face the challenge of tuning their livestock operations to increasingly stringent regulatory requirements [15]. Poultry manure is organic matter, rich in valuable elements such as nitrogen, phosphorus, and potassium, and is traditionally stored and used in agriculture (Table 2). However, direct application to arable fields leads to various environmental problems related to their physicochemical, hygienic, and sanitary properties [16]. This method also causes great irritation on the part of local society because of the unpleasant odor. Stored and unused chicken droppings emit large amounts of methane, carbon dioxide, and ammonia into the atmosphere. It should be emphasized that in the event of storing poultry manure in heaps, the temperature often increases spontaneously, reaching the level of 30–40 °C after the beginning of the aerobic decomposition process. Later, in turn, rapid oxygen consumption and the transition to anaerobic digestion occur. During this time, there are high emissions of methane (even up

to 80 kg per 100 birds per year), which has a 21-fold stronger greenhouse effect than carbon dioxide [17].

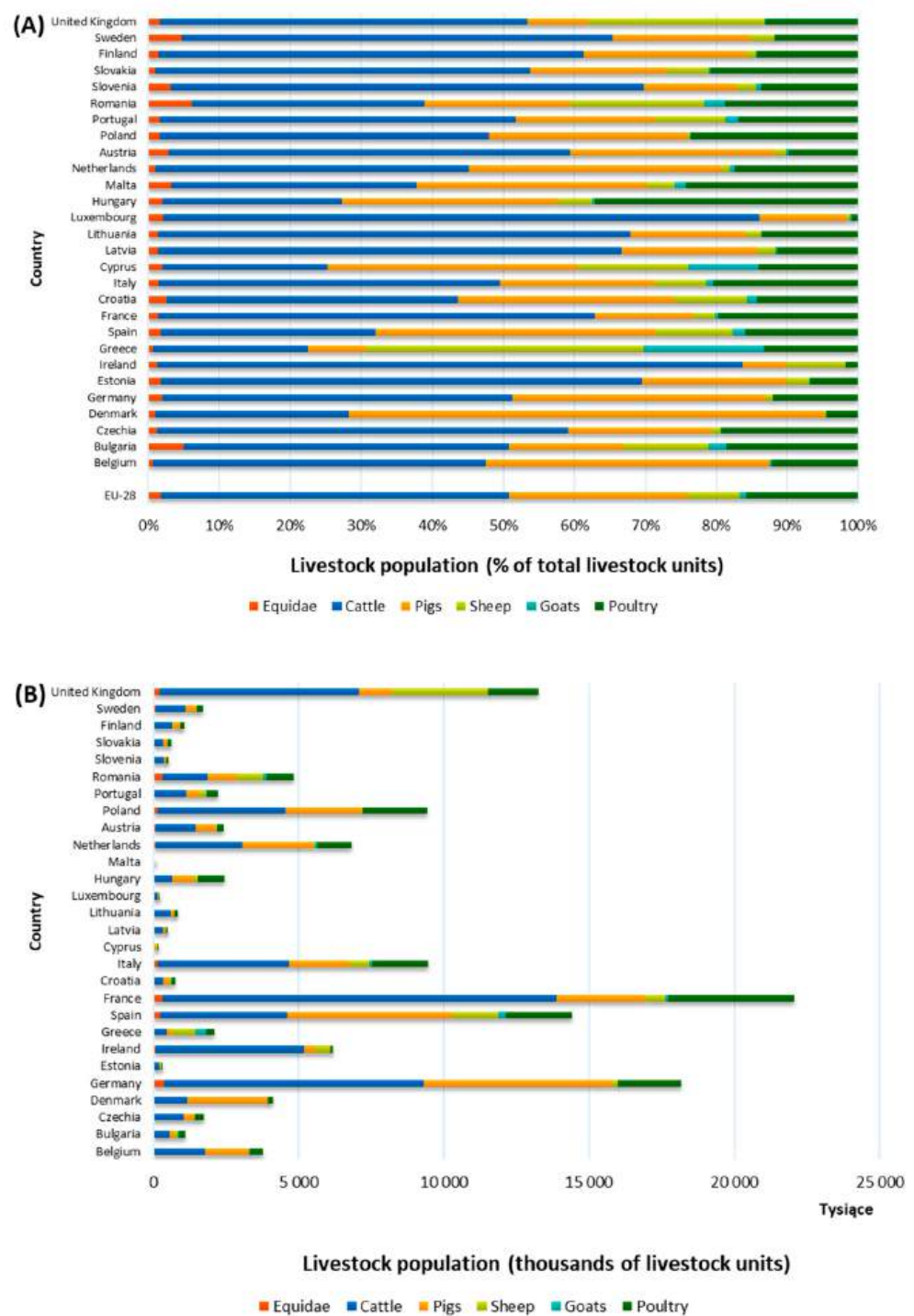


Figure 2. Livestock population in livestock units by type in Europe in 2016: (A) in % of total livestock unit; (B) total livestock unit (heads) [18].

Table 1. The quantities of manure produced by different animals [19–21].

Animal	Unit	Value
Laying hens	kg/(1000 birds·d)	120–150
Chicken broilers		80
Turkeys		200–350
Duck		150–190
Geese		200
Calves	kg/(head·d)	8.0
bovine		20.0
Male bovine		25.0
Dairy cows		53.0
Other cows		25.0
Piglets		0.5
Other pigs		4.5
Sows		11.0
Sheep		1.5
Goat		1.5
Broilers		0.10
Laying hens		0.20
Other poultry		0.3

Table 2. Characteristics of different types of raw manure [22].

Type of Manure	Manure	TS (%)	C (%)	N (%)	P (%)	K (%)
BM	Cattle, Horse, Sheep, or pig	20.9–69.9	11.9–12.0	0.4–2.2	0.2–4.0	0.9–4.0
	Poultry	33.3–78.5	12.6–50.4	1.1–5.9	1.1–3.2	2.0–3.3
SM	Cattle, Horse	24.4–65.0	10.4–48.1	0.6–4.6	0.1–2.5	0.1–3.2
	Pig	28.0–29.0	35.3–41.0	1.3–2.7	1.5–3.2	0.7
	Poultry	33.0–79.4	24.9–46.2	1.7–7.1	0.7–6.7	1.9–5.0
SL	Cattle	0.5–8.3	17.5–36.5	0.2–2.8	0.04–0.1	0.4–0.5
	Pig slurry	0.3–8.3	16.3–41.4	0.1–3.4	0.01–3.1	0.1–2.5
	Cattle, Horse	4.9	NA	NA	0.05	0.2
	Pig	<1.6	NA	0.1	1.0	NA

BM—bedding manure; SM—solid manure; SL—semi-liquid; NA—not available.

However, the high energy potential of animal manure can be used in a controlled manner [9]. The use of biomass for energy purposes and its conversion into fuels—for example, anaerobic digestion—has increased rapidly over the last decade. The anaerobic digestion market is growing at a rate of around 10–12% across Europe and is expected to exceed \$8 billion by 2024 [23]. Anaerobic digestion is now a well-known and well-established technology on a large scale in Europe, primarily in rural areas such as Germany and Denmark [24,25]. The motive for using this method is primarily to save operating costs on farms. However, research on improving the efficiency of operating systems should be intensified, and policies should be implemented to encourage the use of biogas. Animal excrements are an attractive raw material for this process, but they are heterogeneous in size, composition, structure, and properties, and show different degradability by enzymes

or bacteria. They often contain high concentrations of lignocellulose, which reduces their biodegradability and increases the required retention time in the anaerobic digestion process [24,26,27]. Characteristic in the case of animal droppings, especially chicken droppings, is a high content of ammonia, which is a process inhibitor. Diluting manure to 3–6% of the total solids eliminates the problem and ensures good mixing conditions for anaerobic tanks, but the biogas yield (and methane content) is often too low for profitable production due to the larger reactor volumes required, the water consumption, and also the production of large volumes of digestate slurry [28].

Hence, the need to use new solutions related to the proper treatment of the substrate and obtaining greater efficiency of methane production, which can be achieved by adding other substances to the charge (anaerobic co-digestion) [17], among other methods. The anaerobic co-digestion process is increasingly being studied, with the involvement of various materials and wastes, as confirmed by the literature. In the case of animal manure, a large number of results have been published, while only a few papers have attempted to systematize the knowledge and evaluate the prospects of using the method. This work provides an overview of the available information and collected results on manure processing through co-digestion. Particular attention has been paid to identifying opportunities for intensification of biogas production from manure and optimization of the process. In addition, this issue is covered in broad terms, taking into account the factors influencing the high production of animal manure in the world, its processing by biological technologies, and the management of the resulting products.

2. Anaerobic Digestion in the Face of Rational Animal Manure Management

The principles of storage and management of manure are regulated via a variety of legal acts. The correct method of recycling this waste is important for sustainable development and environmental protection. EU member states are primarily obliged to apply EU law, which in this situation prevails over national legislation. One of the legal frameworks that take into account the use of animal manure as fertilizing product is the Nitrates Directive [29], which obliges member states to create a code of good agricultural practices. In Poland, the Code of Good Agricultural Practice is in force, containing a set of environmentally friendly solutions for the use and collection of natural fertilizers [30]. The green light for the effective and safe use of manure as a natural fertilizer came with the entry into force of Regulation (EU) 2019/1009 of the European Parliament and of the Council on 5 June 2019 [31]. This document allows the natural use of manure, classified as category 2 animal by-products, provided it is first properly treated.

Due to the intensive production of animal excrement, there is a problem with their current use, hence the need for their proper storage and use. Slurry storage methods include manure storage on heaps in a field, a deep barn under an inventory, or deposited on a liquid manure pit with a sealed bottom and a slope that allows water to drain off. However, in organic farming, composting or anaerobic digestion is recommended [30,32,33]. For example, the storage of fresh manure takes about 4–6 months. During this time, its humidity decreases even to 20–30%, and gases produced due to microbial activity migrate to the atmosphere. Mroczek et al. [34] report that thermal drying is a better solution, where the dryer capacity fluctuates between 0.8–1.5 tons per hour. Unfortunately, odors are a significant problem in this process. According to Best Available Techniques (BAT), it is recommended to dry poultry manure to a content of 90% dry matter. The resulting product can be stored in closed rooms and further processed in the pelleting process. Pellets obtained from compressed manure can be used for energy or fertilizing purposes. It has the form of a briquette or pellets and has a calorific value similar to wood, and little ash is produced during combustion.

Combustion is a relatively simple method of manure management and is economically advantageous, due to high electricity production. The BMC Moerdijk power plant in the Netherlands processes 430,000 tons of poultry litter per year, generating 285,000 MWh of green energy and 60,000 tons of high-quality fertilizer in ash form [35]. Combustion, despite

its simplicity, is an extremely invasive method in relation to the environment because the process generates compounds such as carbon monoxide, volatile phosphorus compounds, dioxins, and furans, which require the use of special filters. Another disadvantage of this process is the loss of nitrogen [36].

Therefore, more and more emphasis is placed on biological utilization techniques, among which composting and anaerobic digestion stand out. Organic recycling such as the composting process is a natural process of decomposing organic matter by aerobic bacteria, fungi, and nematodes. Thanks to organic recycling, a product with a higher organic nitrogen content and less odor nuisance is obtained. Particularly noteworthy is the vermicomposting technology, which involves earthworms. However, due to the sensitivity of the organisms, in this case, a mixture of feces undergoing the process to obtain a neutral pH is recommended and about 12–16 months of aging of the substrate are required. Among the mentioned methods, the greatest hopes are associated with digestion related to biogas production [34].

Anaerobic decomposition of organic substances is one of the oldest biological processes and the beginnings of its use were associated with a lack of thorough knowledge of the microbiological activity of the organisms involved. Sewage sludge is a common waste subject to anaerobic digestion. The positive effects of processing the waste in this way prompted the development of this technology and the attempt to use other types of organic substrates, both solid and liquid. Virtually any such organic matter can be a raw material for biogas production, provided it does not contain inhibiting substances [37]. Recycling animal waste reduces the amount of manure and uncontrolled greenhouse gas emissions and prevents the migration of pollutants into ground and surface waters. In addition, it contributes to the sustainable development of energy production. Anaerobic digestion is an economically viable process and is socially acceptable due to its environmental friendliness. In 2020, biogas production in Europe reached 191 TWh, of which 32 TWh was upgraded, while the rest was used to produce local heat and electricity [38]. Figure 3 shows the realistic potential of methane production from manure in different European countries based on data from 2018 estimated by Scarlat et al. [21]. The realistic biogas potential, calculated based on total collectible manure and specific biogas yield, is about 16.1 billion m^3 biomethane in the EU and 17.8 billion m^3 in the whole of Europe. For example, this is equivalent to the natural gas consumption of Belgium each year [21]. The economic efficiency of biogas production largely depends on the cost of the substrate, which seems to be a cost-effective solution in the case of waste [39,40]. Figure 4 shows the main advantages of the digestion process [26].

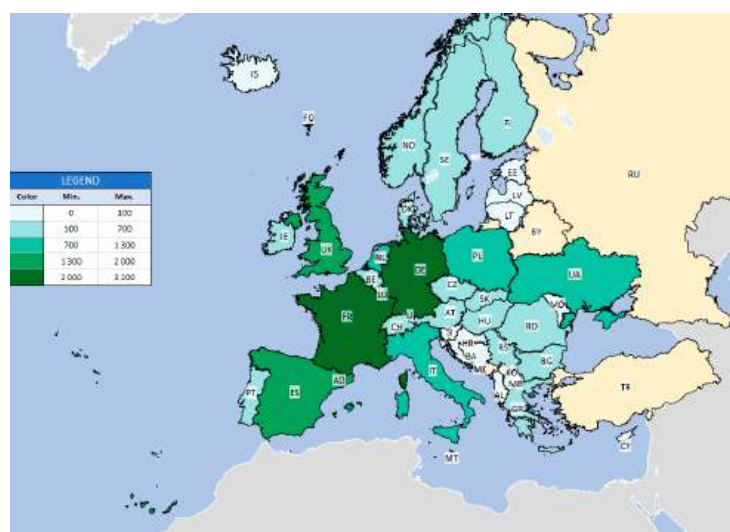


Figure 3. The realistic potential of methane production from manure in different European countries based on data estimated by Scarlat et al. [21]; value at mln m^3 methane (yellow—not available).



Figure 4. Advantages of the anaerobic digestion process [26].

The benefits of using anaerobic digestion to utilize animal manure are primarily waste stabilization, odor control, energy production, reduction of pathogenic organisms, preservation of biogenic elements, inactivation of weed seeds, compliance with progressive legal restrictions, and social acceptance [26]. The carbon in organic waste is part of the renewable carbon cycle so that CO_2 from the combustion of waste biogas does not represent additional GHG emissions, unlike conventional management practices where carbon from waste is oxidized to CO_2 . Therefore, the use of waste-derived biogas should be considered climate neutral, so that replacing fossil fuels with biogas mitigates GHG emissions, provided that fugitive CH_4 emissions are properly managed. Furthermore, during anaerobic digestion, the organic nitrogen present in the feedstock is converted to nitrate (NO_3) and NH_3 and is retained in the digestate residue. Digestate has low levels of pathogens and associated odors compared to untreated animal manure and contains nutrients that are readily absorbed by plants. Sigurnjak et al. [41], in their studies, have shown that digestate can be used to replace synthetic fertilizers without the loss of crop yield. The use of digestate as a fertilizer and soil improver also helps to maintain soil carbon content. In addition to the traditional use of biogas, the process of biogas production can be integrated into other agricultural activities: providing energy to farms and using the digestate to fertilize crops for food, feed, and other value-added by-products [42,43].

In the anaerobic digestion process occur complex biochemical reactions. Nevertheless, from a process engineering point of view, this method is considered relatively simple because no sterilization steps are required (“mixed culture” enrichment of ubiquitous organisms) and there is no need to separate the biogas product as it separates from the aqueous phase [26]. Anaerobic digestion is a relatively simple method of manure management and is economically advantageous, due to high electricity production.

3. The Most Important Factors Affecting the Anaerobic Digestion of Animal Manure

Biogas consists of methane (CH_4), carbon dioxide (CO_2), and trace amounts of nitrogen (N_2), hydrogen sulfide (H_2S), ammonia (NH_3), and water vapor. The exact composition of biogas depends on the biomass sources and the used technology [44]. The effectiveness of anaerobic digestion as a biological process depends primarily on the activity of microorganisms, among which should be distinguished methanogenic species extremely sensitive to environmental conditions. The stages of anaerobic degradation are hydrolysis, acidogenesis, acetogenesis, and methanogenesis [45]. Hydrolysis decomposes polymers such as cellulose, starch, and proteins into monomers by exoenzymes [9]. During acidogenesis, acetate, H_2 , CO_2 , and volatile fatty acids (VFAs) are formed, while acetogenesis produces acetic acid. The last stage, methanogenesis, runs in parallel to the third to convert CO_2 and H_2 into methane [46]. Thus, the course of the process depends largely on the chemical composition of the substrate, its amount and frequency of introduction as well as parameters like temperature, digestion time, pH value, and the presence of toxic substances such as ammonia, ammonium nitrogen, hydrogen sulfide, or heavy metals. Current control

of the above-mentioned factors allows for the effective work of bioreactors and effective biogas production [10]. Below is a more detailed description of the main factors affecting the process of animal manure digestion.

3.1. pH

Organisms participating in individual stages of the anaerobic digestion process show different tolerance to reactions. However, it is assumed that their increase is optimal for a pH value in the range of 6.8–7.5. Undoubtedly, the buffer capacity of the substrate is an important parameter related to the reaction. When the acid–base balance is disturbed, process-inhibiting substances are formed. The buffer capacity of the charge is affected by alkalinity (amounts of carbonates and acid carbonates) and the content of undissociated organic acids. High concentrations of acids in the undissociated form lead to an increase in the carbon dioxide content, resulting in a decrease in the pH value [10]. A sharp drop in pH usually results in a complete collapse of the process. Animal manure is characterized by a relatively high pH (even up to 10), but also a large buffer capacity [47].

3.2. Volatile Fatty Acids, Alkalinity

A useful indicator for controlling acid–base digestion conditions is the ratio of volatile fatty acids (VFAs) to alkalinity. An increase in this ratio precedes the appearance of critically low pH, which can prevent the process from falling faster [10]. For proper digestion, the content of volatile fatty acids is between 100–500 g/m³ [10], while the VFAs/alkalinity ratio should not exceed 0.3 [48]. Wang, Xang et al. [49], in their work on the impact of VFAs on methane production efficiency, also point to the important role of the acidogenesis phase on the anaerobic digestion product. Volatile fatty acids are first converted to acetic acid before the degradation to methane. The incorrect conversion rate of volatile fatty acids can lead to changes to the desired order: acetic acid > ethanol > butyric acid > propionic acid; this change can cause the accumulation of propionic acid, which fails in the methanogenesis stage. Methanogenic species responsible for the production of the final product of anaerobic degradation are therefore the most exposed to the toxic effects of acids [45]. It is also worth mentioning that low pH also increases the mobility of heavy metals, which are process inhibitors [48].

3.3. Ammonia

In the opposite case, namely when the reaction is alkaline, ammonia harmful to methanogens is formed [10]. During anaerobic digestion, some organic nitrogen is bound by organisms in biomass, which depends on the C/N ratio of the substrate. On the other hand, the unbound nitrogen changes into the ammonium form. However, it should be noted that the higher the dissociation rate of ammonia, the lower its toxicity. Low pH causes a higher proportion of undissociated ammonia. At a concentration of 50–200 mg/L, this compound affects anaerobic processes because ammonium nitrogen is the basic element in the synthesis of amino acids, acids, and nucleic acids. In addition, ammonium nitrogen, due to its chemical properties, neutralizes the acids present in fermentative bacteria and thus helps maintain the neutral pH conditions that support cell growth. If ammonia is present in high concentrations, it can lead to the inhibition of the process [23]. It is generally recognized that the inhibition of ammonia consists mainly of the inhibition of the methanogenesis phase [23]. The adaptability of methanogens to the increasing concentration of ammonia depends mainly on the rate of its formation, which is associated with the substrate load, process temperature, and hydraulic retention time [16,50,51]. It is difficult to determine the limit value of ammonia concentration, which causes inhibition of digestion, due to the discrepancy in the results of studies of various authors. The mechanism underlying the inhibition is still not sufficiently defined, with reported concentrations ranging from 1500–7000 mg/L [23]. In aqueous conditions, ammonia occurs mainly in two forms, as

ionized ammonium ion (NH_4^+) stable in the aqueous phase and in gaseous form as free ammonia (FAN), which is represented by Equation (1) [23]:



The relative fraction of free ammonium nitrogen (FAN) relative to the total ammonium concentration is related to the pH and temperature of the solution, as shown in Equation (2) [23]:

$$\text{FAN} = \text{TAN} \times \left(1 + \frac{10^{-\text{pH}}}{10^{-(0.09018 + \frac{2729.92}{T(\text{K})})}} \right)^{-1} \quad (2)$$

FAN = concentration of free ammonium nitrogen (mg L^{-1})

TAN = total ammonium concentration (mg/L)

T(K) = temperature (Kelvin)

Although the inhibitory concentrations of ammonia reported in the literature are different, when converted to free ammonia, they are more consistent, indicating the main reason for the inhibition of this form. For example, for a pH 8 solution, only 4% of TAN is available as FAN at 20 °C; while at 40 °C, 13% becomes available as FAN. The discrepancy in results thus illustrates the difficulty of administering ammonia inhibition based on TAN rather than free ammonia, as total ammonia inhibitory concentrations reported in different studies are not comparable unless pH and temperature conditions are also reported [23].

High ammonia concentration is a major problem in animal manure digestion, especially poultry manure. In addition, longer manure storage results in increased ammonia content, which is associated with the need for rapid waste management or effective removal of this toxic substance [9,10,52]. The simplest method of ammonia neutralization is to dilute the raw material with water; however, this involves a decrease in biogas yield, water consumption, and a large amount of secondary waste, which increases handling costs (pumping, storage, solids/liquid separation, and transport) [28]. Therefore, it seems right to use solutions that are less invasive to the environment, such as stripping with air or water vapor [53], adsorption processes [54,55], filtration techniques, nitrification [56], precipitation [57], ion exchange [58], or anaerobic oxidation [59,60] (Figure 5). Ammonia removal also promotes the recovery of nutrients from the feedstock by capturing ammonia from the carrier gas with scrubbers or traps such as sulfuric acid, creating high-end fertilizers that can be used for agricultural purposes [61]. Due to the possibility of digesting several wastes at the same time, which heterogeneously translates into process economics, the co-digestion technique seems to be particularly promising [11,16].

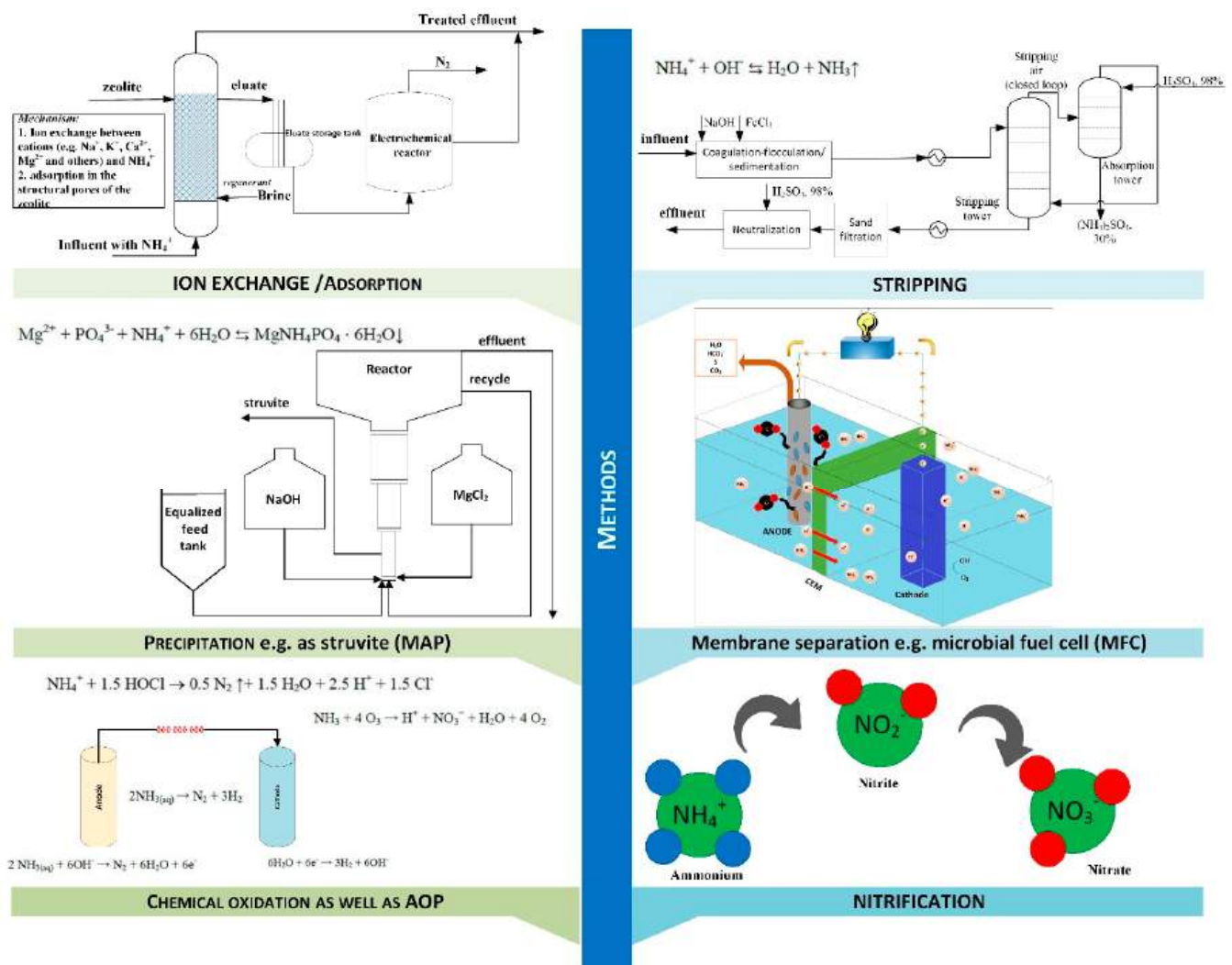


Figure 5. Methods used to remove ammonia from the substrate [53,56–59,62].

3.4. C/N Ratio

The proper ratio of elements such as C and N in the substrate is extremely important from the point of view of the anaerobic digestion process. The optimal ratio of these nutrients is given in the range of 10:1–25:1, while 100:3 is indicated as the maximum value. If the threshold of this ratio is exceeded, nitrogen will be used by organisms participating in digestion, which will translate into a decrease in methane production efficiency. In turn, the decrease in the C/N ratio results in the formation of toxic ammonia and an increase in the pH value [63]. In the case of animal manure, this ratio is usually insufficient for effective anaerobic digestion [64–66]. The appropriate C/N ratio may be the main reason for improving biogas production from this waste [65]. Increasing the amount of C can be achieved by using co-substrates with a high content of this element. There have been many publications in recent years in which the positive impact of a mixture of several wastes on the efficiency of anaerobic digestion has been presented. Wang et al. [67] also pay attention to the interactive effect between the C/N ratio and temperature on methane production efficiency.

3.5. Temperature

Rapid temperature changes cause the death of organisms involved in anaerobic digestion, which is why optimization of this parameter is extremely important in the course of the process. Depending on the psychrophilic (<25 °C), mesophilic (30–40 °C), and thermophilic

(>40 °C) types of digestion, the latter two are commonly used. The influence of temperature on process efficiency is a subject of constant research. Mesophilic conditions favor all operational activities and require less financial effort [27]. Despite the need for heating, thermophilic digestion determines the increased metabolism of organisms, and thus faster degradation of volatile solids and reduction of pathogenic microorganisms [4,68,69]. However, the issue of methane production efficiency at elevated temperatures is divergent. Böske et al. [27] indicate in their research on horse manure digestion 59.8% higher methane yields and 58.1% higher methane rates in the case of thermophilic process in relation to mesophilic conditions. On the other hand, Hansen et al. [70] noticed that methane production efficiency decreases with increasing temperature. Maranon et al. [71] also showed that the co-digestion of cattle manure, food waste, and sewage sludge causes a lower methane gain at 55 °C than at 36 °C. In turn, Mata-Alvarez et al. [40], in their paper presenting a critical review of the literature in the years 2010–2013 regarding anaerobic co-digestion, conclude that the efficiency of the process under thermophilic conditions is slightly higher. However, in fact, maintaining a relatively high temperature in reactors, especially on small farms, can be a problem due to large differences in external temperature. Therefore, the issue of this parameter should be further examined, taking into account other factors that may correlate with temperature, such as mixing intensity, reactor type, or substrate properties.

3.6. Mixing

By mixing the contents of the reactor, a homogeneous fermenting mass is obtained throughout the entire volume of the chamber. The choice of mixing intensity is closely related to the process temperature. At higher digestion temperatures, the mixing of the substrate should also be increased. For mesophilic conditions, the daily capacity of devices used for mixing should be 6–20 times higher than the capacity predicted for the volume of the chamber. In the bioreactor, the difference between the dry matter content of the substrate at different depths should not exceed 5 kg total solids/m³ [10]. In the case of manure, especially horse manure, the mixing process is hindered by the presence of bedding materials that are used to create dry and clean spaces for animals [38]. It is also worth mentioning that mixing should be adapted to the specific type of reactor used [72,73].

3.7. Reactor Type

For most studies on anaerobic digestion, the process is carried out in a CSTR (continuous-stirred tank reactor). However, technology performance can be improved by using other bioreactor configurations [40]. Animal manure is a relatively dry waste and its digestion in a single-stage reactor can be difficult to carry out. Solids tend to float on the surface of the liquid phase, which contributes to the clogging of equipment. For this reason, works are devoted to the separation of individual stages of anaerobic degradation; the aim of which is to optimize the process depending on the substrates used. Smith and Almquist [72], in a study focused on the co-digestion of horse manure and food waste, proposed a technology based on two-stage digestion, where in the first phase the pH value is close to 4 and includes the stages of hydrolysis and acidogenesis, and in the second phase the pH is neutral during acetogenesis and methanogenesis. The main motive of the presented solution was to prevent the accumulation of fibrous biomass in the second phase reactor, which is directly responsible for the production of methane [72]. A similar concept was also presented by Zhang et al. [73] who used a three-stage digester for the co-digestion of horse manure and food waste, broken down into hydrolysis, acidogenesis, and wet methanogenesis. As a result, they obtained greater hydrolytic and acidogenic efficiency of the solid organic substance, thereby accelerating the subsequent stage responsible for the production of methane.

3.8. Hydraulic Retention Time

The presence of the substrate in the bioreactor until it is replaced with a new charge is defined as the hydraulic retention time. This parameter is particularly important at the time of start-up, where the slow increase of the substrate gives the possibility of a maximum growth rate of microorganisms [74]. Changes in hydraulic retention time (HRT) can affect the structure of the microbiome community. The imbalance between fast-growing microorganisms (hydrolytic and acid-forming bacteria) and slow-growing methanogens as a result of inappropriate HRT causes problems such as insufficient utilization of hydrolysis/acidogenesis products in subsequent digestion stages and/or methanogen leaching effects [75]. HRT should be adapted to the type of substrate so that it allows for complete degradation, with different organic substances decomposing at different rates. Usually, the hydraulic retention time is from 20 days in the case of slurry, and up to 60 days for energy crops and hardly degradable compounds such as cellulose or lignin. This parameter depends on the temperature because organic substances decompose faster under thermophilic conditions [76]. As previously mentioned, animal manure digestion is usually carried out at an HRT equal to 20 days. However, Grosser, in her work [77] on the co-digestion of sewage sludge, grease trap sludge, and the organic fraction of municipal waste at different HRTs (12–20 days), indicates that, despite the best process efficiency at the longest HRT, carrying it out at an HRT lower than 20 days is also possible, and daily biogas production was about 46% higher compared to the period with the highest HRT.

3.9. Water Content

Water, as the compound that is the basis of the life of organisms, is important in the course of the process both from the point of view of biological activity and the structure and properties of the charge. To assess the moisture content of the substrate, its dry organic matter content is determined. Depending on the hydration of the biomass, digestion can be divided into wet, semi-dry, and dry, where for the first type the dry matter content does not exceed 15% and the second is about 20%. However, dry digestion occurs in the case of total solid (TS) values above 20%. The optimal amount of TS is considered to be 12–15% because in such conditions the substrates can be easily pumped between devices, and an efficient decomposition process takes place [63,78]. Higher moisture content also promotes the growth of methanogenic bacteria and improves the transfer between substrate molecules and organisms during the last stage of anaerobic digestion [73].

3.10. Pre-Treatment

Due to the structure and composition of animal manure, various pre-treatment techniques can improve its anaerobic digestion and affect the greater efficiency of methane production. Pre-treatment methods include mechanical and chemical techniques as well as biological techniques such as bioaugmentation [39,64]. The selection of the right technique is largely determined by capital expenditure. The year 2011 was a breakthrough in terms of the number of articles published on the preliminary treatments to which digestion substrates are subjected. The increase in publications was undoubtedly associated with research interest in the co-digestion process and the possibility of anaerobic degradation of hardly biodegradable and inaccessible substances [27,40]. Pre-treatment of such substrates is often encouraged to accelerate or increase the methane production potential due to the availability of organic matter or the removal of process inhibitors [64,79].

One of the popular methods in this area is the use of ultrasound, commonly used to break down complex polymers in the treatment of sewage sludge, which can lead to an increase in methane production by up to 34% [71]. Castrillon et al. [80] have shown that the use of this treatment in the case of cattle manure with glycerol causes an increase in the amount of biogas by 121%. However, Maranon et al. [71] noted that the better anaerobic digestion efficiency of cattle manure with food waste and sludge caused by ultrasound treatment does not compensate for the energy that it requires. Similar observations are also made by Azman et al. [81] using ultrasound to treat the manure digestate. Other effective

pretreatment methods associated with large financial outlays and physical manure include aeration [10].

Thermal pre-treatment seems to be a promising solution, often integrated with other treatment methods. Research conducted by Gonzalez-Fernandez et al. [82] has shown that treating pig manure with a temperature of 170 °C for 30 min at a pressure of 7 bar increases methane production by 35%. For comparison, the same authors also pre-treated this substrate using a strong base, but the effect was an increase in methane efficiency by 13%. An interesting strategy was proposed by Rodriguez-Verde et al. [64], combining the thermal treatment of chicken manure at 90 °C with the simultaneous stripping of ammonia (Figure 6). Liquid manure, especially dust, is abundant in nitrogen, hence the removal prevents process inhibition. The solution of the researchers made it possible to reduce the content of ammonia in the substrate and increase its biodegradability.

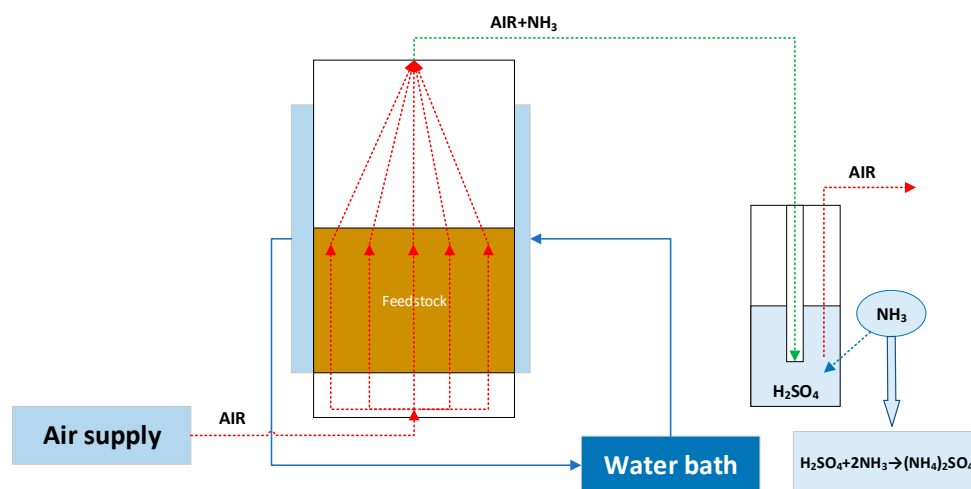


Figure 6. Scheme of thermochemical pretreatment proposed by Rodriguez-Verde et al. [64].

It is also worth mentioning that it is important to properly secure and quickly manage the manure. The long-term storage of animal waste can significantly reduce biogas production. The use of this feedstock after two months results in an almost 6% decrease in gas production, while after four months it's 17% decrease compared to fresh material. [83].

3.11. Other Process Inhibitors

In addition to the ammonia and fatty acids described above, many other substances interfere with the anaerobic digestion process. They include, among others, heavy metals that are extremely dangerous in a mobile and digestible form for methanogenic organisms. The main factors affecting the migration of these elements to cells are pH, oxidation–reduction potential, and the sorption capacity of the substrate. To determine the mobility and bioavailability of heavy metals, speciation analysis is commonly performed, such as the BCR sequential extraction technique, which is based on the elution of elements with reagents of increasing aggressiveness [84]. Hydrogen sulfide, which is an inherent component of biogas, is another compound that can inhibit the digestion process and can induce harmful effects at 50 mg/L [50]. It is worth mentioning, however, that its presence may affect the neutralization of heavy metals in soluble form due to the formation of more soluble metal sulfides [64]. Other elements that inhibit the process also include sodium, potassium, calcium, and magnesium.

4. Importance of Anaerobic Co-Digestion in the Treatment of Animal Manure

The anaerobic degradation process has been investigated in numerous studies, and special attention is paid to the processing of organic waste while maintaining efficiency in biogas production [72]. Figure 7 shows the number of publications on the anaerobic digestion of animal manure from 2001–2023 [85]. However, the anaerobic digestion of

animal manure may not be successful due to the specific properties of this material. Wet anaerobic digestion is commonly used due to the low level of formed sludge, ease of use, and greater methane production efficiency per volatile solids [78]. Animal feces contain a high content of large fibrous particles, causing problems with the clogging of devices [27]. The digestibility and efficiency of horse manure methane production are the lowest compared to other farm animals due to the presence of bedding materials (for example wood shavings) that contain hardly degradable compounds such as lignin or cellulose [73]. Cattle manure is also characterized by relatively low biodegradability [9]. In turn, poultry manure contains a higher concentration of nitrogen compared to other organic waste, which is associated with the risk of process inhibition by the release of ammonia [17,64]. Table 3 presents the basic parameters of anaerobic digestion for the most digested feces: cow, poultry, swine, and other manure.

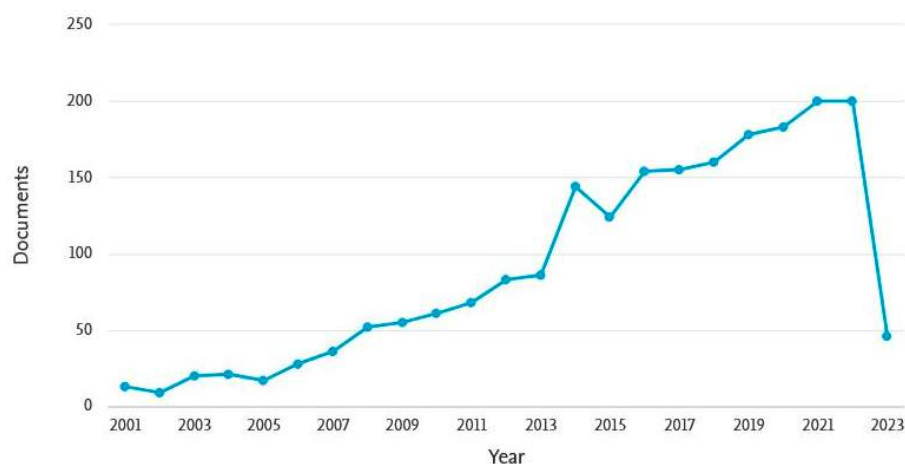


Figure 7. The number of publications on anaerobic digestion of animal manure in 2001–2023 (data status: March 2023) [85].

Due to the potential for energy recovery from liquid manure and its high buffer capacity, attempts were made to remove problems related to process inhibitors. In addition to substrate pre-treatment and stripping of ammonia, joint digestion with animal manure and other materials is a promising method. The main motive for introducing such a solution is processing several wastes at the same time and balancing the content of nutrients in the material, which translates into the optimization of the C/N ratio [52,65,86]. Moreover, this modification of the charge allows for reducing the negative impact of toxic compounds on the process and causes the succession of microbial communities and system stability [87,88]. Co-digestion is therefore defined as a combination of the decomposition of various types of substrates to obtain greater biogas efficiency [24]. Joint treatment of several wastes is also economically advantageous, as it is possible to obtain materials from one source, e.g., the household. Co-digestion is also the most cost-effective and easiest way to improve digestion efficiency for farmers [25].

When choosing suitable co-substrates, factors such as price, access, material composition, methane production efficiency, and pre-treatment and machining costs should be taken into account [9]. Defining the optimal substrate mix is based on trials, but also using modeling of the ratio of co-substrates in batch experiments can maximize methane production [64]. In the case of co-digestion, it is important to properly balance the composition of the substrates and the process parameters. Usually, a solution is observed where the proportion of one of the substrates is above 50%. In agricultural biogas plants, agricultural waste, as well as liquid and solid animal excrements, should be digested first [37]. The anaerobic decomposition of the mixture of municipal bio-waste with liquid manure can also be important in the aspect of sustainable waste management in local conditions [89]. During the processing of animal excrements, co-substrates with a high C/N ratio, low buffer capacity, and, depending on biodegradability, the ability to release large amounts of

volatile fatty acids are sought [40,90]. Table 4 summarizes examples of materials characterized by different C/N ratios [37]. A particularly high carbon-to-nitrogen quotient is found in wood (700), as well as paper (170–800), scobs (200–500), and bark (100–130). Slightly less is contained in straw (80–100), leaves and weeds (90), corn cobs (40–80), and hay (40). Materials with low C/N, are usually those that contain a lot of protein, such as manure (15–18) or legumes (18–20). Below <25 also include kitchen waste, green and food waste, or other non-legumes.

Table 3. Basic technologic parameters for different animal manure digestion.

Substrate	Type of Reactor (Total Volume, L/ Working Volume, L)	Description of Process	VS Removal (%)	Biogas or Methane Production (Increase *)	Methane (%)	Ref.
COW MANURE						
FW + CM	CSTR (140/86)	55 °C; 16 rpm; Recirculation rate: 11.40 m ³ /h OLR: 1, 2, 3, 4 kg VS/(m ³ ·d)	63.01–82.81	0.60–0.8 ¹ (up to 88.6%) ²	61.34–65.89 (up to +4.7%) ²	[91]
CM + barley	Batch (1/0.75)	55 °C; 100 rpm; CM to barley mixing ratio equal 1:1, VS basis; trials inoculated with sewage sludge (SS) last trial co-inoculation of CRF with inoculum	NA	0.278 ¹ (+18%) ³	53–66	[92]
CM + a trace metals solution	Batch (0.120/-)	53 d; 35 °C	NA	0.148 ¹ (+24%) ⁴	NA	[93]
CM	Batch (0.5/0.2)	36.5 °C; I/S 0.5; manure loading a factor was 3.5 g VS/L	58.6	0.204 ²	69.1	[94]
CM + steel slag	Batch (0.5/0.4)	36 ± 1 °C, 35 d concentrations of steel slag: 0.5, 1.0, 1.5, and 2.0 wt%	58.62 ⁵ (+15.5%) ⁶	0.275 ¹ (+153%) ⁶	51.12	[95]
CM + APW	Batch (0.5/0.375)	36 ± 1 °C, APW/DM wet weight ratios: 1:0, 3:1, 1:1, 1:3, and 0:1.	55.9–59.91 (up to +7.4%) ⁷	0.195 ¹ (up to 23.6%) ⁷	61.4–67.1 (up to +12.6%) ⁷	[96]
CM + BS ⁸	CSTR (20/15)	49 ± 1 °C, HRT = 20 d; 5% of shredded straw and 95% of CM of fresh matter	NA	0.213 ¹ (+28.9%) ⁷	NA	[97]
	CSTR (20/15)	49 ± 1 °C, HRT = 20 d; 5% of briquette straw (BS) and 95% of CM of fresh matter		0.217 ¹ (+30.9%) ⁷		
	CSTR (30 m ³ /-)	50 °C, BS concentration— 9% of fresh matter		0.351 ¹ (+33.1%) ⁷		
CM + ESBS-DP	Batch (-/2)	35 ± 0.5 °C ESBC-DP:CM mixture ratios were tested: 0:100, 25:75, 50:50, 75:25, and 100:0	65.3–77.5 (up to +33.2%) ⁷	0.323–0.557 ¹ (up to +24.6) ⁷	NA	[98]
the lactating CM + FeW	Batch (0.05/-)	37 °C added to feed at 30% of the total sample VS weight, 88 d	45.45 (+22.9%) ⁷	0.374 ¹ (−9.4%) ⁷	NA	[99]
CM from young cow + FeW			42.98 (+23.2%) ⁷	0.349 ¹ (+5.1%) ⁷		
Dry CM + FeW			41.60 (+26.9%) ⁷	0.257 ¹ (−5.7%) ⁷		
the lactating CM + WM		37 °C, manure with waste milk was tested at two mixing ratios, 70:30 and 30:70; 88 d	45.44–47.3 (up to +27.9%) ⁷	0.413–429 ¹ (up to +3.9%) ⁷		
CM from young cow + WM			40.03–43.08 (up to +20.9%) ⁷	0.408–0.470 (up to 41.6%) ⁷		
Dry CM + WM			40.22–42.17 (up to +28.7%) ⁷	0.301–0.335 ¹ (up to 22.3%) ⁷		
FR + CM	Pilot scale (-/850)	35 °C, 27% radish and 73% dairy manure (ww); 13% radish and 87% dairy manure (ww),	NA	0.208–0.210 ¹ (up to 38.7%) ⁷	NA	[100]
CM + MS	Batch (1/0.8)	35 ± 1 °C, mixing ratio of 3:1, 2:1, 1:1, 1:2 for CM/MS	NA	0.534–0.614 ¹ (up to +39.8%) ⁷	51.21–58.66 (up to +39.5%) ⁷	[101]
Ss + CM		35 ± 1 °C, mixing ratio of 3:1, 2:1, 1:1, 1:2 for Ss/CM		0.352–0.470 ¹ (up to +7.1%) ⁷	48.4–58.7 (up to +39.6%) ⁷	
POME + CM	SABr (5/3.5)	35 °C, 25:75, 50:50, 75:25, and 100:0 mixing ratios of POME and CM	41–63 (up to +90.9%) ⁷	357–1005 ⁹ (up to +292%) ⁷	NA	[102]
CM + ShM	CSTR (-/2.4)	HRT: 25 d; 37 ± 1 °C, 120 rpm Ratio 1:1	NA	0.179 ¹ (+22.6%) ⁷	61 (+8.9%)	[103]

Table 3. Cont.

Substrate	Type of Reactor (Total Volume, L/ Working Volume, L)	Description of Process	VS Removal (%)	Biogas or Methane Production (Increase *)	Methane (%)	Ref.
CM	Batch (2/0.25)	Mechanical Pre-treatments: shredded (SP), then mixed (MP), and finally blended (BP).	NA	0.216–0.235 ¹ (up to +11.9%) ¹⁰	NA	[104]
CM + WS	Reactor (23.6/20.9 m ³)	35 ± 1 °C, daily flow of feedstock on the level of 0.39 m ³ /d ratio of 1:1 <i>w/w</i> ; Ultrasonic pretreatment		0.460 ¹ (+24.6%) ¹⁰	53 (+1.3%) ¹⁰	[105]
		35 ± 1 °C, daily flow of feedstock on the level of 0.39 m ³ /d ratio of 1:1 <i>w/w</i> ; hydrodynamic cavitation		0.430 ¹ (+16.5%) ¹⁰	54.1 (+3.4%) ¹⁰	
CM + CRS + SBP	Batch (0.5/-)	Mixing ratio: 2:1:1; 39 ± 2 °C Thermal pre-treatments: at 100, 120, 150 and 180 °C with 10, 20, 30, 60, and 120 min	NA	AcD: 0.180 ¹¹ (+11.4%) ⁷ (+100.6%) ¹⁰	NA	[106]
CM + CST	Batch (2/1)	Mixing ratio 1:1 <i>w/w</i> 35 ± 2 °C, 60 rpm Pre-treatment: 1.5% Ca(OH) ₂ and 120 °C	NA	0.290 ¹ (+31.82%) ¹⁰	NA	[107]
	EGSB (3.4/2)	HRT: 1–16 d, 35 ± 2 °C, OLR: 2.18–35.21 kg SCOD/(m ³ d) Mixing ratio 1:1 <i>w/w</i> 35 ± 1 °C, Pre-treatment: 1.5% Ca(OH) ₂ and 120 °C	85.12–96.41 ¹²	0.23–0.31 ¹³	48.21–69.32	
CM + tea waste	Batch (0.6/-)	Mixing ratio 1:1 <i>w/w</i> ; 40 d, 25–35 °C; Pre-treatment: 4% NaOH g/g TS	NA	43.85 ¹⁴ (+55.9%) ¹⁰	NA	[108]
		Mixing ratio 1:1 <i>w/w</i> ; 40 d, 25–35 °C; Pre-treatment: microbial consortium		52.55 ¹⁴ (+86.8%) ¹⁰		
CM:RS	Batch (0.25/-)	Mixing ratio of 1:1, based on TS mass, 35 °C Pre-treatment: limonite concentrations of 1%, 5%, and 10%	NA	1351–1462 ¹⁵ (+18.5–30.3%) ¹⁰	NA	[109]
CM	Batch (0.5/0.4)	37 ± 1 °C, 0.18 wt% microwave pyrolytic carbon material	NA	0.380–0.502 ¹⁴ (up to +70.7%) ⁷	NA	[110]
CM + acorn slag waste	Batch (0.5/0.4)	36 ± 1 °C; 3:1 wet weight ratio Additive: biochar dose: 0.72, 1.08, 1.44, 1.80, and 2.16 g/L	57.4–67.75 (up to +27%) ⁷	0.431–0.581 ¹⁶ (up to +42%) ⁷	62.3–66.4 (up to +11%) ⁷	[111]
CM	Batch (1/-)	38 °C, 30 d Additives: microscale waste iron powder or iron oxide nanoparticles	46.39–55.06 (up to +77.8%) ⁷	0.67–0.222 ¹ (up to +39.6%) ¹⁰	54.33–58.94 (up to +11.6%) ⁷	[112]
CM	Batch (-/0.4)	36 ± 1 °C; Additives: nano-scale tungsten (WC, W2N, and W18O49)	50.08–71.11 ⁵ (up to +73.9%) ⁷	0.426–0.580 ¹⁶ (up to +58.5%) ⁷	NA	[113]
CM + APW	Batch (0.5/0.4)	36 ± 1 °C; 35 d, ratio CM:APW 1:3 <i>w/w</i> Additive: Ti-sphere core-shell structured (0.03 g/L); the magnetic field	53.03–78.25 ⁵ (up to +73.9%) ⁷	0.366–0.510 ¹ (up to +65.53%) ¹⁰	NA	[114]
CM + Cereal crops	Batch (1/0.75)	37 ± 1 °C, 100 rpm Pre-treatment: 10% <i>v/v</i> of <i>Orpinomyces</i> sp. (anaerobic fungus) and spent medium	NA	0.115–0.430 ¹ (up to +33%) ¹⁰	NA	[115]
CM	CSTR (3.0–3.5/-)	37 ± 1 °C, 120 rpm; HRT = 30–40 d, Pre-treatment: bioaugmentation culture containing <i>Bathyarchaeota</i>	NA	0.179 ¹ (+20.1%) ¹⁰	NA	[116]
CM	Batch	A meta-analysis AD, 160 of case studies.	NA	Mean:0.204 ¹ (+38.5%) ⁷	NA	[90]
CM	Continuous mixed	A meta-analysis AD, 72 of case studies.	NA	Mean:0.299 ¹ (+70.9%) ⁷	NA	
POULTRY MANURE						
PM + a trace metals solution	Batch (0.120/-)	53 d; 35 °C	NA	0.407 ¹ (+12%) ³	NA	[93]
PM + B	Batch (0.5/0.2)	36.5 °C; I/S 0.5; manure loading the factor was 3.5 g VS/L	81.4	0.259 ¹	61.1	[94]

Table 3. Cont.

Substrate	Type of Reactor (Total Volume, L/ Working Volume, L)	Description of Process	VS Removal (%)	Biogas or Methane Production (Increase *)	Methane (%)	Ref.
PM + RS	Batch (0.120/-)	SS-AcD, 35 °C; 180 rpm, I/S: 0.5–4.0	80.92–93.25 ¹⁷	0.123–270 ¹	NA	[117]
PM + CC			54.55–88.89 ¹⁷	0.131–0.291 ¹		
PM + PS			56.66–75.94 ¹⁷	0.084–0.157 ¹		
PM + SW			49.89–87.61 ¹⁷	0.098–0.262 ¹		
PM + CH			30.67–81.03 ¹⁷	0.116–0.155 ¹		
PM + SB			33.82–91.7 ¹⁷	0.140–230 ¹		
PM			32.20–89.03 ¹⁷	0.123–0.302 ¹		
PM + CST	CSTR (2.5/2)	HRT:20 d; VS ratios of CST/CM or UPCS/CM were 1:2; OLR: 2.1 g VS/(L d) Pre-treatment: Urea Pretreated CST (UPCS) Additive: 10 g/L of biochar (B)	NA	0.449 ¹ (PM:CST) 0.499 ¹ (PM:UPCS) 0.513 ¹ (PM:CST+B) 0.530 ¹ (PM:UPCS+B)	57.1 (PM:CST) 60 (PM:UPCS) 61.4 (PM:CST+B) 62.5 (PM:UPCS+B)	[79]
PM	Batch (0.5/0.4)	35 ± 1 °C; A: Manure loading (g VS/L):31.0–58.1 Additives: Biochar dosage (%): 1.8–5.2; Cellulose loading (g VS/L): 40.0–158.1	NA	0.177–0.292 ¹	NA	[118]
PM	Batch (0.5/0.4)	37 ± 2 °C; 95 rpm, 35 d Additive: pumice	66.83 ¹⁷	8796 ⁹	68.46	[119]
PM + AWS	Batch (0.5/-)	SS-AcD (TS 20%); 35 ± 2 °C; control AD of PM	NA	0.406 ¹ (+195%) ⁷	NA	[120]
		SS-AcD (TS 20%); 55 ± 2 °C, control AD of PM		0.323 ¹ (+150%) ⁷		
		SS-AcD (TS 20%); 35 ± 2 °C, control AD of TPM Pre-treatment: stripping ammonia from PM		0.562 ¹ (+63%) ⁷		
TPM + AWS		SS-AcD (TS 20%); 55 ± 2 °C; control AD of TPM Pre-treatment: stripping ammonia from PM (treated PM -TPM)		0.298 ¹ (+70%) ⁷		
PM	Batch (1/-)	37 ± 1 °C; enzymatic pretreatment (a mixture of Onozuka R-10 enzyme and Macerozyme R)	NA	0.537 ¹⁸ (+35%) ⁷	NA	[121]
PM + VW	Batch (0.25/-)	SS-AcD, 37 °C, 50 d	NA	0.244 ¹ (+2.8%) ⁷	NA	[122]
PM	CSTR (15/12)	OLR: 1.6 and 2.5 g VS/(ld) 55 °C	42–62	0.094–0.220 ¹⁹	56–67	[123]
		OLR: 1.6 and 2.5 g VS/(ld) 37 °C	44.5–46.1	0.245–0.252 ¹⁹	67–68	
PM	Batch (0.5/0.4)	35 ± 1 °C Additive: biochar made up of wheat straw, discarded fruitwood, and chicken manure at temperatures of 350 °C, 450 °C, and 550 °C	NA	0.214–0.294 ¹ (up to +69%) ⁴	NA	[124]
PM + BPS	CSTR (3/2.5)	4:1 based on VS; OLR: 0.8–3.2 gVS/(ld)	NA	0.193 ¹	NA	[125]
PM	Batch	A meta-analysis AD, 36 of case studies.	NA	Mean:0.260 ¹ (+22.4%) ⁷	NA	[90]
PM	Continuous mixed	A meta-analysis AD, 20 of case studies.	NA	Mean:0.169 ¹ (+71.1%) ⁷	NA	
SWINE MANURE						
SM	Batch (0.25/-)	37 °C, manually mixed once a day, manure loading factors: 8, 16, 32, and 64 g VS/L, pH adjusted to 7.0	54.4 (VS/L = 8) 54.2 (VS/L = 16) 52.2 (VS/L = 32) 49.4 (VS/L = 64)	409.57 ²⁰ (VS/L = 8) 384.66 ²⁰ (VS/L = 16) 361.30 ²⁰ (VS/L = 32) 318.01 ²⁰ (VS/L = 64)	72.8–78.8	[126]
SM	Batch (0.5/0.4)	37 °C, I/S: 1:1, manually mixed once a day Additive: zeolites (natural and sodium), at rates of 0, 10, 40, 70, and 100 g/L of SM	NA	(SM + NZ 40g/L SM) (+35% biogas, and +29% methane)	NA	[127]

Table 3. Cont.

Substrate	Type of Reactor (Total Volume, L/ Working Volume, L)	Description of Process	VS Removal (%)	Biogas or Methane Production (Increase *)	Methane (%)	Ref.
SM	Batch (1/0.8)	Pre-treatment: use of in situ formed graphene in an electric methanogenesis system, 38 °C, 28 d, I/S: 1:5	NA	356.49 ²¹ (+41.49%), 222.17 ²² (+60.89%)	NA	[128]
SM + a trace metals solution	Batch assay (0.120/-)	53 d; 35 °C	NA	0.180 ¹ (+22%) ³	NA	[93]
CM + SM + a trace metals solution	Batch assay (0.120/-)	53 d; 35 °C	NA	0.511 ¹ (+9.7%) ⁴	NA	[93]
SM	CSTR (5.5/4)	196 d, 60 rpm, HRT: 20 d, mesophilic conditions	35.7–41.0	1.06–1.16 ²³	NA	[129]
SM + G	CSTR (5.5/4)	196 d, 60 rpm, HRT: 20 d, mesophilic conditions	74.1–77.7	5.44–5.58 ²³	NA	
SM	Batch (-/0.4)	Additive: ferrous chloride in the amount characterized by final elemental iron concentrations of 5, 10, 25 and 40 mmol/L, 37 °C, I/S: 1:3, 41 d	NA	269.1 ²⁰ (+21.5)	NA	[130]
SM + CST	Batch (1/-)	Substrate combination ratios (SM/CST): 30:70, 50:50 and 70:30 (% w/w); Initial pH values adjusted to 6.0, 6.5, 7.0, 7.5, and 8.0 using 5 mol/L NaOH and 5 mol/L HCL; 35±1 °C, I/S: 1:2.5	7.5 (SM:CST = 30:70) 16.7 (SM:CST = 50:50) 23.8 (SM:CST = 70:30)	11.92 ²⁰ (SM:CST = 30:70) 14.08 ²⁰ (SM:CST = 50:50) 220 ²⁰ (SM:CST = 70:30)	NA	[131]
Dry SM	Semi-continuous (2/1.2)	Additive: wrapped granular activated carbon: 50 g, acclimated sludge (inoculum): 1200 g, HRT: 60 d, 35 ± 1 °C	6.6	1.1–1.67 ²⁴ (+10.6%)	58.8–73.2	[132]
SM	Batch	A meta-analysis AD, 73 of case studies	NA	Mean:0.287 ¹ (+20.6%) ⁷	NA	[90]
SM	Continuous mixed	A meta-analysis AD, 23 of case studies	NA	Mean:0.322 ¹ (+52%) ⁷	NA	
OTHER						
HM	Batch assay (0.5/0.2)	36.5 °C; I/S 0.5; manure loading the factor was 3.5 g VS/L	52.9	0.155 ¹	70.1	[94]
HM	Batch (0.5/-)	35 °C, 35 d, HM solid ratios: 0.5, 1, 2, and 4%	80–90	339 ²⁵ ; 203 ²⁰ (TS: 0.5%) 374 ²⁵ ; 239 ²⁰ (TS: 1%) 370 ²⁵ ; 236 ²⁰ (TS: 2%) 381 ²⁵ ; 247 ²⁰ (TS: 4%)	60 (TS: 0.5%) 64 (TS: 1%) 63 (TS: 2%) 65 (TS: 4%)	[133]
HM + Ss	Batch (0.5/-)	AcD, 35 °C, 35 d, HM TS ratios: 2 and 4%, HM:Ss = 9:1	90	410 ²⁵ ; 270 ²⁰ (TS: 2%) 425 ²⁵ ; 280 ²⁰ (TS: 4%)	65 (TS: 2%) 66 (TS: 4%)	
HM + Ss	Continuous digester (5/-)	AcD, 35 ± 2 °C, TS ratio: 4%, HM:Ss = 9:1	>50	NA	66–68	

Table 3. Cont.

Substrate	Type of Reactor (Total Volume, L/ Working Volume, L)	Description of Process	VS Removal (%)	Biogas or Methane Production (Increase *)	Methane (%)	Ref.
GM	Batch assay (0.5/0.2)	36.5 °C; I/S 0.5; manure loading the factor was 3.5 g VS/L	46.4	0.159	65.8	[94]
RM	Batch (0.25/-)	37 °C, manually mixed once a day, manure loading factors: 8, 16, 32, and 64 g VS/L, pH adjusted to 7.0	49.5 (VS/L = 8) 48.9 (VS/L = 16) 47.5 (VS/L = 32) 46.2 (VS/L = 64)	323.22 ²⁰ (VS/L = 8) 296.87 ²⁰ (VS/L = 16) 261.46 ²⁰ (VS/L = 32) 211.48 ²⁰ (VS/L = 64)	68.3–76.5	[126]

Substrate: FW—food waste; CRF—cow rumen fluid; APW—aloe peel waste; WS—wheat straw; ESBS-DP—dried pellets of exhausted sugar beet cossettes; FeW—feed waste; WM—waste milk; FR—forage radish; MS—maize straw; Ss—sewage sludge; POME—palm oil mill effluent; ShM—sheep manure; CRS—corn silage; SBP—sugar beet pulp; CST—corn straw; SCOD—soluble chemical oxygen demand; RS—rice straw; CC—corn cob; PS—peanut shell; SW—sawdust; CH—coffee husks; SB—sugarcane bagasse; B—biochar; AWS—agriculture wastes; VW—vegetable waste; BPS—banana pseudo-stems; NZ—natural zeolite; G—glycerol; CM—cow manure; PM—poultry manure; SM—swine manure; HM—horse manure; GM—goat manure; RM—rabbit manure; NA—not available. Reactors: CSTR—Continuously Stirred Tank Reactors; SABr—solar-assisted bioreactor; EGSR—expanded granular sludge blanket. Process description: OLR—organic loading rate; I/S—inoculum to substrate ratio; AcD—anaerobic co-digestion; SS-AcD—solid phase anaerobic co-digestion. Other: ¹—specific methane yield, m³/kg VS_{add}; ²—increase in comparison to mono-digestion of FW; ³—AcD of CM + barley; ⁴—without supplementation; ⁵—COD degradation rate; ⁶—control check, namely cow manure and sewage sludge; ⁷—increase in comparison to mono-digestion of CM or PM; ⁸—shredded and briquettes; ⁹—mL; ¹⁰—untreated manure; ¹¹—biogas yield m³/kg TS; ¹²—SCOD removal; ¹³—m³/kg COD; ¹⁴—the accumulation of biogas production, mL/g TS; ¹⁵—methane production, mL; ¹⁶—cumulative biogas yield, m³/kg VS; ¹⁷—COD removal; ¹⁸—methane yield, m³/kg VS_{removed}; ¹⁹—methane yield, m³/kg TS; ²⁰—methane yield, mL/g VS_{add}; ²¹—biogas production, m³/t dry swine manure; ²²—methane yield, m³/t dry swine manure; ²³—biogas production, L/g VS_{add}; ²⁴—biogas production, L/d; ²⁵—biogas yield, mL/g VS_{add}; *—comparison to control reactor.

Despite the fact that the agricultural industry is the most convenient source of obtaining such materials, the need to overcome seasonality and increase the efficiency of methane production has caused great interest in other biodegradable waste [40]. In recent years, a lot of research has been done on the anaerobic co-digestion of animal manure and by-products of various industries. In addition to the aspect of improving process efficiency, an important criterion for selecting appropriate substrates is their availability and production in a given region. For example, rice straw is one of the most abundant wastes generated in Valencia, Spain. Traditional methods of processing this material, namely combustion and landfills, generate high emissions of toxic compounds into the environment and, when stored in soil, an uncontrolled digestion process. Sillvestre, Gómez, et al. [134] used 1, 2, and 5% rice straw addition (on a mass basis) to digest cattle manure, which is also widely generated in Spain [71]. As a result, the largest increase in biogas production in relation to controls (anaerobic mono digestion of cattle manure), amounting to 54%, was achieved with a 5% share of rice straw.

Other substrates used to co-digest cattle manure are food or distillery waste. Zhang, Xiao, et al. [65] showed that with a ratio of food residues to liquid manure of 2 to 41.1% methane production increased. In turn, El-Mashad and Zhang [135] determined that for a digestion time of 20 days, a mixture of 60% food residues and 40% dairy manure is recommended. Callaghan, Wase, et al. [51] also stated that fish and whole solid offal from a brewery could be successfully used for the anaerobic digestion of waste from cattle farming. An interesting experiment was carried out by Westerholm et al. [136] using the joint processing of cattle manure with whole stillage. This waste is also characterized by a low C/N ratio; however, the co-digestion of these substrates has significantly stabilized the process. However, it should be taken into account that the properties of stillage produced in different plants differ, which translates into methane production efficiency.

Table 4. C/N value for exemplary substrates [37].

High C/N Content Materials		Low C/N Content Materials	
Substrate	C/N	Substrate	C/N
Paper	170–800	Kitchen waste	12–20
Scobs	200–500	Green waste	10–25
Wood	700	Fresh grass	12–20
Bark	100–130	Legumes	18–20
Straw	80–100	Non-legume plants	11–12
Leaves and weeds	90	Manure	18
Maize cobs	40–80	Poultry manure	15
Hay	40	Food waste	15

Another waste added to cattle manure digestion may be crude glycerin. This compound is mainly produced in the production of biodiesel, but the market is not able to absorb a large increase in this by-product [40,80,129]. The optimal amount of glycerol as a supplement for digestion is in the range of 4–6% [9]. In their paper, Astals et al. [129] also notice the positive effect of raw glycerin on the processing of swine manure. The authors showed that the addition of this substrate increased the organic loading rate, optimized the C/N ratio, and reduced the free ammonia concentration in the feedstock.

In the case of swine manure, which is characterized by high nitrogen concentration, co-digestion can be performed, for example, with energy crop residues. Cuetos, Fernandez, et al. [24] used for this purpose maize, rapeseed, and sunflower residues. Based on the results obtained, these authors concluded that the best results were obtained with the co-substrate in the form of maize. In the case of the remaining mixtures, however, they received worse results, probably as a result of higher lignin content in rapeseed and sunflower residues. The use of more than two substrates, including other types of excreta, can also be a promising method. Liu, Tang, et al. [33] successfully co-digested swine manure with cattle manure and solid waste. Such an undertaking allows for the treatment of waste in animal husbandry areas.

A lot of the research focuses on poultry manure because it possesses the largest methane gain that can be obtained from 1 kg of dry matter compared to other manure [9]. However, as in the case of waste from pig farming, the proper digestion of this raw material disturbs the high concentration of nitrogen [51]. The suggested share of poultry manure in co-digestion with other materials is from 10–40% of the mixture [137]. As co-substrates for poultry manure, popular agricultural waste, [14,45] like corn stover [78] may be used. Bayrakdar, Molaey, et al. [16] were the first to co-digest poultry manure with used poppy straw, whose annual production in Turkey is around 20,000 tons per year. The result of the research was a methane yield of 0.36 L/g VS when the total ammonium nitrogen concentration did not exceed 4000 mg/L. Borowski et al. [138] also presented satisfactory results in a study on the anaerobic co-digestion of chicken manure and sugar beet pulp residues. By mixing these substrates in a 1:1 weight ratio, a organic loading rate with stable pH and optimal nutrient balance was achieved. A greater proportion of manure in the co-mixture caused process inhibition, mainly as a result of the toxic effect of ammonia and, to a lesser extent, volatile fatty acids. Cocoa pod husk is another waste that can be used for the anaerobic digestion of poultry manure [3]. Cocoa is intensively produced, especially in Ivory Coast and Ghana (over 50% of world production). However, the by-product is difficult to decompose due to the presence of lignin components. Dahunski et al. [3] suggest the pre-treatment of cocoa pod husks with alkaline hydrogen peroxide before co-digestion. In turn, Gelegenis et al. [139] considered whey as a material that could help in the processing of chicken droppings. Whey, produced as a result of precipitation and removal of casein from cheese, is characterized by a high content of organic matter and biodegradability. The results of these authors' research indicate good effects of the co-digestion of whey with chicken droppings; however, this finding only applies in the case of whey as a component percentage below 50% (based on VS). In the case of a 1:1

ratio of these substrates, a decrease in biogas production was observed. Carlini et al. [52], using cheese whey wastewater, obtained the correct course of co-digestion with 50% shares of whey and chicken manure. Additionally, Wang, et al. [25] also presented interesting results regarding the processing of chicken manure. The authors co-digested dairy manure (DM) and poultry manure (CM) with wheat straw, which was added to optimize the C/N ratio. They reached their maximum methane potential at DM/CM 40.3:59.7 by weight and a carbon-nitrogen ratio of 27.2:1.

However, there are still few studies on the possibilities of processing horse manure. The key factor conditioning the course of digestion of this waste is the type and amount of bedding material present in it, such as wheat straw, flax, hemp, and wood chips. For example, softwood bedding hardly decomposes and hinders the anaerobic digestion process, while straw has a higher biochemical methane potential [38,140]. Hadin and Eriksson [141] draw attention to the fact that, despite the low biodegradability of litter, it still makes a positive contribution to the energy balance. In the group of other types of manure, horse manure can have a total solid (TS) content of 20% or more and is therefore exactly suitable as a substrate for handling high-solid or solid-state anaerobic digestion, which usually requires a TS above 15% [38]. Carlos-Pinedo and Wang [38] ran simulations of several scenarios with different feedstock component combinations in a full-scale solid-state process. Their results suggested that the replacement of green waste by horse manure with wood chips as bedding material in a co-digestion mixture with organic waste gave the best improvement in terms of energy turnover.

Undoubtedly, the aspect of using sewage sludge for co-digestion with animal feces deserves special attention. Production of this waste is still increasing and, as in the case of liquid manure, its rational management is important. It is a substrate with non-specific properties, but also with high energy and fertilizing potential [86]. At present, sewage sludge digestion is a thriving process on a global scale. Sludge often contains toxic compounds, so it may be beneficial to dilute it by processing it with other materials. It would seem that, due to the relatively low C/N ratio of sewage sludge, anaerobic co-digestion with animal manure is not a good solution. However, Borowski and Wheatherley [142] demonstrated that a 30% addition of poultry manure to sewage sludge caused an increase in biogas production by 50% and higher efficiency of VS removal. In another work, Borowski et al. [143] also studied the co-digestion of sewage sludge with the manure of pigs and poultry. The experiment showed that a 30% addition of pigsty waste caused an increase in biogas production by almost 40% compared to the anaerobic digestion of sewage sludge alone. However, by supplementing the co-digestion mixture with 10% poultry manure, the efficiency of the process decreased as a result of the high concentration of ammonia. It should be mentioned that the latest EU Regulation [31] excludes the use of sewage sludge as a fertilizer. Nevertheless, research into the possibility of their treatment and a better understanding of the risk associated with their management may contribute to the development of new solutions for their fate.

5. Ecological Potential of Digestate

The product of anaerobic digestion is not only a valuable fuel in the form of biogas but also a post-digestion mass (digestate). Its composition depends primarily on the substrates used in the process. Knowledge of the individual properties of the substrate is important from the point of view of monitoring the quality of the resulting product. The introduction of some co-substrates can lead to the production of unstable substances. The use of digestate as a fertilizer or soil conditioner seems to be the most sensible development direction due to the significant amounts of organic carbon in its composition. The components of the digestion product are mainly organic and mineral compounds as well as the biomass of organisms that have not decomposed [26,65]. However, in areas of intensive animal breeding and manure production, the amount of waste generated often exceeds the plant's nutrient requirements. Therefore, a reasonable solution seems to be the separation of liquid

manure into a liquid fraction that can be managed within the farm and a solid fraction that can be transported to areas poor in fertilizers [24].

Limited soil resources constitute a significant barrier to acquiring new places for plant cultivation. Mineral fertilizers, which are easily available and have good solubility in the environment, are commonly used. However, the negative effect of their application, related primarily to their high nitrogen content, has been noticed. The invasiveness of these substances towards the natural environment is often observed already at the production stage. In addition, their price is not affordable. The advantage of organic fertilizers over mineral fertilizers is not only related to their economic benefits, but also in line with the principles of the circular economy. The digestate contains basic elements and other various micro- and macro-elements necessary for plant development. For example, phosphate rock, which is the only source of P, has been declared a critical raw material by the European Union (EU) (EU Report COM/2014/0297) due to its low substitutability. The EU pays particular attention to critical raw materials within the framework of sustainable development principles. The recovery of phosphorus from phosphorus-rich wastes such as poultry manure, sewage sludge, and their incineration ashes is one of the most promising ways to improve the security of P resources [144]. Anaerobic digestion does not affect P content, meaning that the P content of the digestate is completely determined by the input streams. Similarly, the process does not change the heavy metal content. However, during digestion, dry matter is reduced, resulting in increased P and heavy metal concentrations in the digestate. Only easily degradable organic matter is decomposed, while complex substances such as lignin, remain in the digestate [145]. The high content of organic substances resistant to rapid degradation, which are found in the products of anaerobic digestion, promotes the formation of caries. The use of fresh natural fertilizers such as manure has long been widespread in various European countries. However, this is controversial, especially due to the penetration of toxic compounds, such as pathogens, antibiotics, veterinary drugs, and heavy metals [146] into the soil environment, ground, and surface water, and uncontrolled greenhouse gas emissions. Unlike raw waste, digestate is a stabilized and sanitized material, and its production has no generally negative impact on the environment [147]. Nevertheless, the digestion product still needs to be tested for the presence of antibiotics and their degradation by-products. Dosing manure on agricultural land may also contribute to the dissemination of antimicrobial resistance in the environment through bacterial mechanisms such as transformation, conjugation, or transduction [148,149]. However, the literature suggests that the anaerobic digestion process eliminates, or reduces, the presence of antibiotics and resistance genes in manure [8]. Another contaminant present in manure that has attracted particular attention in recent years is microplastics. Wu et al. [150] demonstrated that the direct application of pig and poultry manure may be a new pathway for this substance in agricultural soils. There is growing evidence that microplastics have a negative impact on the microbial community, as carriers of mobile genetic elements and pathogenic microorganisms promote the persistence of antibiotic-resistant genes. Therefore, greater monitoring of this xenobiotic in digestion-processed manure is suggested.

The high quality of digestate obtained has been confirmed in research by, among others, Recebli et al. [151] using as a substrate a mixture of bovine and chicken manure, or Bohdziewicz et al. [89] in the digestion of swine manure and municipal bio-waste.

The digestate may also be an alternative source of water. For instance, Gao and Li [152] used anaerobic digestate effluent collected from a biogas plant as a source of fresh water and nutrients during bioethanol production. In comparison to the production using fresh water, a higher fermentation yield and ethanol concentration in the product was achieved. Depending on the biogas plant technology, the weight of the digestion product may be less if some of the liquid in the form of process water is recycled to the bioreactors. Usually, however, legal and logistical problems arise in managing such a large amount of digestate. High hydration of the mass also affects its transport costs. These limitations can be overcome by drying and concentrating the product or by separating it into solid

and liquid fractions using centrifuges, screw presses, or sieves. Isolation of a solid fraction can also be achieved using processes such as coagulation, flocculation, or flotation. The dehydrated mass can be directly introduced into the soil or subjected to other treatments, e.g., composting or pelleting [153]. Figure 8 shows the main options for the management of digestate.

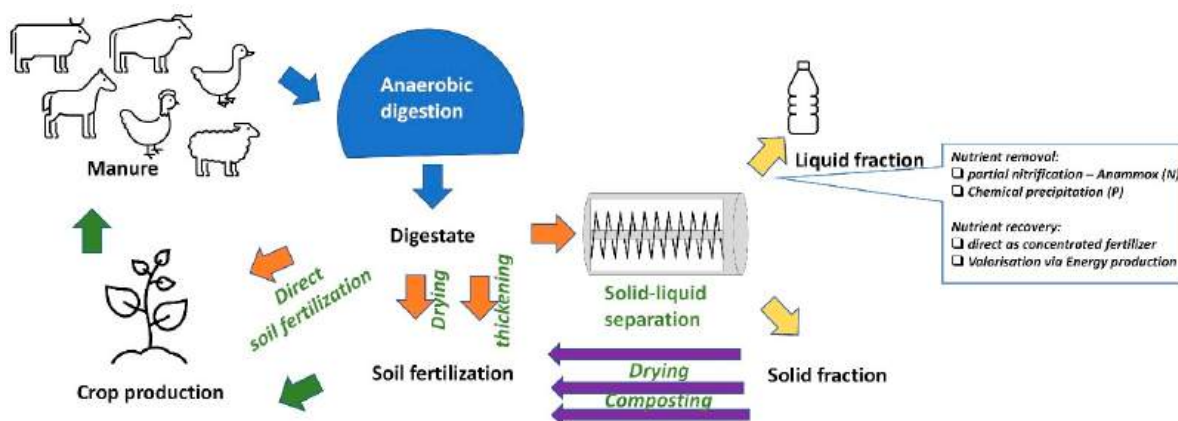


Figure 8. Digestate management methods [153].

6. Conclusions

In connection with the progressive legal restrictions on waste and energy management, it is necessary to implement optimal techniques enabling sustainable development of the agricultural sector. Anaerobic digestion is an attractive solution for processing many raw materials, including animal manure. However, the specific properties of this waste which may disturb the process should be taken into account. The main problem that accompanies the anaerobic digestion of animal excrements is the low C/N ratio and the inhibitory effect of ammonia, which translates into low efficiency of biogas production. The solution in this case may be the adequate pre-treatment of the substrate, but above all its co-digestion with other materials rich in organic carbon. Manure, due to its high buffer capacity, can be successfully decomposed along with raw materials of opposite properties, including onerous waste, such as raw glycerin, stillage, or cheese waste.

The greatest energy potential among all manure is found in poultry manure, which is why a lot of research focuses on it. However, it has a low C/N ratio, which leads to various modifications of the classic anaerobic digestion of this waste. In turn, horse manure seems to be the most difficult to digest because of the bedding materials that accompany it, which is associated with a limited number of publications in this area. Nevertheless, the prospects of conducting the process in different conditions and configurations of bioreactors leave room for further consideration.

Special attention in future research should be paid to the economics of the process based on local conditions and availability of raw materials, as well as a full physicochemical analysis of the substrates used and their biodegradability. Both empirical and modeling methods allow the selection of appropriate process parameters, reactors, and the proportion of individual materials in the feedstock. Due to the progressive regulatory restrictions on the stabilization of biowaste and the possibility of its reuse, it is necessary to characterize the products of the process and assess their further fate based on the available legislation, while keeping in mind other toxic substances such as antibiotics or microplastics, the presence of which in the environment is still subject to research and attempts to establish permissible limit concentrations in the environment.

Among the available methods of managing animal manure, the choice of its anaerobic digestion with other substrates is argued not only by its effectiveness in producing alternative energy but also by obtaining high-quality fertilizer and the possibility of recovering the water and valuable elements, which are part of a sustainable circular economy.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/en16093885/s1>, Table S1: Livestock population in Europe in 2016. Table S2: Total livestock unit (heads) in Europe in 2016.

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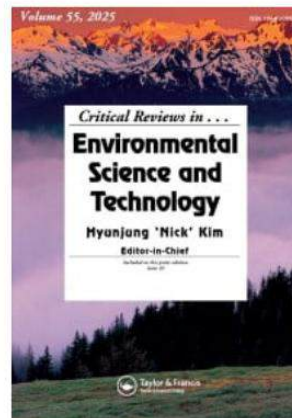
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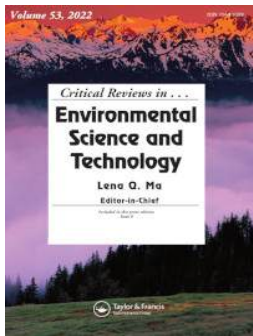
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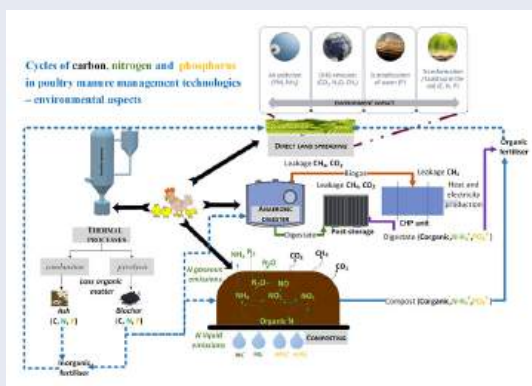
Cycles of carbon, nitrogen and phosphorus in poultry manure management technologies – environmental aspects

Małgorzata Kacprzak^a, Krystyna Malińska^b, Anna Grosser^b, Jolanta Sobik-Szołtysek^b, Katarzyna Wystalska^b, Danuta Drózd^b, Anna Jasińska^b, and Erik Meers^c

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ABSTRACT

Poultry manure (PM) has become a serious environmental problem due to large scale of industrial production and unsustainable management, causing emissions of greenhouse gases (GHGs), odors, leakage of nutrients as well as inorganic, organic and biological pollutants. The main goal of this review was to get a better understanding of carbon, nitrogen, and phosphorous cycles in processing of poultry manure with the most common technologies such as composting, anaerobic digestion, or thermal processes, e.g. pyrolysis. The carbon contained in organic matter (ca. 31%) is mineralized and humified under aerobic conditions (matter recovery) and/or converted into biogas under anaerobic conditions (energy recovery). PM as a feedstock for pyrolysis to obtain biochar may effectively store and prevent C, thus contributing to climate change abatement. During composting, nitrogen is reduced from the compost mixture by leachates in the form of NH_4^+ , NO_3^- or gaseous emissions of NH_3 , N_2O , N_2 . The C/N ratio is also decisive parameter. Most environmental threats of unmanaged PM result from ammonia and nitrous oxide emissions, being higher for PM compared to cattle and cow manure independently of technological processes. The phosphorous in PM is mainly inorganic (32–84%). Using untreated manure as a fertilizer does not allow taking up high doses of phosphorous contained in poultry manure, so the excess accumulates in the soil and then leaches into groundwater. Biochar and struvite are an alternative to storage and source of high concentrations of phosphorous. Cycles of carbon, nitrogen and phosphorous are an integral effect of technologies used for PM management.



1. Introduction

Poultry production is estimated at 40% of the total meat consumption with the biggest producers in 2020: USA (23 million Mg), China (20 million Mg) and Brazil (16 million Mg), (USDA, 2021). Almost 20% of poultry meat within EU-27 countries is produced in Poland (Eurostat, 2021).

Poultry manure (PM) contains about 3-5% nitrogen, 0.9-3.5% phosphorus, and 1.5-3% potassium (Jędrzak et al., 2014). Moreover, the content of calcium, magnesium and sulfur is much higher than in cattle or pig manure (Bolan et al., 2010; Brassard et al., 2018). PM can be directly spreading on soil or treated by different processes to produce added-value products (compost, digestate, biochar). Soil application of PM initiates a series of reactions such as decomposition, hydrolysis, ammonia volatilization, nitrification, denitrification, fermentation and finally could be important source of GHGs: ammonia (NH_3), methane (CH_4), nitrous oxide (N_2O), nitric oxide (NO) and carbon dioxide (CO_2), Li et al. (2012). Moreover, can lead to soil/groundwater contamination by heavy metals - Zn, Cu (Liu et al., 2020). The buffer capacity of the ecosystem may be exceeded and the fertilizer or long-term stored PM become an environmental hazard (Kabelitz et al., 2021). Therefore, any PM processing technology should be based on a thorough analysis of the nutrients (especially C/N/P, properly calibrated application methods based on biogeochemical cycles to prevent GHGs emissions and efficiently use the nutrients for soil improvement. Composting and anaerobic digestion (AD) are biotic-based, however strictly controlled by abiotic factors as temperature, moisture, redox potential, pH, substrate concentration gradient (Li et al., 2012). Biogas production from PM and use of the digestate as an organic fertilizer as renewable energy production and substitution of the mineral fertilizers led to the decrease of GHGs emissions (Kreidenweis et al., 2021). However, improperly processing results in an increase GHGs emissions, mainly due to high ammonia volatilization in the initial step of process.

Thermal conversion of PM is also considered as renewable energy source. The energy obtained is CO_2 neutral and saves fossil fuels (Billen et al., 2015). The life cycle assessments shown that thermal processing of PM generates less GHGs than direct land application and AD (He et al., 2022). Moreover, the solid post-process residues (ash, biochar) enable their environmental use as soil improvement (Piash et al., 2022).

The overall goal of this study was to analyze the current state of the art on biogeochemical cycles of carbon, nitrogen and phosphorus in the most common technologies poultry manure management such as land spreading, composting, anaerobic fermentation, combustion, gasification and pyrolysis in the context of environmental threats and climate changes mitigation.

2. Technologies for management

2.1. Mechanical

2.1.1. Landspreading

Traditionally PM has been used directly as a soil fertilizer (Table S1, [supplementary material](#)). Proper use led to the increase of biomass production and crop quality due to the content of minerals, such nitrogen and phosphorus. However, inappropriate use affected soil and plant quality and contaminated surface and groundwater by potentially toxic elements (PTEs), including cadmium, chromium, nickel, cobalt, copper, barium, lead, (Muhammad et al., 2020). Moreover PM could be a significant source of pathogens. In Australia 100% of broiler litter was contaminated

with *Actinobacillus* and *Salmonella* and in the US - with *Escherichia coli* resistant to amoxicillin, ceftiofur, tetracycline, and sulfonamide (Kyakuwaire et al., 2019).

2.2. Biological

2.2.1. Composting

During PM composting, mineralization, nitrification, incomplete denitrification, and the release of gaseous occur. It follows various phases, i.e. mesophilic, thermophilic (dynamic biological processes involving microorganisms), cooling, and finally the compost maturation. The process requires adequate moisture, a C/N ratio, and the porosity of the compost mixture. To meet these conditions, the manure must be mixed with appropriate bulking materials such as sawdust, grasses, various types of straw, wood shavings, and chips. The temperature of the compost mixture should exceed 55°C and reach 70°C for the hygienization (EU Regulation No 142/2011). This applies, inter alia, to the degradation of live eggs of *Ascaris* sp., *Trichuris* sp., *Toxocara* sp., bacteria *Salmonella* and *Escherichia coli*. The pathogens number can be reduced after reaching high temperatures however requires strict monitoring (Lim et al., 2020). Aeration of the compost mixture is crucial and the minimum 15-20% O₂ is necessary. Lower values may cause the formation of anaerobic zones and contribute to the putrefaction of the mixture, thus hindering the proper functioning of microorganisms. The processing time may be 4-40 weeks, and the final weight of the compost will be reduced by 40-50% in relation to the initial weight. During composting nitrogen, carbon and phosphorous are partially lost: 13-70% for nitrogen, 42-62% for carbon and 28-50% for phosphorus (Hao & Benke, 2008; Harrison, 2008). Nitrogen is lost through leaching of NH₄⁺, NO₃⁻ or releasing gaseous emissions of NH₃, N₂O, N₂. Carbon is lost in the form of CO₂, CH₄ and phosphorus mainly in the leachate of HPO₄²⁻ and H₂PO₄.

Bacteria and fungi are essential for composting. The most common bacteria in the compost mixture includes *Pseudomonas*, *Xanthomonas*, *Actinobacteria*, *Nocardia*, *Streptomyces* which can break down organic matter. *Nitrosomonas* and *Nitrospira* specializing in nitrogen transformation are especially important for composting poultry manure. The fungi such *Aspergillus*, *Acremonium*, *Chrysosporium*, *Fusarium*, *Mortierella*, *Penicillium* and *Trichoderma* occur partially in the first and dominate the last stages of composting. Fungi occur in the mesophilic phase and during compost maturation where the temperature is not as high as in the active phase during which it can reach 50-60 °C (Sánchez et al., 2017).

Compost from PM has a positive effect on reducing soil evaporation, enriches soil with nitrogen, improves the release of nutrients, plant biomass and quality (Al-Bataina et al., 2016; Faucette et al., 2004). For example, the increase starch content of cassava as well as the oil and protein from mustard seeds was observed when plants were cultivated on soil fertilized by PM compost (Amanullah et al., 2010).

The mineralization causes changes in the organometallic complex of heavy metals. The ligands are transformed into forms of ligands with oxygen, i.e., carboxylic or hydroxyl complexes, which affect the mobility of particles and the possibility of their accumulation in living organisms.

2.2.2. Anaerobic processes

Anaerobic digestion (AD) is a complex biochemical process of macromolecular organic substances decomposition contained under anaerobic conditions (ORP < -200mV). Transformations allow for the conversion of biodegradable organic matter mainly into methane and carbon dioxide (Gallert & Winter, 2005) and reduction of nitrate and sulfate to hydrogen sulfide and sulfide, as well as anaerobic ammonification. The overall process of AD can be described by the equation (1) proposed in 1952 by Buswell (Anukam et al., 2019; Meegoda et al., 2018):

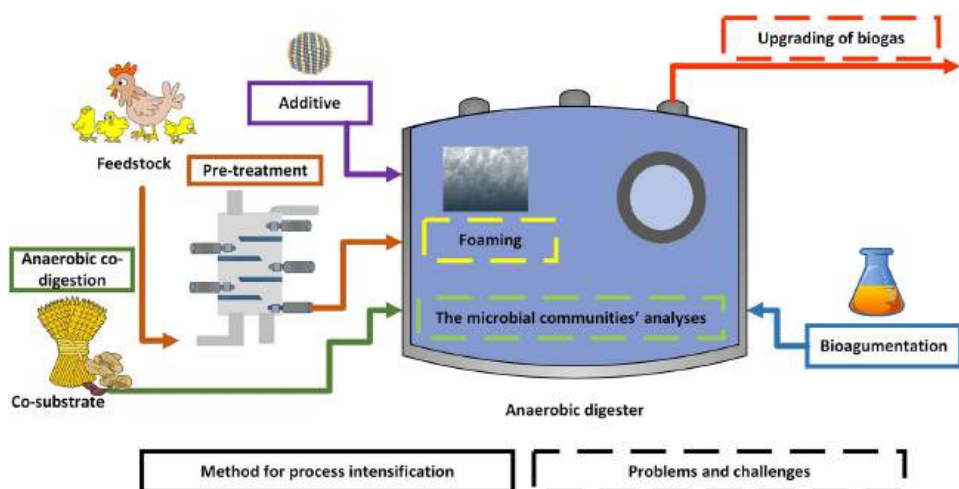
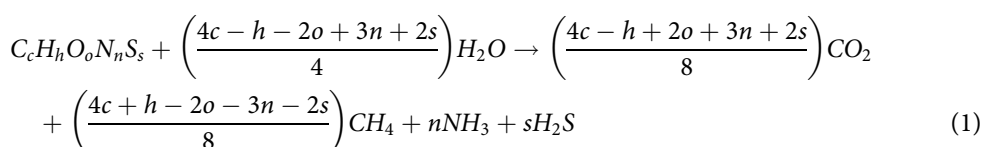


Figure 1. Key issues of research regarding AD of poultry manure, based on (Khoshnevisan et al., 2021).



Based on the biochemical reaction formula and the elementary composition of raw chicken manure (C (35.16%), H (4.83%), O (30.12%), S (0.84%), and N (5.44%)), Qiao et al. estimated that degradation of 1 kg of volatile solids (VS) of chicken manure produced

0.74 m³ biogas, 0.42 m³ methane and 72 g of ammonia nitrogen (87 g free ammonia nitrogen (FAN)) (Shapovalov et al., 2020).

AD is often limited by low C/N ratio, high content of nitrogen, hydrogen sulfide in biogas as well as problem with foaming (Figure 1). Optimization and improvement of anaerobic digestion efficiency is achieved by feedstock pretreatment before introduction to the digestion chambers and/or co-digestion of poultry manure with other organic waste (Drózd et al., 2020; Khoshnevisan et al., 2021; Kreidenweis et al., 2021). Bioaugmentation and the use of additives in the form of nanoparticles or trace elements supplementation also seem to be a promising option (Aguilar-Moreno et al., 2020; Hassanein et al., 2019; Shah et al., 2021). Furthermore, to reduce the risk of failure of the process due to ammonia accumulation, the following solutions can be applied: i) stripping (Guštin & Marinšek-Logar, 2011); ii) addition of ion exchange/adsorption materials such as e.g., zeolite (Ziganshina et al., 2015), natural chabazite (Lin et al., 2016) or bio-char (Ma et al., 2021; Pan et al., 2019); iii) membrane separation (Wang et al., 2018); d) dilution of the feedstock (Duan et al., 2018). Table S2 (supplementary materials) summarizes some results of AD of poultry manure within the above-mentioned solutions.

2.3. Thermal

2.3.1. Combustion

The mostly used PM thermal process is combustion and co-combustion (Junga et al., 2017; Florin et al., 2009), where organic compounds are oxidized with the release of large amounts of heat (Figure 2). The process produces gases such as sulfur dioxide, nitrogen oxides, carbon dioxide, hydrogen chloride and ash. The use of the scaffold technology requires good technical solutions due to high ash content in PM and its low melting point (Kelleher et al., 2002). While the fluidized bed technology enables the reduction of nitrogen oxide emissions (Florin et al., 2009).

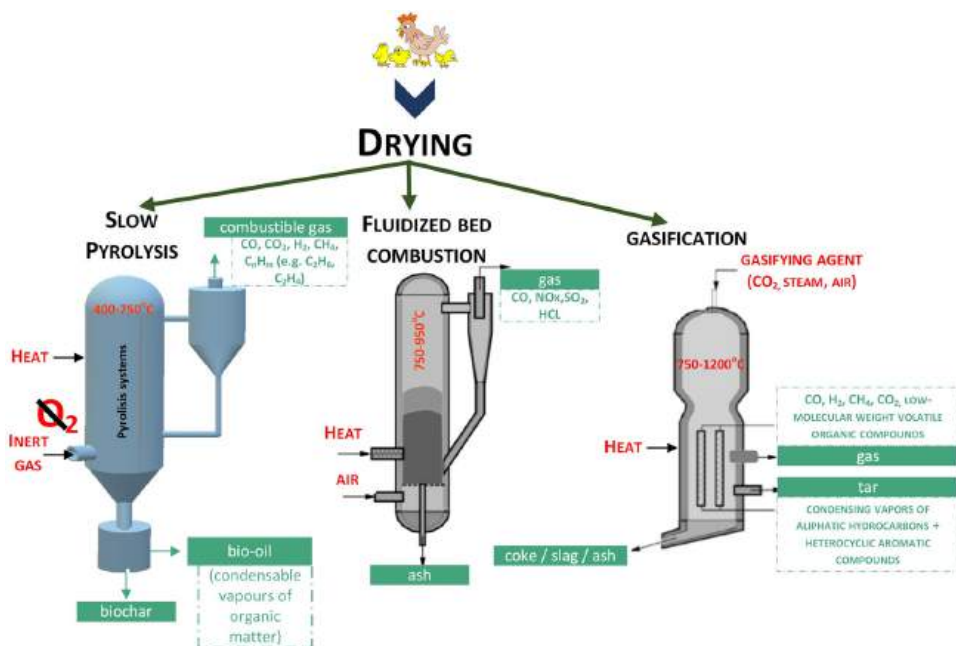


Figure 2. Possibilities of thermal conversion of poultry manure based on own study.

Usually, before combustion, gasification or pyrolysis, manure is pre-prepared by drying (Tańczuk et al., 2019a) which increase its caloric value from 2.6 to 13.5 GJ Mg⁻¹ (Kelleher et al., 2002).

The ash generated by fluidized combustion is odorless, sterile and dry. It can be used as a fertilizer or a soil improver due to the desired phosphorus and potassium content (Billen et al., 2015, Kaikake et al., 2009, Luyckx et al., 2020). Therefore, it can also be used as additive for fertilizers or even chicken feed (Blake & Hess, 2014).

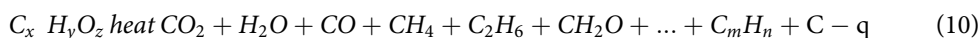
2.3.2. Gasification

PM gasification (Figure 2) is usually carried out in 1000°C in presence of oxidizing agents (e.g. air, oxygen, water vapor). In result, gaseous products mainly hydrogen and carbon monoxide, methane, carbon dioxide, nitrogen and water vapor are creating (equations 2-9) (Wielgosiński, 2016):





Thermal decomposition can be summarized (equation 10):



These chemical reactions are both exothermic and endothermic. Solid residues in the form of ash or slag are also produced. The PM gasification is often analyzed in the option of co-gasification with biomass of plant origin. However, process is rather limited to laboratory and small-scale applications (Tańczuk et al., 2019a) due to technical problems and low quality of the gas produced (Tańczuk et al., 2019b). However, comparing to the gasification of fossil fuels the negative environmental impacts of this process (i.e. greenhouse effect) is reduced (Jeswani et al., 2019).

2.3.3. Pyrolysis

Pyrolysis is a process of transformation of organic matter under anaerobic conditions at high temperatures up to 1000° C (Wielgosiński, 2016) (equation 11):



The liquid products of pyrolysis are mainly a mixture of oils, tars and water with dissolved simple aldehydes, alcohols and organic acids (Figure 2). Moreover, gases, such hydrogen, methane, ethane and their homologues, carbon monoxide and dioxide, and compounds such as hydrogen sulfide, ammonia, hydrogen chloride, and hydrogen fluoride are produced (Wielgosiński, 2016). Solid products containing mainly carbon (Wielgosiński, 2016) are referred to as biochar. Pyrolytic technologies are not as widely used on an industrial scale as is the case of combustion. In the effect the pyrolysis of PM biochar which can be used as a soil improver is produced (Mickan et al., 2016). Moreover, bio-oils can be a potential substrate for bio-grease production. The so-called slow pyrolysis (Ibarrola et al., 2012) during which metals are immobilized and phosphorus is made available, is recommended for the production of biochar (Roberts et al., 2017, Kleemann et al., 2017). Process efficiency is determined by the type of substrate being converted and the temperature of pyrolysis (Bavariani et al., 2019).

PM thermal processing ensures the sanitary safety of post-process products (Maj et al., 2022), (Figure 2) and eliminate potential risk of infection during direct land application (He et al., 2022). The presence of antibiotics or heavy metals in the PM does not interfere with thermal processes. However, the high content of chlorine and alkali metals in the ash can generate many problems, such as high-temperature corrosion, deposit formation on boiler heating surfaces and bed agglomeration in fluidized bed boilers (Maj et al., 2022).

3. Carbon cycle

3.1. Mineralization and humification under aerobic conditions

The 31% of PM is organic matter (Morvan et al., 2021). Mineralization and humification are fundamental for carbon, nitrogen and phosphorous cycles under aerobic conditions. Firstly organic matter is fragmented by invertebrates, chemically degraded, and organic substances are leached (Morgado et al., 2018). Then, during chemical reactions, microorganisms converted the C structures of complex organic molecules toward simpler generally soluble compounds (e.g., carbohydrates, proteins, amino acids) (Morgado et al., 2018). Some of them immediately transform during the primary mineralization process into soluble inorganics and/or gaseous (Figure S1, supplementary material). Simultaneously, the slower humification begins and the formation of complex molecules of colloidal nature and dark color called humus. During humification through several oxidation and hydrolysis reactions - humic acids, fulvic acids, hyalomelanic acids, and humins are created. C:N ratio of initial feedstock material play a crucial role in the process of

carbon fixation associated with enzymatic activity and microbial variation. Products contain higher C, H and lower O compared to the original fresh residues, thus are more stable and resistant to decomposition (Morgado et al., 2018). The C/N value of 17.3 significantly enhanced the composting efficiency and the plant germination index (23.7%) compared to the C/N ratio 9.61, (Liu et al., 2020). Moreover, the suitable C/N ratio increased the relative abundance of Bacilli which played an important role during the mesophilic and thermophilic phases. Bacilli abundance was related to cellulose and β -glycosidase activities, thus improved fiber degradation and humification.

During the secondary mineralization the humus can be partially lost. Addition of thermally treated montmorillonite (M-) and illite (I-) improved the humus formation (Pan et al., 2021). Furthermore, structural equation model and variance partitioning analysis demonstrated that M- and I- treatments promoted precursors to synthesize humic acids (HA) by coordinated regulation of biotic and abiotic pathway and to increase of HA content in the M- and I- treatments mainly through the abiotic pathway.

Microbially dependent mineralization is divided into three stages (Islam et al., 2021): i) an initial with typically present bacteria such *Bacillus*, *Clostridium*, *Proteus* and lactic acid bacteria; ii) an intermediate stage with more specialized *Bacillus*, *Pseudomonas*, *Acinetobacter*, *Arthrobacter*, *Enterobacter* and iii) last stage when the one-carbon-compound-oxidizing bacteria and other specialists release CO₂ from the most refractory of the organic residues. Kinetics of PM mineralization in soil depends on the type of manure. Higher CO₂ emissions (800 mg C kg⁻¹) were found in soils amended with hen poultry manure (PMH) as compare to broiler poultry manure (PMB) – 600 mg C kg⁻¹ (Martín José et al., 2012). Moreover, application of different doses of solid and liquid fermented poultry manure significantly influenced the CO₂ emission from treated soils (Sathya & Maheswari, 2017).

3.2. Conversion into biogas under anaerobic conditions

Anaerobic digestion (AD) generally is divided into four phases such as hydrolysis, acidogenesis, acetogenesis (acetogenesis), and methanogenesis (Figure S2, supplementary materials). During technical scale, AD is divided into two phases: acidic (includes hydrolysis and acidogenesis) and methane (acetogenesis and methanogenesis) (Anukam et al., 2019; Issah et al., 2020).

Phase I - hydrolysis - in which water-insoluble organic polymers such as proteins, lipids, and carbohydrates are decomposed into simple, soluble monomers by extracellular enzymes (hydro-lases e.g., proteases, lipases, cellulases, amylases) released by hydrolytic bacteria. The products of hydrolysis are amino acids, simple sugars, hydroxy-alcohols (main glycerol), and long-chain fatty acids (LCFAs) (Sahota et al., 2018). However, only 50% of the organic matter contained in the feedstock is broken down in this phase. This is linked with a lack of suitable enzymes for their degradation. (Meegoda et al., 2018).

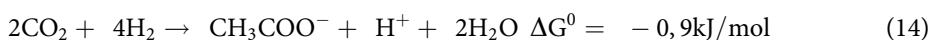
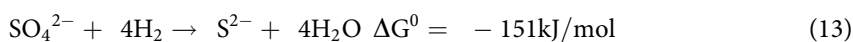
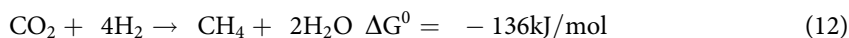
The presence of lignocellulosic material in PM (e.g., straw, wood shavings, rice husk) is the bottleneck of this stage. Generally, lignocelluloses consists of three polymers: cellulose, hemicellulose, lignin and various inorganic materials (Bhatnagar et al., 2022). The proportion of each component depends on the manure type, the region and, consequently, the digestibility of fiber by the animals live in different parts of the world (Orlando & Borja, 2020, Table S2 supplementary material). Hemicellulose fractions are easily hydrolyzed into simple sugars. However cellulose and lignin are more difficult to degrade (Ferraro et al., 2020; Matheri et al., 2018). Lignin hydrolysis is a complicated process mainly due to the numerous carbon and ether bonds between phenolic alcohol derivatives (the basic monomers are trans-p-coniferyl, trans-p-coumaryl and trans-p-sinapyl alcohol). Furthermore, lignin surrounds hemicellulose and cellulose and forms a barrier that limits enzyme accessibility. The breakdown of polysaccharides become hampered (Cai et al., 2021; Orlando & Borja, 2020; Paritosh et al., 2021; Shrestha et al., 2017). Therefore, a

pretreatment is essential to solubilization and degradation of the substrate with a significant content of recalcitrant lignin, such as PM (Figure S3, supplementary material). To accelerate the hydrolysis process after pretreatment of animal manure can be used: i) reapture the crystalline structure of cellulose fraction; ii) increase the digestibility of the feedstocks owing to the reduction of particle size; iii) increase of lignocellulose materials porosity; iv) solubilization of hemicellulose; v) disruption of hemicellulose; vi) depolymerization of hemicellulose; vii) deacylation of hemicellulose; viii) alteration of lignin structure; ix) breakdown of lignin; x) hydrolyzation of fibrous proteins (e.g., keratin); xi) split complex polymer (Orlando & Borja, 2020; Reyes et al., 2021). Also, pretreatment of poultry manure before AD, via enzymes such as peroxidase and laccase enhance the rate of hydrolysis (Bhatnagar et al., 2022).

Phase II – acidogenesis - in which, facultative and obligate acidogenic bacteria convert the products of the hydrolysis to low-molecular organic compounds, mainly volatile fatty acids VFAs (formic, acetic, propionic, butyric, valeric, caproic), alcohols (methanol, ethanol), aldehydes, and gaseous products (carbon dioxide and hydrogen). The key factor impact on the products obtained in the phase is the hydrogen partial pressure formed during the process. At high pressure of the gas, less reduced metabolites such as lactate, butyrate, fatty acids with at least 3 carbon atoms in the chain (C3) are produced. On the other hand, under stable operating conditions (depends on the feedstock and species of bacteria), acetates, carbon dioxide, and hydrogen are produced, which are precursors for the production of methane in the fourth stage of AD (Anukam et al., 2019; Deublein & Steinhauser, 2011). This phase is characterized by a low pH (5-6) and an intense odor. In a balanced system, most of the organic matter is converted into readily available products for methanogens (acetate, hydrogen, and carbon dioxide), while the remainder (about 30%) is broken down into volatile fatty acids (VFAs) or alcohols (Anukam et al., 2019; Issah et al., 2020).

Phase III – acetogenesis - in which, organic acids and some aromatic compounds (e.g., benzoate) are converted into acetic acid, hydrogen, and carbon dioxide (Table S3, supplementary material). For thermodynamic reasons ($\Delta G^0 < 0$), the necessary condition for the decomposition of the compounds is keeping the concentration of hydrogen formed during the process at a low level (Figure S3) (Anukam et al., 2019; Deublein & Steinhauser, 2011; Khanal, 2011).

In a properly functioning system, the partial pressure of hydrogen should be in the range from 10^{-4} Ba (a necessary condition for the reduction of carbon dioxide) to 10^{-6} Ba (oxidation of propionic acid) (Jędrzak, 2007; Ramirez et al., 2009). Therefore, acetogens exist in close symbiosis with other bacterial species between which the so-called interspecies hydrogen transfer (IHR) takes place, (Deublein & Steinhauser, 2011; Figure S.4. supplementary material). The generated hydrogen can not only be utilized by methanogens so-called hydrogenotrophs (Equation 12), but also by sulfate-reducing bacteria (SRB) e.g., *Desulfovibrio desulfuricans* (Equation 13). It is worth emphasizing the fact that sometimes there can be competition for hydrogen between the mentioned groups of microorganisms. In digesters, hydrogen can also be consumed by homoacetogenic bacteria such as *Acetobacterium woodii* and *Clostridium thermoaceticum* (Equation 14) (Anukam et al., 2019; Deublein & Steinhauser, 2011; Gallert & Winter, 2005; Khanal, 2011).



At high ammonia concentrations, synthetic acetate oxidation (SAO) can also occur in this phase (Ramirez et al., 2009). This is a two-step process and involves oxidation by SAOB (syntrophic acetate oxidizing bacteria) of acetates to hydrogen and carbon dioxide (Equation 15) and for thermodynamic reasons the conversion of the resulting products to methane by hydrogenotrophic methanogens (Buhlmann et al., 2019). As reported by Ramirez et al. (2009) the effect of altering

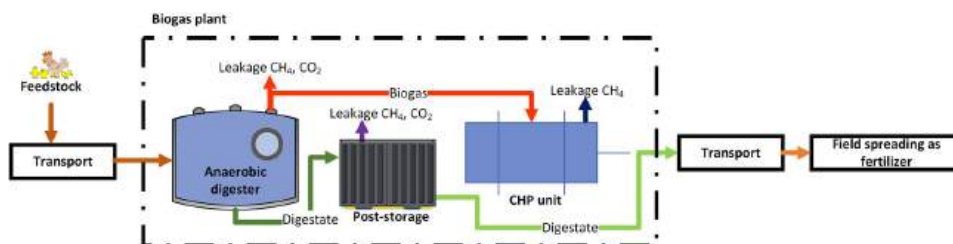


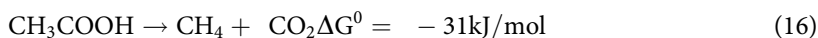
Figure 3. Flow chart of the anaerobic digestion of poultry manure, based on (Khoshnevisan et al., 2021).

the biochemical pathway of acetate degradation results in the reduction of biogas production.



However, the enzymes involved in SAO require trace elements (especially cobalt, selenium, tungsten) for their proper function. Lack of supplementation of TE during AD can lead to SAO inhibition, and accumulation of VFAs in the fermentation broth. Molaey et al. (2018) indicated that supplementation of selenium during AD of PM has a critical role in the stable digestion performance and improves methane production at high organic loading rate and TAN levels.

Phase IV – methanogenesis – 70% of the methane is produced by the decomposition of acetic acid by heterotrophic bacteria (acetotrophic methanogens) (Equation 16). The remaining amount is generated by carbon dioxide reduction, of which only 5 to 6% is obtained from dissolved hydrogen. This phenomenon is explained by interspecies hydrogen transfer, during which hydrogen is not dissolved in the fermentation broth, but transferred directly from acetogens to methanogens (Deublein & Steinhauser, 2011; Jędrzak, 2007).



Methanogens can also produce methane from the following substrates, (Frąc & Ziemiński, 2012): HCOOH , CH_3OH , CO , $(\text{CH}_3)_3\text{N}$, $(\text{CH}_3)_3\text{NH}$, $(\text{CH}_3)_3\text{NH}_2$, $(\text{CH}_3)_2\text{S}$, Me^0 . Some methanogens can only convert one substrate e.g., *Methanobrevibacter arborophilus* or *Methanosaeta* - H_2/CO_2 and acetate, respectively. While others are more versatile e.g., *Methanospirillum hungatei* and *Methanobacterium formicicum* and capable of taking up formate, or genus *Methanosarcina* can convert a wide range of compounds e.g., H_2/CO_2 , acetate, methanol, methylamines, acetic acid, carbon monoxide (Sousa et al., 2007).

However, determining the environmental impact of AD and analyzing the carbon cycle during this process requires a broader view. Apart the biogas production, losses of methane during production and transmission, losses of gases during digestate storage as well as emission from co-generation units should also be taken into account (Figure 3). Table S4 (supplementary material) summarizes the formulas that can be used to estimate gas emissions during the AD (Kreidenweis et al., 2021).

A separate issue is the fate of micropollutants and emerging contaminants during AD. The removal of antibiotics, antibiotic resistance genes (ARGs), and heavy metals under anaerobic conditions are debatable (Riaz et al., 2020). Zhang et al. (2019) demonstrated that microwave pretreatment of manure and activated carbon enhanced ARGs removal and methane production during mesophilic AD of PM. Enrofloxacin in dose $8\text{ mg kg}^{-1}\cdot\text{TS}$ promoted the AD, while higher than $16\text{ mg kg}^{-1}\cdot\text{TS}$ inhibited the biogas production rate (Du et al., 2022). However lack is explanation why most of these pollutants are still present in the digestate (Gurmessa et al., 2020). Riaz et al. (2020) and Gurmessa et al. (2020) suggested that following factors play important role in the degradation: i) the operational parameters of the AD (e.g. temperature, hydraulic retention time, etc.); ii) microbial community structure; iii) the physicochemical character of the substrate; iv) genetic elements. Despite that the digestate before application to the soil is treated (e.g.,

composting or solid-liquid separation (SLS) which reduce manure contaminants (Congilosi & Aga, 2021) still the attention must be put to optimization of post-treatment processes as well as aspects related to feedstock preparation (Gurmessa et al., 2020).

3.3. Biochar production and carbon sequestration potential

Pyrolysis temperature and biochar substrates have impact on the stability of C in biochar and determine its application (Tab.1).

Table 1 selected biochar properties described by (Novak et al., 2009, Song & Guo, 2012, Srinivasan et al., 2015, Khan et al., 2016, Qi et al., 2017, Li et al., 2018, Bavariani et al., 2019, Wystalska et al., 2021)

The C content (86.70%) in biochar from PM is significantly higher, than in other biochar types produced at similar temperatures, due to a high proportion of litter material in the substrate converted into biochar (Srinivasan et al., 2015). Presence of stable C cause that biochar can also be used to sequester carbon in the soil. Using pyrolysis, more than half of the C of biomass can be converted into permanent C-sinks instead of lost as CO₂ (European Biochar Certificate 2020a) (Figure 4). Application of biochar to soil or feed additives average degradation rate is about 0.3% per year. This means that 100 years after biochar application to soil, biochar would still sequester about 74% of the original carbon (European Biochar Certificate 2020a). Moreover, the use of biochar on cultivated soils reduced CH₄ emissions and improved the soil ability to absorb greenhouse gases. The use of biochar from chicken manure for maize cultivation caused reduction in CO₂ of 3.2 kg per 1 kg of biochar (Panwar et al., 2019)

Biochar with a high aromatic carbon content is probably suitable for long-term sequestration of C (Novak et al., 2009). The stability of biochar depends on the O/C molar ratio, the lower its value, the potentially longer the half-life period of biochar and the higher its usefulness for

Table 1. Selected biochar properties described by (Novak et al., 2009, Song & Guo, 2012, Srinivasan et al., 2015, Khan et al., 2016, Qi et al., 2017, Li et al., 2018, Bavariani et al., 2019, Wystalska et al., 2021).

Substrate	Temperature °C	pH –	ash	C	H	N	S	P	K	Ca	CEC cmol kg ⁻¹	BET m ² g ⁻¹
					%							
Poultry litter/ poultry manure	200-700	7.2- 12.8	11.16- 60.78	32.30- 86.70	0.3- 5.62	0.12- 4.90	0.4- 3.53	2.27- 6.38	1.04- 9.15	7.17- 9.40	29.2- 86.5	1.1- 86.7

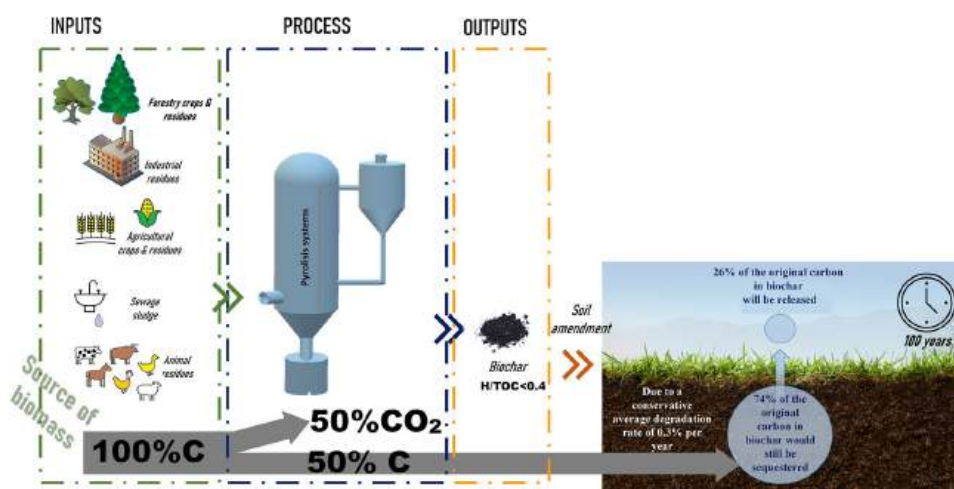


Figure 4. Scheme of transformation of C contained in biomass into C-sinks, based on (European Biochar Certificate 2020a).

sequestration of C in soil. The O/C ratio of biochar studied by Srinivasan et al. (2015) was low and equal to 0.12, similarly in the study reported by Novak et al. (2009), the O/C ratio was 0.14 and < 0.01 . Higher O/C in biochar, i.e. 0.46 and 0.48 was reported by Li et al. (2018). The $O/C > 0.6$ indicates the half-life of the biochar < 100 years. A range of O/C values of 0.2-0.6 may indicate a half-life of 1000-100 years (Srinivasan et al., 2015, Zhang et al., 2017). The $O/C < 0.2$ extends the half-life > 1000 years. In turn, high values of H/C ratio suggest that biochar will degrade quicker in the soil which will result in an increased emission of CO_2 (Van Zwieten et al., 2010). The greatest potential for sequestration of C is displayed by biochar with the H/Corg below 0.4 (Brassard et al., 2018) and may achieve the value H/C ratio of 0.013 (Srinivasan et al., 2015). The low H/C ratio, indicating the high aromaticity of the analyzed biochar, affects the potential of C sequestration. Among biochar produced from different substrates at $680^\circ C$, only biochar from pine sawdust had a higher potential to longer storage in the soil than poultry manure biochar.

For the assessment of C sequestration potential, the overall carbon balance should include all direct and indirect emissions from e.g., biomass production (planting, cultivation, harvesting, transport, crushing), the processing of biomass into biochar and its final treatment (e.g. grinding, mixing, packaging), transport, and soil application (European Biochar Certificate, 2020b), (Figure 4).

CO_2 equivalent (CO_2eq) covers all GHGs in effect of production and treatment of the biomass, its conversion in a pyrolytic installation, and the processing of biochar (European Biochar Certificate, 2020a). The calculation example for the C-sink potential based on guidelines of EBC (i.e. European Biochar Certificate, 2020b) is shown in Table S5 (supplementary material). If renewable energy is used in the production of biochar, CO_2eq is considered to be zero and similarly when electricity corresponding to at least its consumption is generated in the production of biochar in the pyrolysis process (European Biochar Certificate, 2020b). Only biochar produced from C-neutral biomass, e.g., biomass that is waste or by-product that meets certain requirements (European Biochar Certificate, 2020b), is eligible for C-sink certification.

4. Nitrogen cycle

4.1. Forms of nitrogen in poultry manure

High nitrogen content (3-5% PM dry weight) in the form of uric acid (40-70%), feed protein (10-40%), urea (4-12%) and ammonium (4-20%) (Bhatnagar et al., 2022) depends primarily on animal diet, type and age (Nahm, 2003) when applied to soil undergoes a number of transformations (Figure 5). During PM storage organic N is converted into ammonia which leads to volatilization and N loss into the air. When poultry manure is applied to soil for plant uptake N from organic forms is converted through mineralization to N mineral forms. This depends mainly on pH, temperature and moisture content.

4.2. Ammonia emission

Effect of poultry production is gaseous emissions of carbon dioxide, ammonia, hydrogen sulfide, methane, nitrogen oxides, dust and others. Ammonia emission from poultry manure is higher than from cattle and cow manure and during the three-month storage period was 2.94 kg Mg^{-1} , pig manure 2.40 kg Mg^{-1} and cow's 0.45 kg Mg^{-1} , respectively. It also depends on the type of poultry, feed and a breeding system. The highest amount of ammonia is obtained from turkey manure - over 950 kg/year per 1000 birds, from ducks at 680 kg/year from 1000 heads goose at 350 kg/year per 1000 birds whereas smallest broiler chickens generated about $220 \text{ kg ammonia/year}$ per 1000 birds. Stable ventilation of farm buildings, can reduce the amount of ammonia by

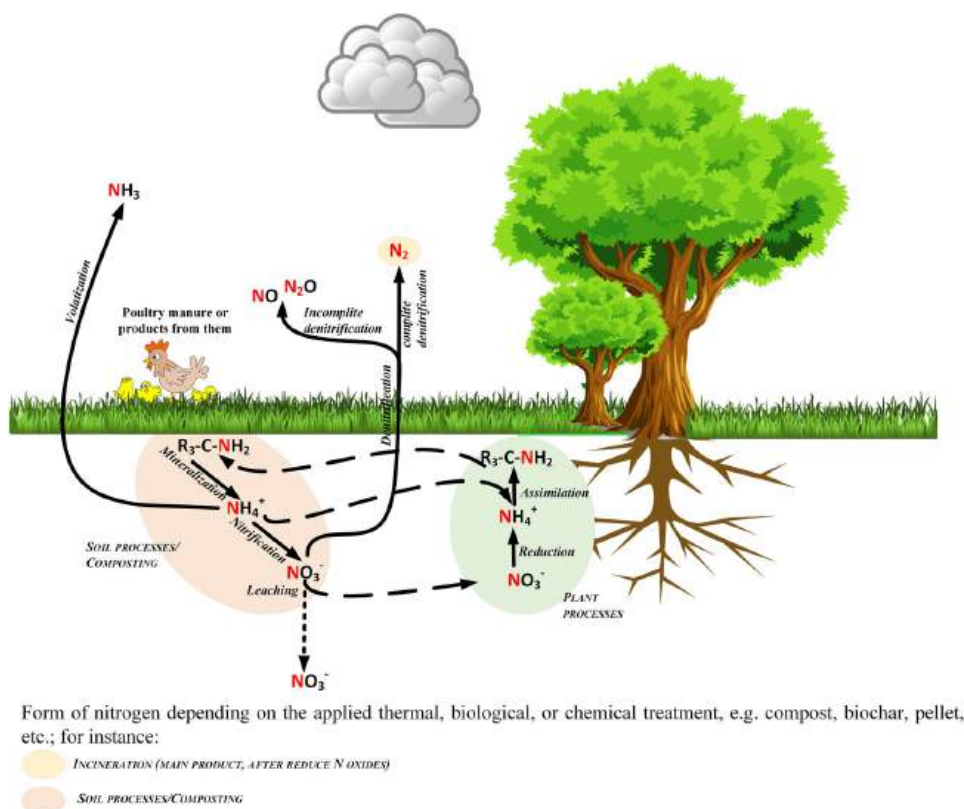


Figure 5. Transformation of nitrogen based on poultry manure in soil based on (Ros et al., 2020).

about 20% by decrease the interior temperature, thus slowing down the uncontrolled processes of organic matter decomposition (Bittman et al., 2014). The decrease of ammonia generation is also achieved by acidification of the liquid part of the manure, e.g. with sulfuric acid to a pH of 3.5-5.5.

During composting too fast or absent thermophilic phase and an unfavorable C:N ratio (range 40-50: 1) results in the formation of slow-acting organic combinations of nutrients. During this time, the mesophilic microflora develops excessively, which allows for the maintenance of the chemical activity of urease causing excessive ammonia emissions (Krawczyk & Walczak, 2016).

Ammonium ion (NH_4^+) and/or free ammonia nitrogen (FAN) are the main inorganic products of decomposition of organic nitrogen fraction in phase II – acidogenesis of anaerobic digestion (AD). The equilibrium state is established between the forms mentioned above of nitrogen, and their share in total ammonia nitrogen (TAN – sum FAN and NH_4^+) depends on the pH. As the pH increases, free ammonia nitrogen (FAN) increases. At neutral pH, the dissociated form prevails; only 0.5% of nitrogen is in the form of free ammonia, while at pH equal to 8, its (FAN) share increases to 5% (Deublein & Steinhauser, 2011). Accumulation of TAN in the digester can lead to inhibition of the process. The inhibition mechanism caused by ammonia can be related to: i) inhibition of specific enzymatic reactions (blocking enzymes); ii) pH imbalance inside the cell and thus the disturbance of the acid-base balance and/or potassium deficiency; iii) the accumulation of VFAs (there is an interaction between FA, pH and VFAs; disturbance of the process balance leads to accumulation of VFAs in the chamber, resulting in a decrease in pH and thus decrease FAN concentration). Moreover, ammonia inhibits the process of acetic acid uptake (Jiang et al., 2019, Fuchs et al., 2018). It is difficult to indicate the TAN limit value for inhibition of the AD of PM because it depends on the characteristics of feedstock, inoculum characteristics,

process conditions (temperature, pH), form of nitrogen (FAN is more toxic than ammonium ions, process inhibition for FAN is observed at concentrations higher than 45–100 mg l⁻¹ and for ammonium ions higher than 1700 mg l⁻¹) and a degree of adaptation of bacteria to TAN (generally, a gradual increase of ammonia concentration promotes adaptation to it). For this reason, a wide range of inhibiting TAN concentrations has been reported in the literature (from 1.7 g l⁻¹ to 14 g l⁻¹) (Duan et al., 2018; Reyes et al., 2021; Yin et al., 2021). However, it is important to note that adaptation of microorganisms to high ammonia concentrations can result in high ammonia concentrations in the digestate. According to Ortakci et al. (2019) the concentration of ammonia nitrogen in such a digestate can be higher than 5000 mg dm⁻¹.

4.3. Nitrous oxide and nitric oxide emission

Nitrous oxide (N₂O) and nitric oxide (NO) can be emitted as a result of nitrification where NH₄⁺ is a substrate for aerobic respiration or alternatively – denitrification when oxygen is limited and NO₃⁻ is used for anaerobic respiration as an electron acceptor by microorganisms.

N₂O has a global warming potential of 298 times that of CO₂ (IPCC, 2019) and can be the main GHG contributor (Ziganshina et al., 2015; Zhu et al., 2020). Meta-analysis results showed that PM considerably increased CO₂, CH₄, and N₂O emissions than pig and cattle manure (Shakoor et al., 2021). Soil application of PM during autumn season resulted in N₂O emission at the level of 1.52% total N applied (Thorman et al., 2020). Indirect N₂O emissions may arise from the re-deposition of volatilized NH₃ and NO_x onto nearby soil, providing a substrate for nitrification and denitrification. The important factor is C:N value (Charles et al., 2017). Nitrogen are lost to surface water via leaching of NO₃⁻ which is later denitrified (Thorman et al., 2020).

Moreover, many other factors influenced emission, such as soil properties (texture, drainage, bulk density, water holding capacity), SOC, N_{tot}, crop rotation, duration of the land management and climatic factors. Soil nitrogen cycle is closely related to soil C cycle and therefore increased SOC sequestration might even cause increase N₂O emission. The process can be mitigate by biochar or non-pyrogenic C amendment application (Janczak et al., 2017; Guenet et al., 2021).

4.4. Leaching and runoff lost

Nitrogen losses from livestock production through leaching and runoff loss can reach up to about 50%. Nitrogen losses through leaching occur mostly in the form of nitrates (NO₃⁻) which is favored by high soil moisture, heavy rainfall, excessive irrigation, and poorly developed root systems of plants. Uncontrolled losses of NO₃ to groundwater can be harmful to human health in dose higher than 50 mg l⁻¹ NO₃. Light soils, i.e. fawn, podzolic, and rusty podzolic soils, created on the basis of sands, are very permeable, airy, and quickly heat up, contributing to the rapid leaching of nitrates and their seepage into the soil profile and further into the groundwater. On the other hand, heavy soils, i.e. mainly clays and loams, poorly permeate water and oxygen to the deeper parts of the soil profile. They limit the leaching of nitrates, but plants growing on such soils have a problem with the properly growth through a too compact structure. A soil with a sand and clay fraction is optimal, which makes it good for plant growth and does not allow significant amounts of nitrogenous compounds to pass into groundwater (Soroko & Strzelczyk, 2009).

5. Phosphorus cycle

5.1. Forms of phosphorus in poultry manure

The content of phosphorus in PM is 2 to 4 times higher than in other manure and ranges from 13.6 to 25.4 g P₂O₅ kg⁻¹dm (Table S.6, [supplementary material](#)). Because in cereals and

others (barley, wheat bran, soybean, rapeseed meal), phosphorous occurs in form of inaviable phytin, hence, the phytic form is present in significant amounts in poultry excreta (Toth et al., 2011, Ashworth et al., 2020). Available phosphorus sources for birds are mineral supplements added to feed (Sharpley et al., 2007). The inorganic phosphorus in PM is in the range of 32-84% wt (Othake & Tsuneda, 2019) and organic in the range of 14-68% wt, with the potential to be mineralized to a plant-available form (He et al., 2008). Pagliari and Laboski (2014) found that the solid phase of phosphorus is not entirely organic, but is combination of minerals and organic compounds. The inorganic phosphate fraction includes calcium phosphate, dibasic calcium phosphate, and poorly bound water-soluble phosphorus (He et al., 2016). Both fractions of total phosphorus (orthophosphates and organic phosphorus) are essentially immediately labile. Phytates are an exception. Compared to other animal manures, poultry manure contains proportionally more of the stable form of phosphorus, which ranges from 22 to 58% of total phosphorus (Dail et al., 2007).

Between 12 and 20% of the phosphorus in PM is in a water-soluble form and is washed away in rainfalls after being applied to the soil (Guo et al., 2009). Combustion of poultry manure to produce nutrient-rich ash for agricultural applications is the most environmentally friendly methods (Fahimi et al., 2020; Kaikake et al., 2009). The phosphorus content in ash ranges from 8.3 to 13% and is comparable to that of some natural phosphate rocks (Desmidt et al., 2015). The amorphous phase of phosphorus in poultry ash is a source of a bioavailable form of phosphorus, and depends on the combustion temperature (Vance, 2019). The main mineral phase of the ash is potassium-sodium-calcium phosphate $(K,Na)Ca_2(PO_4)_2$ and in the form of calcium phosphates (hydroxyapatite and whitlockite) - from 8.8 to 14.8% P_2O_5 . Bioavailable forms in ash may achieve to 31% d.w. P_2O_5 of total content (Table S7, [supplementary materials](#)) Song and Guo (2012) demonstrated that the conversion of poultry manure to biochar by slow pyrolysis at $\geq 350^\circ C$ reduced the proportion of water-soluble phosphorus from 19.5% to less than 6.9%. This will help maintain a steady and long-term supply of nutrients in the soil preventing rapid nutrient loss during runoff. Such an effect can be achieved by converting poultry manure by pyrolysis into biochar and its use as a soil additive (Guo et al., 2020; Haider et al., 2021). Wang et al. (2015) found that pyrolysis immobilized the water-extractable phosphorus contained in raw PM by converting it into more stable, less available minerals (Mg and Ca phosphates) present in biochar (Figure S6, [supplementary materials](#)). Phosphorus content in biochar from PM was higher than that of unprocessed manure and increased with pyrolysis temperature from 3.391% in $200^\circ C$ to 6.38% in $500^\circ C$ (Table S8, [supplementary material](#)), Bavariani et al. (2019).

Experiments on sequential leaching of biocarbon from raw poultry manure produced at $600^\circ C$ showed that phosphorus was released slowly but in much higher (43.2 mg g^{-1}) amounts compared to other biochars of animal and lignocellulose origin (Table S9, [supplementary material](#)), (Hadroug et al., 2021). The total amount of released phosphorus was about 95% of its original content in the biochar.

Negassa and Leinweber (2009) evaluated the balance of nutrients (including phosphorus) in poultry litter used as a fertilizer for five different treatments: direct application on the ground; composting; composting and pelleting; co-digestion with feed corn; thermal conversion. Final products of all the treatment methods contained the same amount of phosphorus (in the form of P_2O_5) per Mg of processed litter. In the case of ash, the annual efficiency of P_2O_5 , depending on the crop, varies from 37% to even 100%. In contrast, it is around 70% for other products. The effectiveness of P_2O_5 , depends on the crop and soil type where poultry litter ash is used as a fertilizer. Figure S5 (Nusselder et al., 2020, [supplementary materials](#)) shows the fertilizer equivalents available in the first year to plants, delivered from 1 Mg of poultry litter, treated using various methods.

5.2. Poultry manure struvite

Struvite ($\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$), i.e. hydrated ammonium-magnesium phosphate can be an alternative source of phosphorus contain the two macronutrients (phosphorus and nitrogen) needed for plant growth and development. Struvite is formed by the following reaction (17):



According to low solubility (Rech et al., 2018) the fertilizing components are released slowly and the plants themselves stimulate the intensity of release which reduces the need for frequent fertilization. No leaching is observed, either into surface water or to deeper soil layers where phosphorus could become unavailable to plants. Struvite is as effective as compound fertilizer, providing plants with the right doses of nutrients N, P, K, Ca, and Mg (Krishnamoorthy et al., 2021; Zhang et al., 2020).

Struvite produced from the acidified PM ash extract after supplementing with Mg and adjusting the pH to about 8.5 contained 90% of the phosphorus originally present in fresh chicken manure (Poblete-Grant et al., 2020). The process yielded a multi-nutrient fertilizer composed of struvite- NH_4^+ , struvite - K, and significant amounts of potassium sulfate and hydroxyapatite, with a high content of macroelements (P, K, Mg, S), low content of microelements, and lower levels of heavy metals compared to fresh manure (Poblete-Grant et al., 2020).

Poultry manure is a good source of phosphorus for the production of universal agricultural fertilizer (Rivera et al., 2022; Sarvi et al., 2021). Yilmazel and Demirer (2013) confirmed the recovery of nutrients (N, P) by precipitation of struvite from poultry manure using HCl. However, it further led to the dissolution of metals along with nutrients and crystallization of other minerals along with struvite. It was also found that high concentrations of Ca^{2+} ions allow complete recovery of phosphorus from a solution due to calcium phosphate precipitation.

5.3. The mobility of phosphorous in environment

The biogeochemical cycle of phosphorus is very sensitive to agricultural and industrial activity, especially to poorly planned or implemented agrotechnical procedures that modify the natural P cycle (Figure 6).

The content of total phosphorus in soil ranges from 100 to 3000 mg P kg^{-1} (Sönmez et al., 2016), but is mostly in the range of 500-800 mg P kg^{-1} soil and depends on the type of bedrock, the degree of weathering, and the content of organic matter. This amount exceeds many times the average nutritional needs of plants. However, only 0.03-0.5 mg P kg^{-1} is actually available to plants, as most of the phosphorus binds in the soil with various chemical forms of other elements (e.g. 2- and 3-valent metal cations, i.e. Mg, Ca, Fe and Al), forming hardly soluble compounds. Phosphorus content decreases with the depth of the soil profile. The active phosphorus in the soil solution is phosphorus in the form of phosphate ions (H_2PO_4^- , HPO_4^{2-}), taken up directly by plant roots. When the amount of active phosphorus decreases due to intensive plant uptake, mobile phosphorus is converted to its bioavailable form, making up for the deficiency. Another form of phosphorus is reserve phosphorus in the form of hard to dissolve minerals, i.e. apatite, phosphate rock and variscite (Mulqueen et al., 2004). Therefore, the phosphorus cycle in the soil is influenced by its biological activity and high enzyme production/activity associated with organic phosphorus hydrolysis. The following extracellular phosphatases are mainly involved in phosphorus metabolism in the soil: acid phosphomonoesterase, alkaline phosphomonoesterase, and phosphodiesterases, (Waldrip et al., 2011). Manure application increases the concentration of total and soluble phosphorus in the soil and the concentration of stable organic groups of this element (Waldrip-Dail et al., 2009).

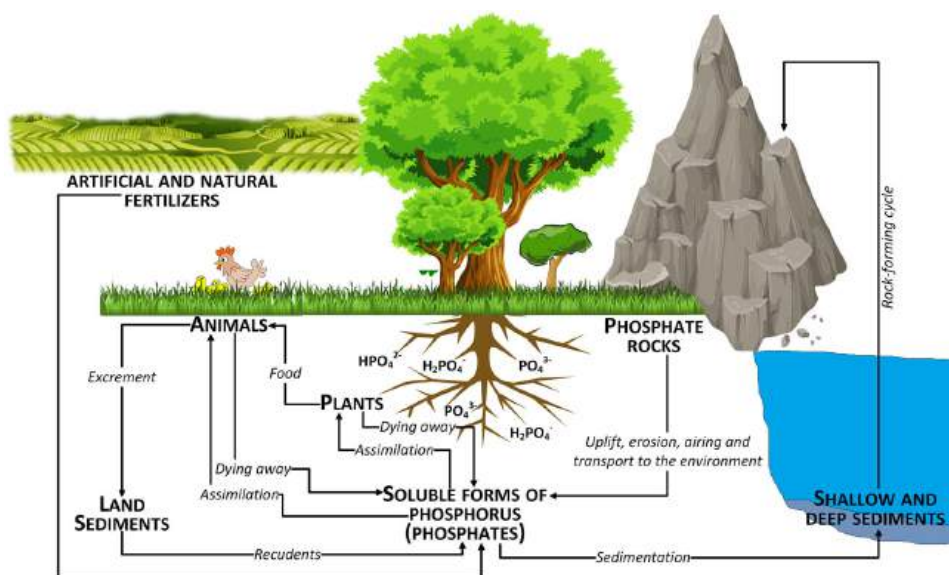


Figure 6. The cycle of phosphorus in environment based on own study.

Depending on the form of occurrence (type of binding) and variation of soil and water conditions (i.e. soil reaction, the organic matter, Fe and Al), phosphorus may be released to soil solution and ground water and as a consequence, through leaching, move deep into the soil profile or undergo the process of leaching to surface waters (McDowell et al., 2011). The following mechanisms are responsible for phosphorus transfer from soil to water: i) chemical dissolving; ii) physical separation and removal of soil particles through erosion; iii) incidental migration from soil to water after application of phosphorus fertilizer or manure in conditions that are not conducive to its permeating into the soil structure, (Roy, 2017).

The release of phosphorus from the soil in the presence of organic carbon, is associated with leaching of its soluble forms into groundwater which with high mineralization efficiency and low phosphorus utilization by plants results in contamination of groundwater (Figure S7, supplementary material). Phosphorus bound to mineral and organic soil particles accounts for 60 to 90% of phosphorus transported through surface runoff. Dissolved forms include phosphorus desorbed from the soil, leached from plant residues, and released from sources such as mineral fertilizers and manure. In catchments in agricultural areas, the level of phosphorus leaching may be about 0.5 kg ha^{-1} . The process of phosphorus leaching is a type of the flow through privileged pathways of underground flow, i.e. the networks of regular cracks and macropores, huge cracks in the soil, chasms and channels carved by the soil fauna, root channels and wedges, lenses and layers that are characterized by improved filtration parameters than other parts of the soil. These migration pathways are responsible for the intensity and time of solution transfer (including phosphorus) during precipitation and thawing (Weiler & McDonnell, 2007).

Excessive or inappropriate application of manure and litter can result in increased accumulation of phosphorus in the soil and subsequent increase in surface runoff, leading to eutrophication of reservoirs, (Tiessen et al., 2011). Transport of phosphorus contained in PM after its application in the soil shown that dry mass of the manure decreased over time, but the concentrations of phosphorus in the manure were essentially constant over time, meaning that it was removed from the manure at the same rate as it decomposed Vadas et al. (2007). Numerous studies have also examined the effects of drying conditions on phosphorus content in PM (He et al., 2006). For broiler manure, freeze-drying or oven-drying (105°C) yielded lower concentrations of

inorganic and total phosphorus than for air drying (Akinremi et al., 2003). The drying process can cause the relatively stable phosphorus in PM to shift to more labile, or conversely, insoluble forms (Dail et al., 2007). Crops are unable to absorb the high doses of phosphorus contained in PM, so the excess accumulates in the soil. The P availability for plants can be increased by mixing with calcium-rich opoka rock (Kacprzak & Sobik-Szoltyssek, 2022). Other possibility is adding sulfur-phosphate mixtures. This results in the oxidation of sulfur to sulfuric acid through the activity of *Thiobacillus spp.* bacteria. The reaction with acid led to the partial decomposition of phosphates and the formation of mono- and dicalcium phosphates, which are more readily available to plants (Figure S7, [supplementary material](#)).

6. Conclusion

Poultry manure is historically used as a fertilizer, however positive impact of application on the increase of the plant yield or the improvement of soil properties depends on many factors, such dose, soil type or temperature. Generally, increase of organic matter content, nitrogen ammonification, pH value, content of P, K, Cu, Mn, Fe and Zn available to plants is noted. However, PM can affect enzyme activity and introduce contaminations (e.g. pathogens; antibiotics, heavy metals). Moreover, ammonia, NO_x emission or unbalanced C:N ratio in soil poses serious environmental problem.

Technological processes of PM has both positive and negative effect on carbon, nitrogen and phosphorous transformations and gases emissions. During aerobic processes (composting) nitrogen is lost by leachate of NH₄⁺, NO₃⁻ or by emissions of NH₃, N₂O, N₂; carbon by emissions of CO₂, CH₄ and phosphorus by the leachate in the form of HPO₄²⁻, H₂PO₄, respectively. In turn, the main challenge of anaerobic digestion is optimization toward high methane production. PM is not an easily biodegradable substrate, hence modification of technological parameters and co-substrates additives is required to increase the process efficiency and to obtain a digestate can be used as valuable fertilizer. Finally, thermal treatment requires energy for PM drying and monitoring of specific emissions. However from other hand products resulting from thermal processes have great potential for carbon (biochar) and phosphorous (ash) long-storage.

Regardless of the technology used, PM can be a suitable substrate for the production of valuable products that have a positive impact on the environment.

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D.3

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Review

Environmental Impacts and Contaminants Management in Sewage Sludge-to-Energy and Fertilizer Technologies: Current Trends and Future Directions

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Abstract: The increasing focus on sustainability and the circular economy has brought waste-to-energy technologies to the forefront of renewable energy research. However, the environmental impacts and management of contaminants associated with these technologies remain critical issues. This article comprehensively reviews the environmental impacts of converting sewage sludge into energy and fertilizers, focusing on managing potential contaminants and assessing the implications and ecological risks. It also highlights the latest trends in waste-to-energy technologies, waste-to-soil amendment, and their integration into circular economy frameworks. The discussion encompasses challenges and opportunities in optimizing these processes in wastewater treatment plants to minimize pollutants and enhance sustainability. Addressing these challenges is essential for ensuring the long-term viability and acceptance of waste-to-energy solutions, making this topic highly relevant and timely.

Keywords: environment; emerging contaminants; waste to energy; wastewater treatment plants; sewage sludge; green transformation; risk assessment



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1. Introduction to Sewage Sludge

Sewage sludge is an integral part of the operation of wastewater treatment plants, and the amount of sludge is increasing every year as a result of the development of municipal infrastructure, the sewerage network, and the increase in the capacity of the treatment plants themselves [1]. Sludge is generated by treating domestic and industrial wastewater delivered to the wastewater treatment plant by the sewerage system. Its composition varies significantly, depending on factors such as the degree of industrialization of the city, the number of residents, average water consumption, and seasonal cycles, among others. Thus, the final composition of rural and urban sewage sludge can differ significantly [2]. Being organic–mineral and solid, sludge is a rich source of many biogenic elements and is a good raw material for further management [3]. Most wastewater treatment plants are described as producing raw, untreated sludge with a characteristic strong odor, primary sludge resulting from the sedimentation of suspended solids (in primary settling tanks), secondary sludge formed after biological treatment of wastewater (usually in activated sludge flow devices), and mixed sludge resulting from a combination of primary and secondary sludge.

Considering the high water content, and possible micropollution by heavy metals, microplastics, parasite eggs, or pesticides, it is necessary to properly process the sludge further to ensure that it becomes environmentally safe [4]. Directions for the management of finished sludge are strictly standardized by law, whether for use in agriculture, soil remediation, thermal treatment, or temporary storage [5,6].

The introduction of regulations in Europe for the sustainable management of non-renewable resources and waste management aims to ensure their efficient processing in an environmentally safe manner. Achieving climate neutrality regarding greenhouse gas emissions and efficient use of secondary resources is also a major goal of the European Green Deal. One of the proposed solutions is using sewage sludge in agriculture, due to its high content of elements such as nitrogen, phosphorus, potassium, and calcium, while maintaining low levels of heavy metals and pathogens. The use of sludge can enhance soil fertility, porosity, aggregate stability, microbial biomass, and biological properties of the soil [5]. Thus, sludge treatment has therefore become crucial for its reuse. An ecological approach begins at the sludge drying stage, where standard procedures like thermal or mechanical drying are energy-intensive and contribute to fossil fuel consumption and greenhouse gas emissions. Therefore, solar drying is proposed as a solution, despite the longer processing time [7,8]. For many authors, the main obstacle to the use of sludge in agriculture is the content of heavy metals such as cadmium or copper, and, as they point out, the degree of toxicity is determined by their speciation rather than the concentration itself [9]. Heavy metals are not biodegradable, so repeated fertilization of cultivated soil with sludge leads to accumulation and increased ecotoxicity, negatively impacting soil fertility. Heavy metals can eventually contaminate surface and groundwater, entering the human body [10,11]. In recent years, many effective methods have been proposed for removing heavy metals from sewage sludge, such as ion exchange extraction, thermal conversion, chemical treatment, and electrokinetic techniques, with conversion considered an effective method for reducing the bioavailability of heavy metals in plants [12].

Despite the diversity of wastewater treatment plant designs, the regulatory-based process sequence looks similar in most cases. Typically, wastewater flows into the treatment plant via interceptors from different parts of the region and combines in a connection chamber to enter the treatment plant via a collection pipeline. The first stage involves using rare earth grids to remove larger contaminants such as tampons, stones, and bottle caps, and from there, the wastewater is directed to a pool equipped with pumps that take it to a higher level so that further treatment processes can occur by gravity flow. Some treatment plants have two such basins that can be directly accessed by pumps to retention tanks in case of heavy rains or wastewater accumulation. The next stage of filtration is dense sieves with a mesh diameter of 0.5 cm where smaller contaminants are retained; these, in turn, are transported by feeder to the operation, which recovers excess water and reduces their volume significantly. The pre-treated wastewater is directed to a sand pit, where the flow rate is about 0.3 m/s to retain fines and grease effectively. Thus, prepared wastewater goes to settling tanks, where the organic fraction is separated, from which sludge will be formed [13].

Biological treatment is associated with removing phosphorus and nitrogen compounds from wastewater, which can lead to environmental eutrophication. An effective method is using a Bardenpho bioreactor, where the levels of these elements are reduced in the anaerobic, hypoxic, and aerobic zones [14]. The first anaerobic stage occurs in a chamber equipped with an agitator to maintain the activated sludge in suspension. Under anaerobic conditions, phosphorus bacteria get rid of phosphorus accumulated in their cells, and the energy gained allows them to take up and store acetic acid as a spare substance. In the aerobic chamber, the bacteria again take up phosphorus, however, in a much larger amount than in the anaerobic process, and by removing the final biomass from the wastewater, the phosphorus level in the treated wastewater is significantly reduced. The nitrogen removal process is similar and uses the processes of nitrification and denitrification to make the bacteria that take oxygen from nitrates release pure nitrogen into the atmosphere. After leaving the bioreactor, the wastewater flows to a settling chamber, where activated sludge is separated, and the treated wastewater can be discharged into a river or a retention basin. Activated sludge is often reused in the bioreactor for nitrogen and phosphorus removal processes [14].

In the chemical treatment of wastewater, iron sulfate is most often used, which, reacting with phosphates contained in sewage, leads to the formation of chemical sludge, reducing the amount of phosphorus. This process supports biological filtration, increasing the amount of final sludge. The resulting sludge can have many avenues of application, depending on its chemical composition; after appropriate mechanical and thermal treatment, it is thickened and dehydrated. Quicklime can assist in dewatering, which further reduces the amount of sludge. After leaving the dewatering centrifuges, the sludge can be stored or used in this form for biofuel production. Application in agriculture requires additional qualitative and quantitative analyses [15,16].

European Trends in Sewage Sludge Generation

The scale of sludge production is upward, but estimating exact quantities seems difficult. Water consumption from 2015 to 2030 will increase by 24%, and by 2025 by 51%, sewage networks in Europe have an estimated length of 3.3 million kilometers, an increase of 10% in recent years. In addition, the increase in the urban population and the decrease in the rural population should be taken into account; this together translates into the amount of sludge produced [17]. It is also important to mention the rapidly growing market of Asia, and China, which has built more than 39,000 treatment plants since 2020 and plans to renovate 80,000 km of sewer pipes, which is expected to translate into an increase in treatment capacity by 7.3 cubic kilometers of wastewater by 2025 [3,18]. In total, production is probably led by 12–18 million tons per year, India 10–15 million tons, Russia 5–10 million tons, the European Union 12–14 million tons, Japan 2–3 million tons, and the UK 1.6–1.8 million tons [4]. In China, 29.3% of municipal sewage sludge was treated by agricultural route, 26.7% by incineration, and 20.1% by sanitary landfill. The dominant technologies were incineration, composting, thermal hydrolysis, and anaerobic digestion, with the capacity of 27,122 t/d, 11,250 t/d, 8342 t/d, and 6944 t/d, respectively [3]. In the USA, nearly 43% of these biosolids were applied to land, while the rest were either disposed of in landfills, incinerated, or handled through other means such as storage or deep well injection [19]. In the European Union, the vast majority of sludge is incinerated (25%), used in agriculture depending on the law (27%), composted (21%), or stored periodically or permanently (9%). As studies indicate, sludge incineration leads to the formation of ash, which often contains metals, and the cost of disposal is high [20].

In Europe, the total reported amount of sludge generated in 20 selected EU countries was 6464.37 thousand tons in 2014, which then increased to 6792.94 thousand tons in 2018. The year 2021 already shows a total decline, which is difficult to interpret, but this result is significantly influenced by the lack of reporting data for Spain. In 2021, we had a declared decrease in EUROSTAT data to 5451.98 thousand tons. The decline in sewage sludge production may also reflect the impact of improved environmental policies and technological advances in wastewater treatment, which focus on waste reduction and improved plant efficiency and recovery of possible nutrients (Figure 1).

In addition, there is a global trend in sewage sludge management towards a circular economy, such as resource recovery from sludge and production of energy and fertilizers, which can contribute to reducing the overall volume of sludge. Differences in trends between countries underline the variability of policy implementation, population and demographic changes, adaptation of technologies to new climate and energy challenges, and investments in wastewater infrastructure across Europe.

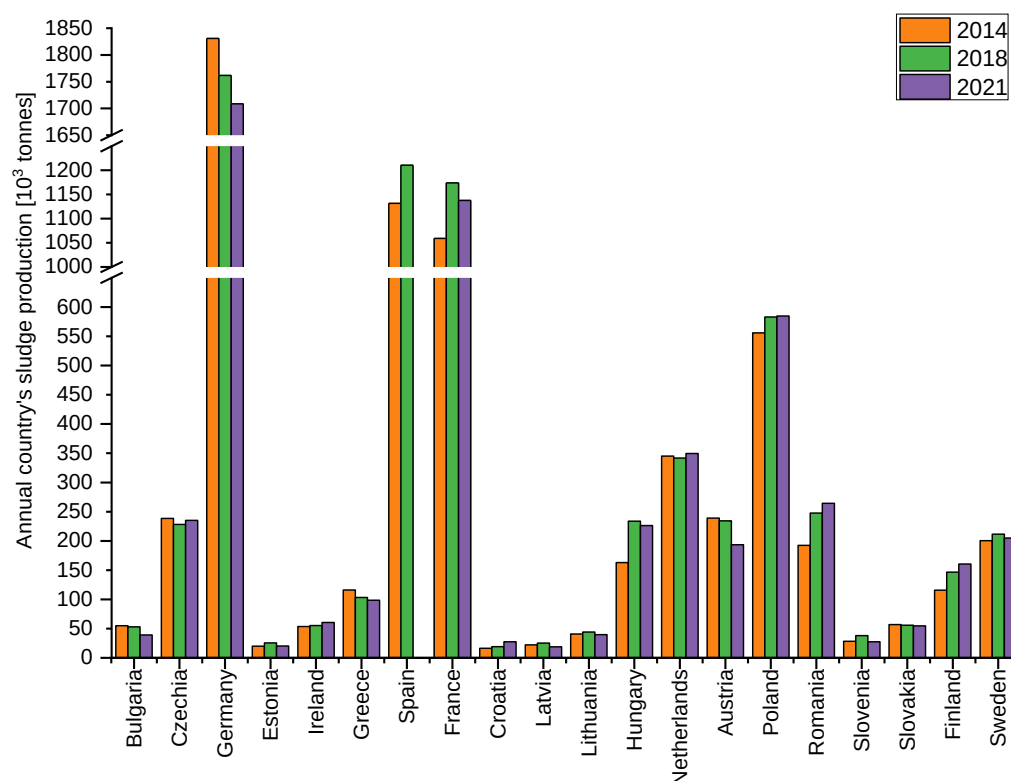


Figure 1. EU selected countries' sludge production in thousand tons from urban wastewater treatment plants in 2014, 2018 (2017 for France, no data for 2018), 2021 (2022 for the Netherlands, no data for 2021) (Based on Eurostat data); total reported amount (2014) 6464.37, 2018 (6792.94), 2021 (5451.98) thousand tons from 20 EU countries [21].

2. Sewage Sludge Treatment and Reuse Technologies—Need for Development in the Context of Circular Economy, Sustainable Development, Human Health, and Energy and Fertilizer Saving

Sewage sludge treatment and reuse technologies are essential to modern waste management systems. Currently, these technologies stabilize sludge, reduce its volume, and remove harmful contaminants, making it safe for reuse or disposal. Commonly used methods include anaerobic digestion, thermal hydrolysis, composting, landfilling, and advanced oxidation processes [22,23]. These techniques enable the extraction of valuable resources such as biogas, fertilizers, and water, which can be repurposed for various applications [24,25]. However, developing these technologies is necessary to enhance efficiency, increase resource recovery rates, and address emerging environmental and health challenges. By advancing sludge treatment and reuse technologies, we can better support sustainable development goals, protect public health, and promote energy and resource conservation.

Walter's definition of a circular economy (CE) states that "A 'circular economy' would turn goods that are at the end of their service life into resources for others, closing loops in industrial ecosystems and minimizing waste" [26]. It is crucial to fully integrate the CE with sustainable development, ensuring that they share common goals. This integration necessitates a comprehensive rethinking and broadening of CE's focus beyond mere recycling and short-term economic benefits. To contribute to sustainability, CE must evolve into a holistic economic model that enhances social well-being and environmental quality [27]. In this context, sewage sludge treatment and reuse technologies are indispensable, requiring a focus on environmental sustainability and social well-being.

One of the primary benefits of these technologies is the recovery of valuable materials from sludge, such as phosphorus (P), nitrogen (N), and organic matter. Phosphorus, for example, is a critical component of agricultural fertilizers and is essential for food production.

However, natural phosphate rock reserves are finite and depleting. Technologies such as struvite precipitation allow for phosphorus recovery from sludge, which can then be used as a fertilizer. In addition to struvite precipitation, thermal treatment technologies, such as incineration, pyrolysis, and thermal hydrolysis, are employed to recover phosphorus and reduce the volume and toxicity of sewage sludge. These methods enhance resource recovery while minimizing environmental impact [28–30]. Thanks to the development of technology and process optimization, it is possible to recover phosphorus much more effectively and, consequently, to use it further. For example, struvite precipitation, which is a commonly used method for phosphorus recovery, can be improved in terms of efficiency. Further development of low-energy recovery systems and scaling these technologies will be key to making phosphorus recovery more viable and cost-effective. This provides a sustainable source of phosphorus and reduces the environmental impact of mining activities.

Moreover, the treatment and reuse of sewage sludge can contribute significantly to reducing greenhouse gas emissions. For instance, anaerobic digestion of sludge produces biogas, a renewable energy source [25,31,32]. The use of biogas can replace fossil fuels, leading to a reduction in carbon dioxide emissions. The development of biogas technology and improvement of this process is necessary to meet the growing demand for energy and mitigate climate change [33,34]. Additionally, the application of treated sludge in agriculture can enhance soil carbon sequestration, further mitigating climate change [35]. Examples from various regions highlight the potential of these technologies. In Denmark, for instance, nearly all sewage sludge is treated and reused, primarily as fertilizer in agriculture. This not only closes the nutrient loop but also supports sustainable farming practices [36]. Lessons learned from these examples emphasize the importance of regulatory frameworks and public acceptance. Robust regulations ensure the safe and sustainable application of treated sludge, while public awareness campaigns can foster acceptance and support for these practices. Collaboration between stakeholders, including governments, industry, and communities, is crucial for successfully implementing sludge treatment and reuse technologies.

Sewage sludge, if not properly treated, can pose serious health risks due to the presence of pathogens, heavy metals, organic pollutants such as pharmaceuticals, parabens, and microplastic [24,37]. Therefore, it is necessary to constantly develop and improve sewage sludge treatment technologies to minimize the release of these pollutants into the natural environment and to minimize exposure to the toxic effects of these pollutants for humans. The modern treatment processes aim to reduce or eliminate these contaminants, making the sludge safe for reuse.

Pathogens in untreated sludge, such as bacteria, viruses, and parasites, can cause diseases if they enter the food chain or water supplies [38]. Treatment technologies like thermal hydrolysis, composting, and advanced digestion processes significantly reduce pathogen levels, ensuring the safe reuse of sludge [39]. For example, composting stabilizes organic matter and generates heat, which destroys pathogens, resulting in a safe and nutrient-rich soil conditioner.

Heavy metals, including cadmium, lead, and mercury, can accumulate in the food chain and pose long-term health risks. Advanced treatment methods, such as chemical precipitation and adsorption, can effectively remove heavy metals from sludge [40,41]. Ensuring that these metals are below permissible levels before applying sludge to land is crucial for protecting human health.

Pharmaceuticals and microplastics in sewage sludge also present significant concerns for human health. Residues of pharmaceuticals, including antibiotics, hormones, and other drugs, often persist through wastewater treatment processes and can accumulate in sewage sludge [24,42]. When this sludge is used in agriculture or disposed of improperly, these substances may enter the food chain or water sources, potentially leading to adverse health effects such as antibiotic resistance and hormonal disruptions. Similarly, microplastics—tiny plastic particles that result from the breakdown of larger plastic debris or are introduced through personal care products and industrial processes—are increasingly

found in sewage sludge. These particles can carry harmful chemicals and pathogens, and their environmental persistence risks human health. Microplastics in the soil can enter the food chain through crops, and their potential effects on human health are a growing area of concern [43,44]. Addressing these issues requires advanced treatment technologies and regulatory measures to effectively remove or mitigate the presence of this micropollutant in sewage sludge. Enhanced treatment processes, such as advanced oxidation, activated carbon adsorption, and membrane filtration, are essential to reducing these contaminants. Developing and implementing robust guidelines for sludge management and disposal can also help minimize their impact on human health.

In addition, emerging contaminants, such as PFAS (per- and polyfluoroalkyl substances), present a growing challenge, requiring adaptation of existing treatment technologies or the development of new approaches. PFAS are highly persistent in the environment and resistant to conventional treatment processes, making their removal difficult [18]. To address this, the development of targeted treatment methods is essential. Innovative solutions such as PFAS-specific adsorption techniques, using advanced materials like engineered activated carbons or biochar, as well as destruction technologies like plasma treatment, AOPs, and electrochemical degradation, will be critical. These methods can effectively break down or capture PFAS compounds, preventing their accumulation in agricultural soils and mitigating potential risks to the environment and human health. Additionally, integrating these solutions into existing sewage sludge treatment systems will be vital to ensure comprehensive contaminant removal while maintaining the efficiency of resource recovery processes [18].

Sewage sludge contains significant organic matter, making it an excellent feedstock for energy production through various technologies. Anaerobic digestion is one of the most widely used methods, stabilizing the sludge and producing biogas—a renewable energy source. Biogas can be used to generate electricity and heat or upgraded to biomethane as vehicle fuel [45]. Optimizing anaerobic digestion processes to improve biogas yields while reducing emissions is crucial. Incorporating combined heat and power (CHP) systems can help maximize energy recovery from sludge. Furthermore, integrating renewable energy sources with these technologies could reduce reliance on fossil fuels, lowering the overall carbon footprint. Other technologies for energy recovery include thermal treatment methods like incineration, pyrolysis, and gasification. These processes convert organic material into heat, electricity, and syngas. Incineration reduces sludge volume and produces ash that can be further utilized, while pyrolysis and gasification offer the potential to produce cleaner energy forms with fewer emissions. Improving these technologies is crucial for maximizing energy recovery and efficiency. Emission control remains a priority for technologies like pyrolysis, gasification, and incineration. Innovations such as advanced flue gas cleaning systems can significantly reduce air pollutants like nitrogen oxides (NO_x) and sulfur oxides (SO_x), volatile organic acids, CH_4 , and others. Additionally, optimizing these processes, to reduce the production of ash and other byproducts, can lessen their environmental impact. The use of sewage sludge for energy purposes reduces the need for its storage and, at the same time, reduces the burden on the environment by reducing greenhouse gas emissions and other pollutants. Enhancements could include optimizing anaerobic digestion conditions, integrating combined heat and power (CHP) systems, and developing more efficient thermal processes. Advancements are necessary to increase the economic viability and environmental sustainability of energy recovery from sewage sludge, meet rising energy demands, and contribute to climate change mitigation efforts. Also, extracting nutrients from sewage sludge for use as fertilizers presents significant energy savings and environmental benefits [39]. The production of synthetic fertilizers is energy-intensive, involving mining raw materials and chemical processes. Recovering nutrients like nitrogen and phosphorus from sludge reduces the need for artificial fertilizers and their associated energy consumption. Technologies such as struvite precipitation and advanced nutrient recovery systems extract these valuable nutrients. Using treated sludge as a soil amendment also improves soil health, enhancing its ability to retain water and

nutrients. This can lead to better crop yields and reduced need for additional fertilizers. The holistic approach of using treated sludge in agriculture supports sustainable farming practices and conserves energy. Advancing fertilizer recovery technologies involves refining processes to increase nutrient recovery rates and ensure the safety of the end products. Research and development efforts should focus on improving the efficiency and scalability of these technologies. Additionally, policies and incentives encouraging the use of recovered nutrients in agriculture can drive wider adoption and reduce reliance on synthetic fertilizers [46].

Developing and implementing sewage sludge treatment and reuse technologies are critical for advancing the circular economy (CE), protecting human health, and achieving energy and fertilizer savings. These efforts promote resource efficiency, reduce environmental impacts, and support resilient agricultural systems, contributing significantly to sustainable development goals. To fully understand and optimize the environmental and health impacts of sewage sludge-to-energy and fertilizer technologies, life cycle assessments (LCAs) should be incorporated into the design and operation of these systems. This can help identify key stages in the process, where improvements can be made to reduce energy consumption, emissions, and contaminant levels.

3. Anaerobic Digestion and Biogas Production

Anaerobic digestion is considered an effective and environmentally friendly technology that allows for the utilization of sewage sludge and the production of significant amounts of biogas which contains up to 75% methane [47]. Therefore, improving this technology contributes to implementing the assumptions of the closed-loop economy and, consequently, reducing the amount of waste generated. This process stabilizes sewage sludge, reduces pathogens, and produces biofuel with high potential for use as a renewable energy source. To improve the process, pre-treatment of sludge is increasingly used, which allows for enhanced methane production and efficiency of substrate decomposition in biodegradation processes (Figure 2). Moreover, this results in a reduction in solid mass [48].

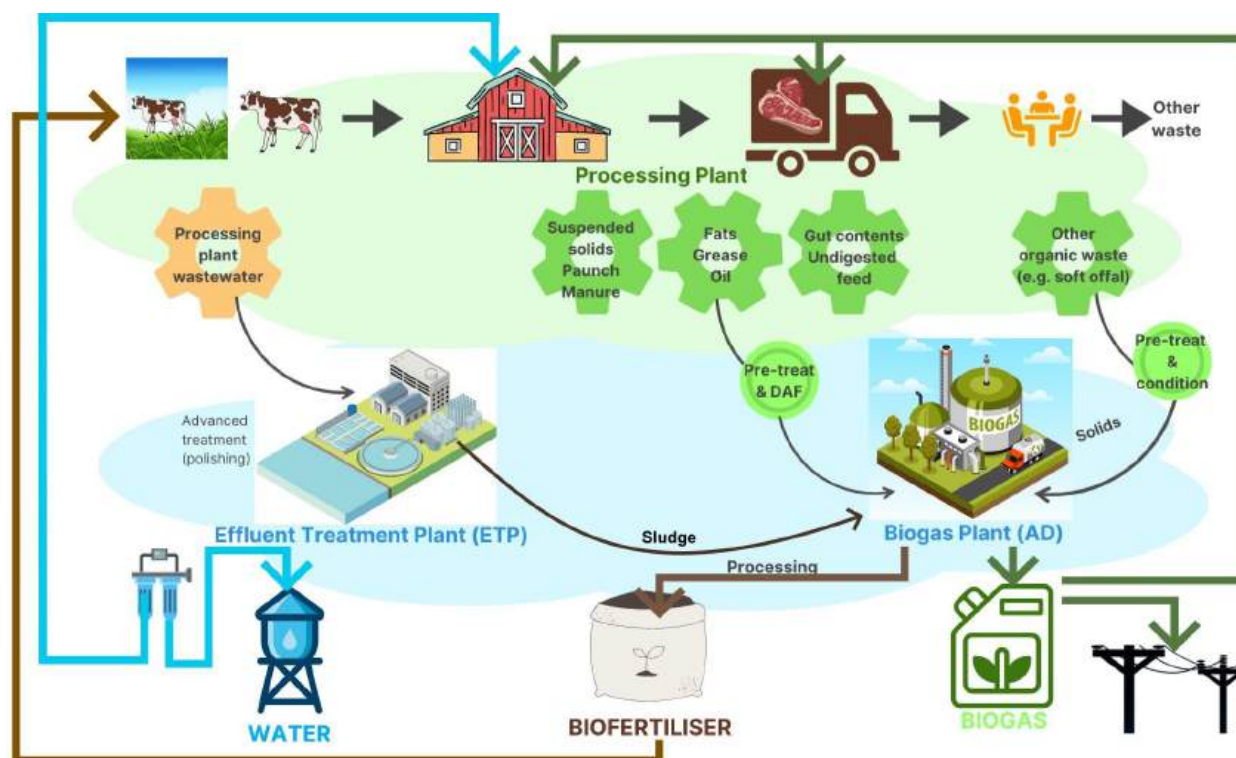


Figure 2. Stages of biogas production in organic waste-to-energy concept.

Improving wastewater treatment processes has often led to expanded biogas production from sewage sludge. One of the flagship treatment plants using organic substrates, such as food waste in fermentation, is the East Bay Municipal Utility District (EBMUD) in Oakland, California. The amount of renewable energy produced by this treatment plant is higher than the demand for this source (Home: East Bay Municipal Utility District, n.d.). The United States Environmental Protection Agency and EBMUD pilot project confirmed that implementation of food industry waste increased the methane potential and generated enough energy to power 1400 houses from biogas obtained from processing 100 tons of food waste [49].

Another technologically advanced wastewater treatment plant is the Blue Plains Advanced Wastewater Treatment Plant in Washington DC with an average treatment capacity of 1.45 million m³ per day [50]. It uses hydrothermal carbonization (HTC) technology which leads to the formation of hydrochar. This effectively increases the amount of energy produced by improving the quality of substrates. Additionally, a reduction in fermentation inhibitors is observed. HTC is a treatment that transforms organic waste in process water into biogas with the optimal temperature being in the range of 180–200 °C [51]. Analyses conducted by Aragón-Briceño et al. [52] showed that the use of hydrothermal carbonization in sewage sludge processing leads to obtaining 10 times more energy than in conventional fermentation. In Sweden, anaerobic digestion of sewage sludge contributes to over 0.7 TWh of biogas annually. The pasteurization process is often used in sewage treatment plants due to the effective increase in dissolved organic carbon (DOC) concentrations and generally increasing methane potential [53].

Research conducted by Ahn (2021) confirms the effectiveness of mechanical pretreatment of sewage sludge with subsequent low-temperature treatment in increasing biogas production. The high potential of using this method also results from the acceleration of the biodegradation rate [54].

Microplastics, according to modern terminology, are defined as plastic particles with a diameter of less than 5 mm, which enter the environment in an uncontrolled manner, and their amount is closely related to the number of inhabitants, population density, and environmental awareness [55]. Modern measuring equipment can detect and characterize plastic particles below 10 µm. Still, the constant modernization of the plastic production process and the enrichment of plastics with dyes, resins, or stabilizers make it difficult to determine the origin of each particle tested [15]. Based on current publications, it can be concluded that the efficiency of wastewater treatment plants in removing microplastic ranges from 16.5% to 98.4% for primary treatment and 78.1% to 100% for secondary treatment [56]. Current wastewater treatment technologies partially remove contaminants such as microplastics (MPs) but are not 100% effective. Microplastics are mainly removed during primary and secondary treatment stages, where physical processes such as sedimentation and filtration allow for the retention of about 80–90% of MPs in sewage sludge. Primary treatment removes 78–98% of MPs, while secondary treatment reduces MPs by only 7–20%, and tertiary treatment has little effect on microplastic removal. However, these treatments are not specifically designed to remove microplastics, but mainly to retain them in sewage sludge, leading to their accumulation in sewage sludge, which is often used as fertilizer in agriculture, posing a risk to the environment. The efficiency of microplastic removal is influenced by various factors, such as sewage flow, temperature, and type of treatment. Studies have shown that anaerobic digestion and other sludge treatment methods may not effectively break down MPs. Almost 100% efficiency of microplastic removal in anaerobic digestion process is achieved only for microplastics derived from natural, biodegradable polymers such as PLA. The article emphasizes that although sewage treatment plants are the last barrier against MP particles entering the environment, there is an urgent need to develop standard protocols and conduct further research to optimize MP particle removal and reduce their impact on the environment [57].

Most microplastic removed by conventional means is ultimately trapped in sewage sludge. In many countries, there are no standards for the amount of plastic in the volume

of sludge to be allowed for soil fertilization [54]. Thus, the study showed that in the studied glen, which was regularly fertilized with sludge, the number of detectable particles increased from an average of 4 pcs/kg to 542 pcs/kg (North America, New York area) and in other samples detected 71,000 pcs/kg in Denmark, 10,200 pcs/kg in Chile, 1900 pcs/kg in Mexico, and 38 pcs/kg in Iran, among others. A comprehensive analysis of more than 150 sites worldwide further proved that microplastic concentrations in industrial areas were four times higher than others, in the case of sewage sludge by up to 70 times. The highest documented concentration of microplastic in sludge-fertilized soil was described in China, in the Wuhan area, and was 690,000 pcs/kg, with more than 82% of the particles being less than 250 μm in size and including film fines and synthetic fibers [58].

Sewage sludge processing technologies differ in their efficiency in removing specific microplastics. Composting of sewage sludge leads to the removal of polyethylene (PE) at a level of 13%. In the case of hyperthermophilic composting of sewage sludge, the results are more diverse depending on the type of microplastics—for PHA (polyhydroxyalkanoates) degradation is 29%, for PVC (polyvinyl chloride) only 3%, while for PS (polystyrene) it is 43.7%, which leads to a significant reduction in these microplastics. Traditional composting of sludge with green waste shows lower efficiency, especially for polystyrene, the degradation of which is only 4.5%. On the other hand, composting with the addition of biochar from animal manure results in a reduction in PHA at a level of 50%. Aerobic processes used for sewage sludge lead to low degradation of PE (4.1%) and PLA (polylactide) (3.7%), but are more effective for PCL (polycaprolactone), which is degraded by 34.8%. Vermicomposting is particularly effective in removing PP (polypropylene) and HDPE (high-density polyethylene), with degradation rates of 22–78% and 31–56%, respectively. However, the highest efficiency is demonstrated by anaerobic digestion at 55 °C, with degradation rates of 90%, 80%, and 75%, respectively, for biodegradable microplastics such as PHB (polyhydroxybutyrate), PCL, and PLA [5,18,55].

Screening studies of sewage sludge show that sewage sludge contains significant amounts of microplastics, among which fibers and fragments dominate (Table 1). In terms of polymer types, polyethylene (PE), polypropylene (PP), and polyethylene terephthalate (PET) are predominant, originating mainly from consumer products. We know more and more about the potential use of sewage sludge as fertilizer for agricultural soils. Unfortunately, microplastics remain in these soils, infiltrating plants and soil organisms. It turns out that microplastics migrate horizontally to adjacent fields and penetrate deep into the soil profile [59]. Agricultural application of microplastic-rich sewage sludge leads to further unchecked contamination. It should also be noted that the sewage sludge currently produced has significantly different parameters than those used 20 or 30 years ago. Currently, consumers have different preferences, and the market offers many synthetic products. As a result, the consumption of synthetic plastic products compared to natural products in cosmetics and everyday items is dominant compared to previous decades. The majority of microplastics from wastewater are captured in sewage sludge; therefore, the concentration of microplastics in sewage sludge is expected to increase in the coming years.

Due to the composition of sewage sludge, effective technologies should be used to reduce pollutants in the digestate. The presence of compounds such as naphthalene, polycyclic aromatic hydrocarbons (PAHs), toluene, nitrobenzene, or the presence of pathogens, filamentous bacteria, and heavy metals, affects the quality and possibilities of using the digestate and poses a threat to human health and environment. Therefore, selecting the appropriate method for these pollutants increases the process's effectiveness and minimizes the pollutants' content in the digestate. Co-fermentation technology is used more often, leading to the stabilization of feedstock, optimization of biogas production, and improvement of the C/N ratio [47]. However, it is associated with determining the optimal proportion of co-substrates and adjusting the process conditions.

Table 1. Microplastics in sewage sludge from municipal WWTPs in selected countries.

Location	Sampling Site	Concentration (pcs/kg)	Shape	Polymer	Reference
Germany	Dewatered sludge	1200–9400	Fragments, films	PE, PP, PA	[58]
China (11 provinces)	Dewatered sludge	1600–56,400	Fibers, fragments	PE, PP, PET	[59]
Spain (Barcelona)	Primary and secondary sludge	7500–25,000	Fibers, fragments	PP, PET, PA	[60]
United Kingdom	Primary sludge	37,700–286,500	Fibers, fragments	PES, PVA, PE, others	[61]
Finland	Tertiary treated sludge	2000–12,000	Fragments, beads	PE, PET, PS	[55]
Norway	Dewatered raw sludge	6000	Fragments, beads	PE, PET, PS, PVC	[62]
USA (Midwest)	Dewatered sludge	10,000–30,000	Fibers, films	PE, PP, PVC	[59]
Italy (Milan)	Dewatered sludge	5800–37,600	Fragments, fibers	PS, PE, PP	[18]

PE: Polyethylene, PES: Polyester sulfone, PET: Polyethylene terephthalate, PP: Polypropylene, PS: Polystyrene, PVC: Polyvinyl chloride; PVA: Polyvinyl alcohol.

Generally, the methods for reducing the content of contaminants in digestate include mechanical, chemical, and biological techniques. One of the effective methods for eliminating pathogenic microorganisms is the preliminary treatment of sludge using microwave reduction. As a consequence of this process, it is possible to remove such species as *Escherichia coli* bacteria effectively. A similar effect can be achieved by alkaline treatment, which allows the destruction of *E. coli* and *Salmonella* species. Another chemical method of reducing pollution is the oxidation process, which contributes to the removal of pathogens and converts complex compounds into substances with higher biodegradation abilities [47]. Other commonly used chemical methods for digestate treatment are flocculation, ion exchange, and precipitation [63]. In turn, biological methods include bioaugmentation, which involves the application of microorganisms to stimulate the anaerobic decomposition of sewage sludge. This technology is effective in reducing the content of heavy metals in digestate. Research conducted by Montusiewicz A. et al. [64] proved that microorganism vaccination increases fermentation efficiency and reduces metal content in digestate. However, no significant differences were observed in the amount of biogas produced with and without the addition of microorganisms [64]. Among biological methods, the use of enzymes to reduce contaminants also deserves attention. Typically, proteases and lipases are used to degrade polymeric substances [47].

4. Thermochemical Conversion: Pyrolysis and Gasification and Combustion

Thermochemical conversion, including pyrolysis and gasification, is a process that can be used to convert sewage sludge into energy and chemical products. These are modern technologies that allow for effective waste management and limiting their impact on the environment. Both processes have advantages in the context of sewage sludge management. Pyrolysis and gasification reduce the volume of waste, limit the emission of pollutants into the environment, and enable the recovery of valuable energy and chemical products. Additionally, with proper management, they can be part of a sustainable municipal and industrial waste management system.

In the thermochemical conversion of sewage sludge, such as pyrolysis and gasification, many technological innovations have been introduced to improve process efficiency, reduce costs, and minimize environmental impact. Recent years have shown increased interest in sewage sludge treatment thermochemical methods. Team Rijo et al. [65] reviewed various thermochemical conversion processes of sewage sludge aimed at generating products

with positive value such as biochar, biofuels, and renewable gases. Research indicates the effectiveness of using catalysts such as Ni, Cu, Mg, and Al in pyrolysis and gasification processes, which leads to increased hydrogen production efficiency and reduction of undesirable pollutants. The authors also characterize other processes: hydrothermal carbonization (HTC), hydrothermal liquefaction (HTL), and hydrothermal gasification (HTG). The operation of HTC is conducted at temperatures in the range of 180–250 °C with the pressure at 2–25 MPa allowing it to reach the product of biochar, hydrochar, or char. In turn, the performing conditions of HTL are as follows: temperature 250–374 °C, and pressure at 2–25 MPa allowing to achieve biooil, or aqueous phase oil. The HTG is performed at a temperature above 375 °C, and pressure of 18–25 MPa with gaseous products (such as CH₄, H₂, CO₂, C₂H₄, etc.) as final products [66]. Pyrolysis and gasification have high energy recovery but involve significant capital and operating costs. In contrast, HTC, HTL, and HTG have lower energy requirements, but product quality control and process optimization pose problems.

A fascinating comparison of the two technologies was presented in the research by Giwa et al. [67]. By combining plasma pyrolysis with anaerobic fermentation, the efficiency of the process increases. Sewage sludge is pre-processed in the plasma pyrolysis process. This makes the substrate available to bacteria. These two processes rely on high temperatures, which can cause problems with energy demand. According to the authors, these challenges are offset by the energy generated, which can power the treatment plant or be sold to the grid. Plasma pyrolysis waste can be converted into biochar, organic fertilizer, or soil conditioner.

There are also several publications on optimizing the sewage sludge gasification process. One of them is [68]. The publication's authors assessed the impact of the operating parameters of the gasification agent heating temperature and the equivalence coefficient on converting sewage sludge into synthesis gas through gasification and combined heat and power generation. This approach allows reducing CO₂/kg of sewage sludge emissions.

Thermochemical processing of sewage sludge, such as pyrolysis and gasification, may produce pollutants, including dioxins, furans, and solid particles. Dioxins and furans are toxic and can be emitted during high-temperature processes if improperly controlled. Particulate matter, which contains heavy metals and other harmful substances, can also be emitted, affecting air quality. Emission controls, including filters and catalytic converters, are key to minimizing these pollutants [69]. Dioxins and furans are recognized as highly toxic organochlorine pollutants with significant detrimental effects on public health and major economic repercussions. These compounds include polychlorinated dibenzo-p-dioxins, dibenzofurans, and dioxin-like substances such as polychlorinated biphenyls and carbazoles. Classified as persistent organic pollutants, they are featured in the Stockholm Convention's "Dirty Dozen" list. They are also identified by the International Agency for Research on Cancer as Group 1 carcinogens, indicating that they are known to cause cancer in humans [70]. Figure 3 below shows the main sources of dioxins in the environment. Therefore, many studies focus on the formation and control of dioxin and furan emissions during the pyrolysis of sewage sludge. One of them is research conducted by Cones's team [71]. The author aimed to estimate the emission of dioxins and furans under various decomposition conditions. This made obtaining an approximation of waste distribution in real conditions possible. A group of researchers from Iceland [72] analyzed the formation of dioxins in the gasification process and the operational parameters that can be controlled during the process to minimize the formation of dioxins. The authors note that using a high-temperature reactor at temperatures above 800 °C, combined with rapid gas cooling and the absence of oxygen, significantly reduces the formation of dioxins. This method is effective even for fuels with high chlorine content, demonstrating that high-temperature gasification can mitigate dioxin production under challenging conditions.



Figure 3. Sources of dioxins in the environment, based on [69,70].

Several studies and simulations have been conducted to reduce the emission of hazardous compounds such as dioxins, furans, or solid particles. This is evidenced by publication [73], in which the authors try to optimize the sewage sludge processing processes most efficiently. An interesting solution was proposed in the research presented in the publication by Pacheco's team [74]. The study aimed to contribute to developing sustainable waste-to-energy technologies, namely plasma gasification, by using sewage sludge as a valuable resource for syngas production.

Dioxins, furans, and particulate matter emissions seriously threaten human health and the environment. Protection against these pollutants requires monitoring emission sources, implementing appropriate emission reduction technologies, and taking action to improve air quality.

Thermochemical treatment of sewage sludge is a process that can generate various hazardous pollutants. There are several strategies to control and reduce these pollutants. It is essential to properly optimize each process, either by controlling the temperature or determining the exposure time. Another important factor is implementing monitoring systems to track pollution levels in real time continuously. Monitoring pollutant emissions allows for ongoing adjustment of process parameters. The composition of sewage sludge itself is more important. Therefore, before processing the sludge, its composition should be analyzed to identify potential sources of pollution and adjust the processing. Pretreatment methods such as pre-incineration, composting, or chemical stabilization are also recommended to reduce the amount of pollutants before the main thermochemical process [75]. Pollution control and reduction strategies are of increasing interest. It is becoming increasingly important to ensure that processes comply with national and international environmental standards regarding pollutant emissions [76].

The limited availability of landfill areas has led to the increasing incineration of sewage sludge. The result of burning the organic matter contained in sewage sludge is its conversion into carbon dioxide and water vapor, while simultaneously generating energy. Incineration significantly reduces the mass and volume of sewage sludge. In recent years, research on the co-incineration of sewage sludge has progressed. Co-incineration of sewage sludge with fossil fuels is a viable way to reduce environmental pollution. One of the most extensively studied mixtures is coal and biomass. The use of such a mixture helps to reduce the amount of coal (by supplementing it with sewage sludge biomass) and contributes to the reduction of greenhouse gas emissions. It turns out that adding sewage sludge improves the ignition and combustion properties of anthracite and bituminous coal. Additionally, it has been observed that sewage sludge improves the ignition properties of coal slurries in power plants. Co-incineration of biomass with fossil fuels is considered a promising method for reducing fossil fuel consumption. Efforts are also being made to

introduce sewage sludge into natural gas-powered power plants. The use of renewable energy seems economically promising, especially given high natural gas prices.

The document “Guidance on Best Available Techniques for Waste Incineration” issued by the European Commission contains the best available techniques (BAT) for waste incineration, including technologies reducing dioxin and furan emissions [77]. Similar information can also be found in the report “Sewage Sludge Management: Best Practices for Thermochemical Treatment” issued by the U.S. Environmental Protection Agency (EPA) [78]. The report presents best practices for sewage sludge management in thermochemical treatment.

Effective emission management during the thermochemical treatment of sewage sludge requires using complex strategies including process optimization, advanced treatment technologies, monitoring and control, reduction of pollution sources, and compliance with appropriate norms and standards. Implementing these strategies helps minimize negative impacts on human health and the environment.

5. Fertilizers from Sewage Sludge

Sewage sludge as a byproduct of wastewater treatment is rich in organic matter and essential nutrients such as nitrogen (N), phosphorus (P), and potassium (K) [79]. The concentration of those elements in sewage sludge organic fertilizers is comparable to traditional organic fertilizers. Additionally, fertilizer from sewage sludge is long-acting and contains micronutrients like copper, zinc, and iron, which are vital for plant health [80]. However, in inadequate concentrations, those trace elements are harmful. The sewage sludge properties depend on the wastewater type, treatment process, and often on the seasons [81]. The heterogeneous nature of sewage sludge forces its usage for fertilizer purposes and requires strict legal regulation and continuous monitoring of the content of trace elements [82]. Fertilizer from sewage sludge may contain many pollutants that pose a threat to human health and the environment, such as heavy metals [83], pathogens [84], pesticides, herbicides [85], dioxins [70], hormones [86], antibiotics [9], and microplastics [15]. Pharmaceuticals found in sewage sludge may have a negative impact on the functioning of soil microorganisms and increase the resistance of pathogens to antibiotics [9]. Moreover, sewage sludge may contain biogenic elements such as nitrogen and phosphorus that are too high, which, when flowing into water reservoirs with precipitation, may contribute to eutrophication processes. The organic matter in sewage sludge enhances soil structure, improves water retention, and promotes the growth of beneficial microorganisms [87], which play a crucial role in nutrient cycling and disease suppression. Sewage sludge as an organogenic substrate contains high amounts of humic acids and proteins. Therefore, sewage sludge is known to improve the quality of organic matter in the degraded soil, which is promising for soil carbon sequestration limiting the emission of CO₂ from the soil [88].

Using sewage sludge as an organic fertilizer recycles essential nutrients back into the soil, reducing the need for synthetic fertilizers [79]. This not only conserves natural resources but also lowers the environmental footprint of agricultural practices. Due to the organic and inorganic components, sewage sludge can be used for soil remediation purposes, improving soil quality and increasing soil productivity [89].

By harnessing the nutrient-rich properties of sewage sludge, we can reduce dependency on synthetic fertilizers, improve soil health, and address environmental challenges associated with waste disposal [13]. Using sewage sludge in soil fertilization is either due to reuse or relatively low costs.

6. Emerging Trends and Future Directions

The assessment of environmental risk associated with using sewage sludge for fertilizer and energy purposes is complex and involves various analytical and modeling tools. These tools help to identify, quantify, and assess potential environmental and health risks associated with contaminants present in sewage sludge. Wastewater contaminants are mainly biodegradable during the treatment process, while the resistant ones are retained in

sewage sludge to the greatest extent. Wastewater contains high levels of MPs, and most of them ($\approx 99\%$) remain in sewage sludge [18]. Many are not biodegradable or only slightly biodegradable and have high water solubility. Therefore, pharmaceuticals and personal care products (PPCPs), endocrine disrupting chemicals (EDCs), such as bisphenol A (BPA) and phthalates, per- and polyfluoroalkyl substances (PFASs), known for their persistence in the environment, pose a major threat. For this reason, the presence of these contaminants in sewage sludge is associated with a potential threat, both in ecological and health terms (Figure 4). Therefore, it is important to monitor the total content of these contaminants in sewage sludge and their intermediate forms of decomposition. Emerging pollutants in sewage sludge, including pharmaceuticals, personal care products, endocrine-disrupting chemicals, and microplastics, pose increasing risks to human health and the environment [90]. Various scientific and technical analytical tools evaluate these contaminants in sewage sludge (Table 2).

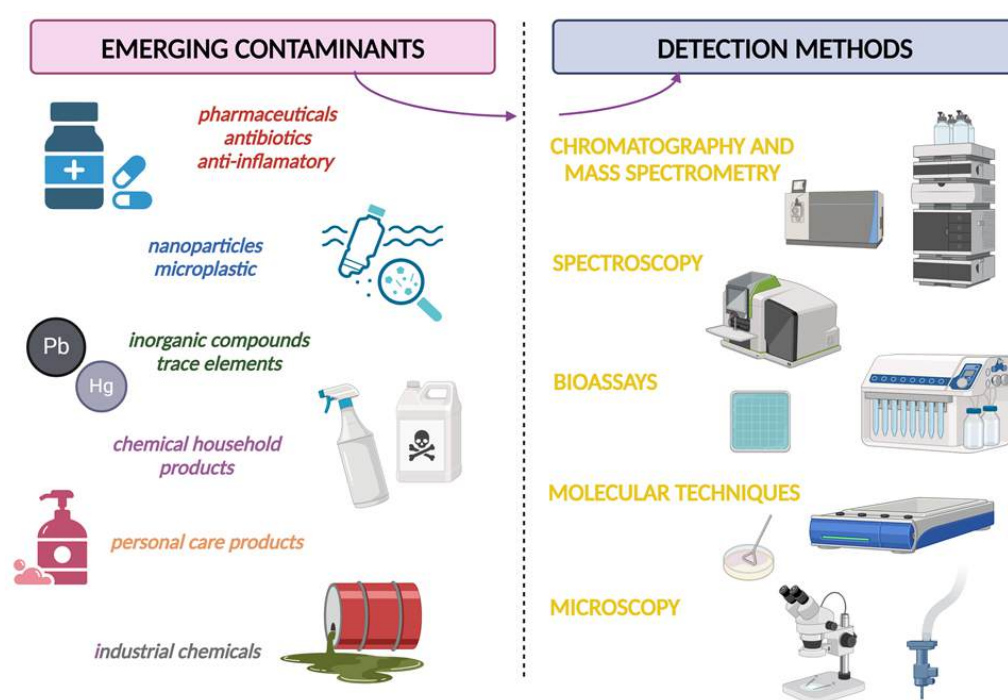


Figure 4. Emerging contaminants in sewage sludge and detection methods.

Table 2. Tools and techniques used for emerging contaminants assessment.

Technique	Description	Application	Reference
Chromatography and mass spectrometry			
High-Performance Liquid Chromatography (HPLC)	Separates, identifies, and quantifies mixture components	pharmaceuticals, steroid hormones, and endocrine-disrupting personal care products	[91] [42]
Liquid Chromatography-Mass Spectrometry (LC-MS)	Identifies non-volatile and thermally labile compounds by combining liquid chromatography and mass spectrometry	- Surfactants - thiabendazole, acesulfame, fenofibric acid, valsartan, irbesartan, salicylic acid, diclofenac, carbamazepine, 4-amino antipyrine (4-AA), 4-acetyl amino antipyrine (4-AAA), 4-formyl amino antipyrine (4-FAA), venlafaxine and benzoylecgonine	[92] [93]

Table 2. Cont.

Technique	Description	Application	Reference
Chromatography and mass spectrometry			
Gas Chromatography-Mass Spectrometry (GC-MS)	Detects volatile and semi-volatile organic compounds	22 organic compounds used in industrial personal care products (PCPs)	[94]
Ultra-Performance Liquid Chromatography (UPLC)	Enhanced form of HPLC with higher resolution and sensitivity	60 emerging pollutants in sewage sludge	[95]
Spectroscopy			
Fourier Transform Infrared Spectroscopy (FTIR)	Identifies organic, polymeric, and some inorganic materials	Polymer analysis, microplastics,	[96]
Nuclear Magnetic Resonance (NMR) Spectroscopy	Provides molecular structure and dynamics information	Structural biology, organic chemistry, drug discovery	[97]
Bioassays			
Toxicity Tests	Uses organisms like <i>Daphnia magna</i> , fish embryos, or algae to assess biological effects	Environmental toxicity assessment, pharmaceutical testing, ecological studies	[98]
Genotoxicity Assays	Detects genetic damage potential; Comet Assay	Carcinogenicity testing, drug development, environmental health	[99]
Molecular techniques			
Polymerase Chain Reaction (PCR)	Detects specific DNA sequences for bacteria or antibiotic resistance genes	Clinical diagnostics, genetic research, microbial identification	[100]
Metagenomics	Offers insights into microbial communities and their functions	Environmental microbiology, human microbiome studies, biodiversity assessments	[101]
Microscopy			
Scanning Electron Microscopy (SEM); Transmission Electron Microscopy (TEM)	Provides detailed surface images for identifying microplastics and nanoparticles Offers high-resolution images for micro- and nanoparticles	Nanoparticles' transformation in sewage sludge	[102]

The tool that facilitates the decision on whether a given sewage sludge can be used for natural purposes, especially in agriculture, is the analysis of the potential ecological risk. Such an assessment requires many procedures, tools, and models (Table 3). These models simulate the movement and transformation of contaminants in the environment, helping to predict their distribution, persistence, and potential impacts on ecosystems and human health.

Table 3. Table summarizing the monitoring and risk assessment tools for sewage sludge usage.

Tool/Method	Description	Application	Reference
Quantitative Structure–Activity Relationship (QSAR) Models	Predict contaminant properties and effects based on chemical structure	Understanding the sorption of pharmaceuticals to sludge in risk assessment	[103]
Exposure Models	Estimate human and environmental exposure levels	Public health risk assessment, environmental exposure studies, regulatory compliance; estimation of the human toxicity potential associated with chemical contaminants released upon the application of sewage sludge to agricultural land	[104]

Table 3. Cont.

Tool/Method	Description	Application	Reference
Fate and Transport Models	GMS (Groundwater Modeling System): used to simulate groundwater flow and contaminant transport in subsurface environments; HYDRUS version 5.01: a software package for simulating water, heat, and solute movement in variably saturated media; USEPA's PRZM (Pesticide Root Zone Model): originally developed for pesticides;	- model the leaching of contaminants from sludge into groundwater; - model the transport of contaminants in soil following sludge application; - model can be adapted to simulate the fate and transport of contaminants in the root zone of crops fertilized with sewage sludge;	[105] [106] [107]
Risk Quotient (RQ) Method:	Defined as the ratio between the maximum measured environmental concentration and the predicted no-effect concentration (PNEC)	The ecotoxicological potential of the studied nine PhACs in the wastewater discharged from the sewage treatment plant (Egypt)	[108]
Life Cycle Assessment (LCA)	Semi-quantitative assessment methodology for selecting appropriate sludge treatment options based on a lifecycle assessment approach.	analysis of the technological unconventional system of a small wastewater treatment plant with modifications generating fertilizer, biomass, or peat substitution ("biosolids factory" concept)	[13]
Risk Assessment (RA)	Scientific process in which the risks posed by inherent hazards involved in the process or situations are estimated either quantitatively or qualitatively	Environmental hazard indicators: the accumulation index of heavy metals in soil (Igeo), potential environmental risk indicator (PERI), risk assessment code (RAC), environmental risk factor (ERF); assessment of natural usage of sewage sludge.	[109]
Environmental Risk Assessment (ERA)	Identifying and evaluating the adverse effects on the ecosystems, animals, and people, exposed through the environment, resulting from technological activities.	Potential Ecological Risk Factor (ER), Risk Index (RI), Risk Assessment Code (RAC), Individual Contamination Factor (ICF), Global Risk Index (GRI), Individual Ecological Risk (IER), Global Ecological Risk (GER); to demonstrate the differences between the level of ecological risk posed by the different heavy metals in sewage sludge	[110]
Ecological Risk Assessment (ERA in the USA)	process that "evaluates the likelihood that adverse ecological effects may occur or are occurring as a result of exposure to one or more stressors"	Diclofenac, ibuprofen, and carbamazepine, 17- α -ethinylestradiol, β -estradiol, residues in sewage sludge; potential ecotoxicological effects	[111]
EcoFate, tool	EcoFate is a software package for conducting ecosystem-based environmental and ecological risk assessments of chemical emissions by point and non-point sources in freshwater and marine aquatic ecosystems, including lakes, rivers, and marine inlets. EcoFate is available online [112]	EcoFate is designed to assess the cumulative impact of chemical inputs in terms of contaminant concentrations in water, sediment, and biota of an entire ecosystem.	[113]
Geographic Information Systems (GIS)	Spatial analysis and mapping of contamination sources and patterns	Environmental monitoring, urban planning, public health surveillance, disaster response; to select sites suitable for land application of sewage waste.	[114]

By utilizing these tools and sources, researchers and practitioners can effectively assess the presence and impact of emerging contaminants in sewage sludge, facilitating informed decision-making and the development of mitigation strategies.

The integration of artificial intelligence and smart technologies for real-time monitoring and control of pollutants represents a significant advancement in environmental protection and management [115]. AI can play a pivotal role in controlling pollutants through several advanced techniques and systems [116]. Machine learning algorithms can predict contaminant levels based on historical data and operating conditions of wastewater treatment plants [117]. Additionally, AI can analyze data from sensors monitoring soil parameters such as pH, moisture, nutrients, and heavy metal levels, quickly alerting you to sudden changes [118]. Integrating AI and smart technologies enables ongoing monitoring of environmental pollution and soil condition monitoring, indicating areas requiring attention and quick response in a compatible application. Precise application of sewage sludge can be carried out using AI which, based on GPS data, determines areas requiring fertilization, thus limiting the possibility of over-fertilization and groundwater contamination. AI can assess the risks associated with using sewage sludge by analyzing data on the content of heavy metals, pathogens, and other contaminants [119]. AI can create early warning systems to inform farmers and authorities about the potential risks associated with the use of sewage sludge. Artificial intelligence can be helpful at the sewage sludge's composition level. AI may control pollution by offering a range of prediction and forecasting models. Models available include, among others, a forecast model based on linear regression for determining the content of heavy metals in plants fertilized with sewage sludge, allowing the determination of the mobility and accumulation of heavy metals in soil fertilized with sewage sludge [120]. Another model provides for controlling and estimating sludge efficiency at the sewage treatment plant using machine learning models [121]. In turn, neural network models can be used to study plants' uptake of toxic organic substances. Artificial intelligence also allows you to control the sewage sludge composting process before its use as fertilizer and can be helpful to decision-makers [122]. Using AI to fertilize soil with sewage sludge can significantly improve the efficiency and safety of this process. AI provides tools to monitor sudden increases in the concentration of emerging contaminants, allowing for quick response and prevention of ecological disasters. In turn, Ai et al. [123] proposed a model for rapidly identifying microplastic in soil using hyperspectral imaging technology. Such a tool may be useful for quickly controlling the enormous concentration of microplastics in soil treated by sewage sludge.

Currently, legal provisions in force in the European Union govern sewage sludge management. In the EU, sewage sludge is regulated mainly by three legal acts: the Waste Framework Directive (2008/98/EC) [124], which contains general principles applicable to all waste (e.g., treatment by the waste hierarchy); The Urban Waste Water Directive (91/271/EEC) [125], which contains detailed rules on how to deal with residues from sewage treatment; and the Sludge Directive (86/278/EEC) [126]. The latter two pieces of legislation were considered outdated by industry. The European Commission published a roadmap for evaluating the Sludge Directive (86/278/EEC) concerning the use of sludge in agriculture. The Directive requires that sludge is used in such a way as to take into account the needs of plants and not to deteriorate the quality of soil and surface and groundwater, and to take into account the aspect of new micropollutants such as pharmaceuticals and microplastics. Many countries have guidelines and standards for sludge application, including risk assessment protocols for emerging contaminants. Currently, the main goal of many WWTP operators is to reduce sludge production to maintain affordable disposal costs. Operators are also exploring innovative stabilization processes that may still allow agricultural use by eliminating pathogens and minimizing bad odors. In Europe still, overall 50% of sewage sludge is applied to the soil [21].

Work was carried out on changes to Directive 91/271/EEC. The version voted on in April 2024 is revolutionary in changing the regulations. The changes to the directive are based on the "One Health" approach aimed at permanently balancing and optimizing

the health of humans, animals, and ecosystems sustainably. The directive will cover agglomerations above 1000 population equivalent. By 2045, Member States will have to ensure the use of tertiary treatment (i.e., nitrogen and phosphorus removal) in larger treatment plants. Pharmaceutical and cosmetic manufacturers and Member States will have to finance the costs of additional treatment of micropollutants. Reuse of treated municipal wastewater will be promoted. By 2035, municipal wastewater will be subject to secondary treatment (i.e., removal of biodegradable organic matter) before being discharged into the environment, in all agglomerations of 1000 population equivalent (PE) or more. By 2039, tertiary treatment (i.e., nitrogen and phosphorus removal) will be applied in all wastewater treatment plants covering 150,000 PE and more, and by 2045 in plants covering 10,000 PE and more. Additional treatment removing a broad spectrum of micropollutants (quaternary treatment) will be mandatory for all plants above 150,000 PE (and above 10,000 PE based on a risk assessment) by 2045. Various public health parameters will be closely monitored, such as pathogens, chemical pollutants, so-called “forever chemicals” (per- and poly-fluoroalkyl substances or PFAS), microplastics, and the resistance to antimicrobials. The Act introduces extended producer responsibility (EPR) for human medicines and cosmetic products. This covers the costs of quaternary treatment (removal of micropollutants from municipal wastewater). Producers will cover at least 80% of the costs, and national funding for this process will also be introduced. EU countries must promote the reuse of treated wastewater from all municipal wastewater treatment plants, especially in areas with water shortages. The directive also introduces an energy neutrality goal. This means that municipal wastewater treatment plants will have to increase the share of renewable energy used each year gradually. This is 20% by 2030; 40% by 2035; 70% by 2040; and 100% by 2045. Member States have until 2026 to adapt their regulations and submit an appropriate implementation program. Europe is preparing for a revolution in water and wastewater management: legislative resolution of the European Parliament of 10 April 2024 on the proposal for a directive of the European Parliament and of the Council on wastewater treatment.

7. Sewage Sludge-to-Energy and Fertilizer Technologies for Enhanced Sustainability

Previously seen as very burdensome and problematic, the sewage treatment plant sector has recently begun to undergo a green, sustainable transformation towards energy and material recovery. Sewage sludge treatment poses several challenges, especially in terms of managing contaminants such as heavy metals, organic pollutants, and pathogens. Although technologies such as thermal hydrolysis, incineration, and anaerobic digestion are commonly used to address these issues, their complete elimination of harmful substances is not always guaranteed. Therefore, to protect public health and the environment, effective monitoring and prevention of municipal wastewater mixing with highly polluted industrial wastewater is needed. Nowadays, rising energy prices are forcing treatment plants to optimize treatment technologies and introduce sludge technologies such as gasification, pyrolysis, and advanced anaerobic digestion. Sludge treatment plants can improve their energy efficiency while reducing their environmental impact. These methods help to recover valuable resources such as biogas and minimize the ecological footprint of sewage sludge treatment. However, integrating these technologies into existing infrastructure requires significant investments. The circular economy framework provides a key opportunity to increase sustainability by recovering essential nutrients such as nitrogen and phosphorus from sewage sludge, which can then be reused as fertilizer. Although land-based application of sewage sludge supports nutrient recycling, it carries risks such as soil contamination, making advanced nutrient recovery technologies such as struvite precipitation and composting safer alternatives that also promote agricultural productivity and environmental safety. It is essential to balance the environmental benefits of sludge-to-energy technologies, such as reducing greenhouse gas emissions, with the potential health risks associated with residual contaminants when sewage sludge is used as fertilizer. Optimizing these processes involves adopting sustainability indicators that assess energy

efficiency, pollution reduction, and long-term environmental impacts. Public health and environmental impacts should be at the forefront of any technology deployed.

Sewage sludge management is developing significantly. The life of this waste no longer ends at the landfill. Sustainable sewage sludge management is the short-term goal of many sewage treatment plants today. We are moving towards maximizing all nutrients, carbon, energy, and other substances while minimizing the negative impact on the environment. The achievement of all sustainable goals requires new technologies development, which can lead to the possible energy and material recovery through specific processes [127]. However, we still face barriers that affect the implementation of new technologies in sewage sludge management. Overcoming the technical, economic, and social obstacles can minimize the environmental impacts of sewage sludge management [128]. Among the main technical barriers are infrastructure and technology [129]. Sewage sludge node at the sewage treatment plant is unfortunately still visible as a short, side technological line, in most treatment plants ending with the dewatering of the sludge, often with lime stabilization, and sending it to the landfill. The sludge node as an important element of the treatment plant in terms of sustainable development must stop being perceived this way. Investments and implementation of new technologies are necessary, including the transformation of sludge, at the place of its generation, into valuable products. Access to modern, efficient, and environmentally friendly treatment technologies is becoming less limited. Among currently available sewage sludge processing methods we can provide the basic ones like anaerobic digestion process, incineration, and composting, as well as the more advanced ones like anaerobic co-digestion, pyrolysis, gasification, hydrothermal treatment (hydrothermal carbonization (HTC), hydrothermal liquefaction (HTL), hydrothermal gasification (HTG), plasma gasification, and torrefaction. We can provide future methods for material recovery, hydrogen generation, and biofuel production. Implementing the selected technology for a given sewage treatment plant to process sewage sludge requires solving many technical and location issues. The selection of the appropriate technology is therefore crucial to ensure its optimization and functioning and the implementation of economic and environmental aspects (Table 4). It also requires modern pollution monitoring, ensuring the safety of the environment and users, including end users and intermediate users. Developing robust pollutant monitoring systems is essential for tracking pollutant levels in sludge, ensuring safety and compliance with environmental standards. Utilizing cost-effective and locally appropriate technologies ensures sustainability and practical application.

Table 4. Pros and cons of various sludge treatment technologies.

Technology	Advantages	Disadvantages	Microplastic Removal Efficiency	Inorganic Pollutants (Heavy Metals) Removal	Pharmaceuticals Removal	References
Composting	- Low cost - Produces organic fertilizers	-Slow degradation of some MPs - Emission of greenhouse gases (CH ₄ , CO ₂)	Low (4–13%)	Low to moderate (depends on compost quality)	Low to moderate	[5,18,37]
Thermophilic composting	- Faster organic material degradation - High temperature helps eliminate pathogens	- Requires high temperatures, increasing energy costs - Low effectiveness for some MPs	Moderate (29–43%)	Moderate (Temperature-dependent)	Moderate	[5,18,106]

Table 4. Cont.

Technology	Advantages	Disadvantages	Microplastic Removal Efficiency	Inorganic Pollutants (Heavy Metals) Removal	Pharmaceuticals Removal	References
Anaerobic digestion (55 °C)	- High MP removal efficiency - Biogas production	- Requires specific temperature control - Infrastructure required for biogas processing	High (75–91%)	Moderate to High	Low to moderate	[5,63]
Sludge drying	- Volume reduction	- High energy cost - Low MP removal efficiency	Low	Moderate (Stabilizes but does not remove metals)	Low to Moderate	[5,26,61]
Sludge incineration	- Complete destruction of MPs and pathogens - Energy generation	- High capital and operational costs - Greenhouse gas and other pollutant emissions	Very High (100%)	Very High (Complete metal destruction)	Very High (Complete destruction)	[5,18,26,55]

Overcoming economic barriers plays a crucial role in green transformation as well. In a real wastewater treatment plant case study, different scenarios with different sludge train technologies were assessed using the DSS-WWTP simulator. It was shown that although both thermal utilization and thermal hydrolysis showed the highest economic efficiency, thermal hydrolysis brought additional benefits in improving the heat balance of the entire wastewater treatment plant, even when combined with other technologies [113]. Implementing financial incentives such as subsidies, tax incentives, and funding programs can encourage adopting best practices and technologies. Public–private partnerships are also beneficial, as they leverage private sector investment and expertise, bringing innovative solutions and enhancing the overall effectiveness of sludge management programs. Focusing on cost-effective solutions, and prioritizing the implementation of technologies that offer significant benefits without high costs, ensures broader adoption and greater overall impact. Currently, in Europe, treatment plants pay for the collection of sludge by entities that deal with its storage or disposal. Therefore, the main goal of many wastewater treatment plant operators is to reduce sludge production to keep the costs of its disposal at an affordable level. In addition, rising energy costs force treatment plants to invest in new sludge nodes to maximize profits. Additional trends in Europe are the progressive reduction of landfill availability for waste disposal, increased local opposition to agricultural use, anticipated stricter standards for metals and emerging micropollutants under the upcoming European directive, and rising disposal costs for off-site solutions [130]. They are further trends that are largely generated by social barriers. Social approaches are equally important. Engaging communities in planning and decision-making promotes ownership, ensures solutions are tailored to local needs, and increases acceptance and compliance. Awareness campaigns to educate the public about the benefits and safety of proper sludge management can lead to greater community support and participation. Strengthening policies and regulations to ensure compliance and incentivize best practices is also essential. Robust policies and regulations, with incentives for compliance and penalties for non-compliance, ensure adherence to high standards in sludge management [22].

Addressing these barriers requires a multifaceted approach integrating technological innovations, economic incentives, and strong community and institutional engagement (Figure 5).

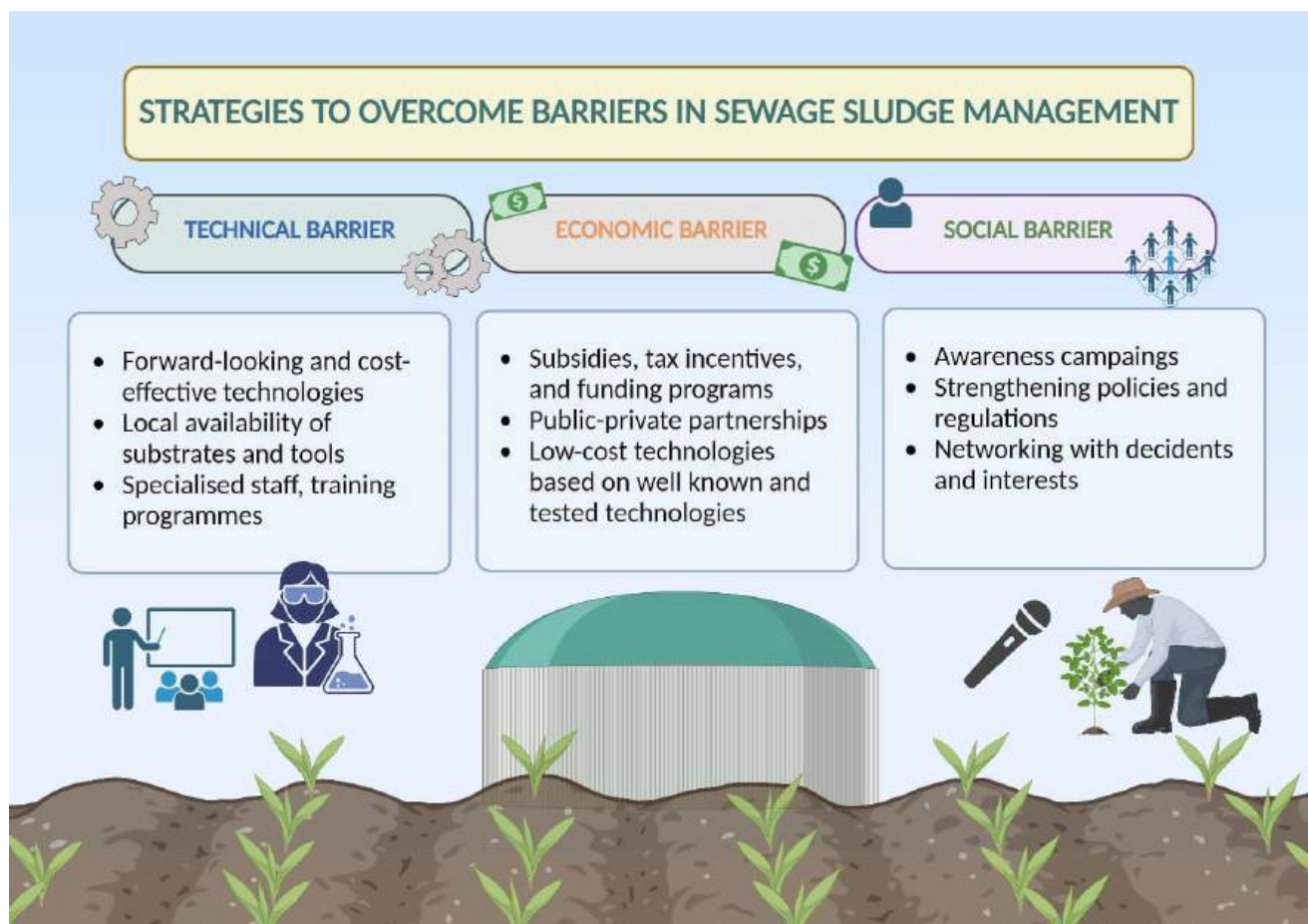


Figure 5. Strategies to overcome barriers in sewage sludge management.

8. Conclusions

Wastewater treatment plants can meet “net zero” emission commitments in the future and have ambitions to go even beyond net zero emissions. These include solar photovoltaics, biomass, hydropower, solar thermal, wind power, anaerobic digestion, combined heat and power (CHP), and wastewater heat collection. These initiatives are also possible in partnership with local industry and the community where other sources of organic and biodegradable waste are also generated and there is a demand for next-generation waste products.

In Europe, current challenges in sludge management are driven by several significant trends, including the progressive reduction of landfill availability for biodegradable waste disposal, increased local opposition to agricultural use, anticipated stricter standards for metals and emerging micropollutants under the upcoming European directive, and rising disposal costs for off-site solutions. WWTPs can significantly reduce climate emissions, green growth, and value creation in the region.

The reluctance to use sludge directly for agriculture results from the growing knowledge of the pervasive problem of microplastic contamination and emerging contaminants in sewage sludge worldwide. Despite progress in wastewater treatment, microplastics and recalcitrant or undiscovered contaminants remain a persistent environmental challenge, with potential implications for soil health and food safety when contaminated sludge is applied to agricultural land. The results underscore the urgent need for innovative treatment technologies, improved regulatory frameworks, and public awareness to address this growing problem effectively. As research evolves, these insights will be crucial to informing policies and practices to mitigate microplastic pollution and protect ecosystems. It is therefore important to emphasize that increasing the sustainability of sewage sludge treat-

ment requires multifaceted solutions, that include implementing next-generation advanced technologies, rigorous pollutant monitoring in the interests of health, and transforming the economy into a circular rather than linear economy. By aligning these efforts with public health and environmental safety goals, the challenges of sewage sludge treatment can be transformed into opportunities for resource recovery and energy generation.

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Abbreviations

The following abbreviations are used in this manuscript:

CE	Circular economy
OWtE	Organic waste-to-energy
WtE	Waste-to-energy
GHGs	Greenhouse gasses
RRfW	Resource recovery from waste
OSW	Organic solid waste
AF	Anaerobic fermentation
ETP	Electron transport phosphorylation
AD	Anaerobic digestion
VFAs	Volatile fatty acids
MSW	Municipal solid waste
COD	Chemical oxygen demand
HTC	Hydrothermal carbonization
CHP	combined heat and power systems
HTC	Hydrothermal carbonization
HTL	Hydrothermal liquefaction
BAT	Best available techniques
PAHs	polycyclic aromatic hydrocarbons
HTV	Hydrothermal vaporization
HTG	Hydrothermal gasification
DCWG	Supercritical water gasification
(EPA)	Environmental Protection Agency
PPCPs	pharmaceuticals and personal care products
EDCs	endocrine-disrupting chemicals
BPA	bisphenol A
PFAS	per- and poly-fluoroalkyl substances
LC-MS	Liquid Chromatography-Mass Spectrometry
GC-MS	Gas Chromatography-Mass Spectrometry
UPLC	Ultra-Performance Liquid Chromatography
FTIR	Fourier Transform Infrared Spectroscopy
PCR	Polymerase Chain Reaction
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy

QSAR	Quantitative Structure–Activity Relationship
RQ	Risk Quotient Method:
PNEC	predicted no-effect concentration
LCA	Life Cycle Assessment
RA	Risk Assessment
PERI	potential environmental risk indicator
RAC	risk assessment code
ERF	environmental risk factor
ERA	Environmental Risk Assessment
ER	Potential Ecological Risk Factor
RI	Risk Index
RAC	Risk Assessment Code
ICF	Individual Contamination Factor
GRI	Global Risk Index
IER	Individual Ecological Risk
GER	Global Ecological Risk
GIS	Geographic Information Systems
AI	artificial intelligence
PE	population equivalent

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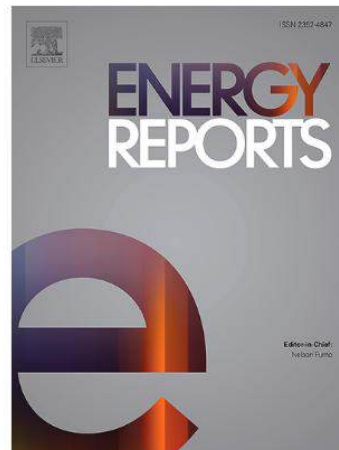
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D.4

**Anna Jasińska, Anna Grosser, Erik Meers,
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*Energy recovery from poultry manure in the process
of semi-continuous anaerobic co-digestion with
sewage sludge*



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Research paper

Energy recovery from poultry manure in the process of semi-continuous anaerobic co-digestion with sewage sludge

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ABSTRACT

In the face of significant legislative changes in renewable energy sources and conservation of natural resources, bio-waste must be managed efficiently. Developing wastewater treatment technologies generate a large amount of sewage sludge requiring appropriate use. A well-known and practiced process is the anaerobic digestion of sewage sludge, but due to its characteristics, the production of biogas is not sufficient enough to ensure the energy self-sufficiency of a wastewater treatment plant. Another waste whose management is a challenge is poultry manure, whose production in Poland is high due to intensive poultry farming. This study investigated the possibility of co-digestion of poultry manure and sewage sludge and its effect on increasing biogas production compared to processing sludge alone. The process was performed in two continuously stirred-tank glass reactors at mesophilic conditions, with 20 days of hydraulic retention time. In the first stage, the batch assay with sewage sludge was applied to promote the development of an anaerobic community. The second stage involved feeding the reactors on a semi-continuous regime with sewage sludge for the control sample and a mixture with the gradual addition of poultry manure from 2.5 % to 60 %. During the experiment, the most important parameters affecting the process and the quantity and quality of the obtained products in the form of biogas and digestate were monitored. The study's results indicated an advantage of the co-digestion process of poultry manure and sewage sludge over the digestion of sludge alone in terms of process efficiency. The highest biogas production was obtained with a co-substrate ratio of 42 % manure to 58 % sewage sludge (ratio based on volatile solids). Co-digestion had no significant effect on gas quality; in both cases, the methane content was more than 60 %. Moreover, a digestate with fertilizer potential was obtained for each sample.

1. Introduction

Due to the high nutritional value of meat, its consumption is increasing worldwide (Ghosh and Saha, 2020). In European countries, in particular, the poultry industry is growing rapidly. Fig. 1 shows the production of poultry meat in the EU based on data collected from 2011 to 2022 (Eurostat, 2023). Poland occupies a leading position in the depicted list, reaching a production of more than 2.7 million tons in 2022, nearly 190,000 tons more compared to 2021 and nearly twice as much as in 2011. It is worth mentioning that the total production of poultry, including broiler chickens, egg-laying hens and turkeys, is

estimated there at an average of 4 million tons per year (Drózd et al., 2020).

The successive increase in the number of poultry farms results in the generation of more animal manure, one of the main by-products of farming. In 2017, the amount of poultry manure (PM) produced in Poland was estimated at 2121750 Mg per year (Drózd et al., 2020). According to the legal definition, manure, as a by-product of animal husbandry, is any excrement and/or urine of farmed animals other than farmed fish, even with bedding. This waste must undergo appropriate treatment based on technologies that are available, economical and as environmentally safe as possible. Improper manure management poses

Abbreviations: A, alkalinity; AcD, anaerobic co-digestion; AD, anaerobic digestion; CPI, co-digestion performance index; CSTR, completely stirred tank reactors; D-AcD, digestate from anaerobic co-digestion; D-Con., digestate from anaerobic digestion of sewage sludge; FA, free ammonia; HRT, hydraulic retention time; N-NH_4^+ , ammonium nitrogen; OLR, organic loading rate; PM, poultry manure; SD, standard deviation; SS, sewage sludge; TC, total carbon; TKN, total Kjeldahl nitrogen; TS, total solids; VFAs, volatile fatty acids; VS, volatile solids; WWTP, wastewater treatment plant; Y_B , biogas yield; Y_M , methane yield.

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the risk of undesirable consequences, such as the production of odours, attraction of rodents, insects and other pests, release of animal pathogens, contamination of soils and groundwater, surface water runoff and emission of greenhouse gases (Dróżdż et al., 2020; Bőjti et al., 2017).

Poultry manure is characterized by a large amount of organic matter and contains in its composition many nutrients and trace elements, such as potassium, copper, zinc, cobalt, iron, selenium, molybdenum, boron and manganese. Unlike other types of animal manure, it has a higher content of nitrogen, phosphorus, calcium, magnesium and sulfur (Dróżdż et al., 2020; Kacprzak et al., 2023a). Due to its significant share of valuable nutrients, poultry manure can be used as a soil additive to improve soil properties and fertility. However, due to the excessive amount of this waste, Poland lacks sufficient agricultural land for such poultry manure management. This method is also fraught with the risk of environmental problems related to the physicochemical and hygienic-sanitary properties of the manure. Hence the need for a suitable strategy for its appropriate further processing, transportation or storage (Dróżdż et al., 2020; Bayrakdar et al., 2017; Abouelenien et al., 2014).

The promising strategy for managing poultry manure is anaerobic digestion (AD) while obtaining a valuable energy source in the form of biogas. This waste is an interesting substrate for the process due to its high organic matter content, 63–80 % of total solids (TS), and its buffering capacity (Dróżdż et al., 2020). Anaerobic digestion is a long known and used process, economically viable and socially acceptable due to its environmental friendliness (ACWilkie, 2005). Due to the biological character of this method, its efficiency depends primarily on the activity of microorganisms, which show varying tolerance to process conditions and the presence of toxic substances such as ammonia, hydrogen sulphide and heavy metals (Magrel, 2002a; Murto et al., 2004). In the case of poultry manure digestion, a particular problem is the high nitrogen content in the ammonium form, which transforms into an inappropriate

carbon to nitrogen ratio in the substrate. Intense accumulation of ammonia released by the decomposition of uric acid and undigested proteins usually results in process inhibition. Hence, inadequate preparation of the feedstock in the form of manure is the reason for the failure of anaerobic decomposition (Kacprzak et al., 2023b).

There are many methods to reduce the negative effects of ammonia on the digestion process. The simplest of these is to dilute the feedstock with water, but then biogas production decreases, a large amount of secondary waste is generated, and technological water consumption increases (Jiang et al., 2019; Carlini et al., 2015; Ellersdorfer et al., 2020). Among the more economical techniques are the stripping process using air or steam, based on the principle of mass transfer, or the use of adsorbents such as biochar or zeolite (Limoli et al., 2016; Malińska, 2015). In recent years, researchers have focused on the intensification of anaerobic digestion of manure through the parallel use of other organic substrates in the process. Co-digestion (AcD) of several appropriately selected materials simultaneously makes it possible to increase the degradation rate of the processed materials, higher biogas yields, improve the nutrient balance, optimize the C/N ratio of the substrates and dilute toxic compounds. This strategy creates new opportunities for processing such organic wastes that are difficult to digest separately (Dróżdż et al., 2020; Borowski and Weatherley, 2013a).

Due to the high C/N ratio of agricultural materials such as corn silage, the agricultural industry is the most convenient source of co-substrates for processing poultry manure. However, the necessity to cope with seasonality and increase methane production efficiency has led to significant interest in other biodegradable waste (Mata-Alvarez et al., 2014). Undoubtedly, the aspect of using sewage sludge (SS) for co-digestion with poultry manure deserves special attention. Currently, no sludge-free wastewater treatment technology has been developed, nor is there a known solution to completely eliminate sludge from the environment. Although there is a progressive modernization and

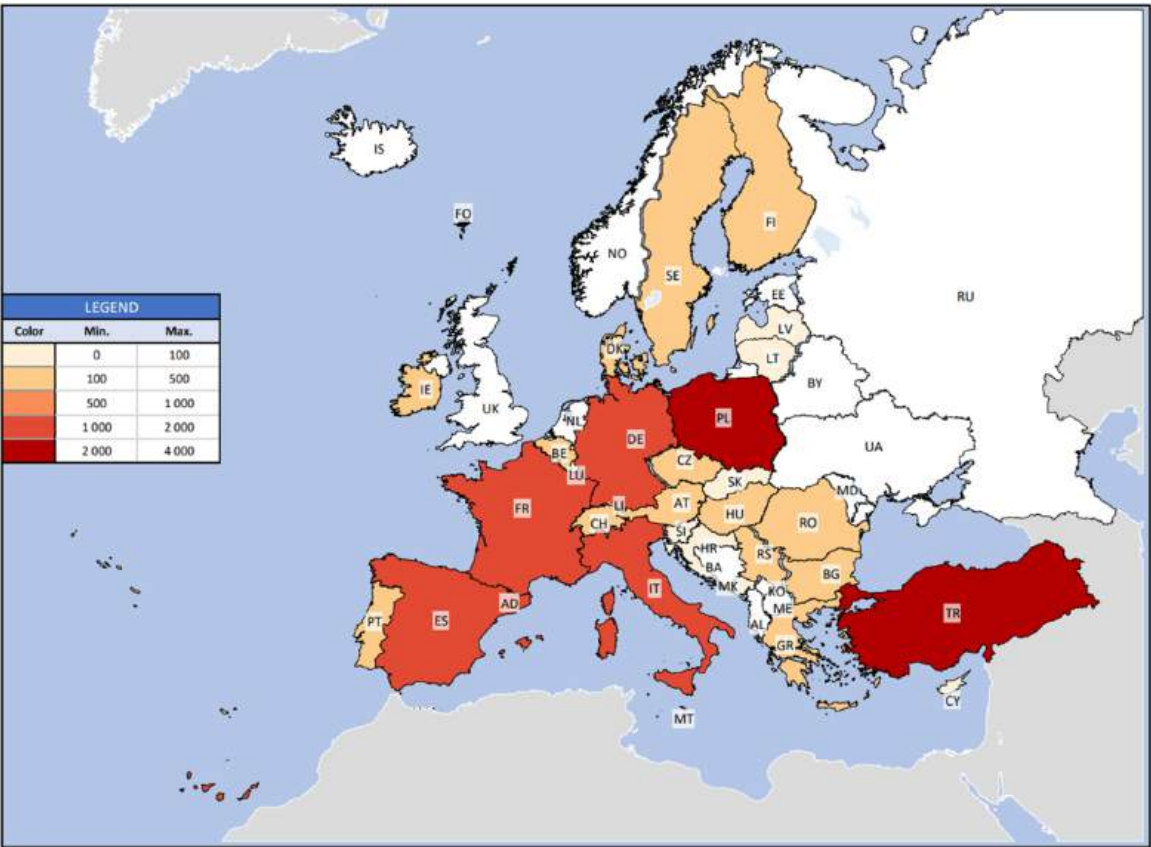


Fig. 1. Poultry meat production in Europe in 2022 (mln Mg) (Sauthor1\$ et al., 2023).

expansion of wastewater collection and treatment infrastructure, most digesters are oversized, so the organic load in them is low (Ghosh and Saha, 2020; Jasińska, 2018). Therefore, co-digestion of sewage sludge with other wastes appears to be a favourable strategy due to the possibility of increasing methane yield, as well as fully utilizing the available facilities at wastewater treatment plants (Xu et al., 2020; Yang et al., 2019).

This paper discusses the possibility of co-digestion of poultry manure and sewage sludge, with a particular focus on biogas production. To the best of the authors' knowledge, to date no one has carried out the process with such a high content of manure (up to 60 % based on volatile solids) in the co-digestion mixture and focused on the quality of the digestate, which is the novelty of the study. Only a few papers have been written on the co-digestion of these two materials, so the issue presented here provides a basis for a broader understanding of the possibilities associated with the aforementioned method of their management. Animal manure is most commonly processed by co-digestion in: (i) centralized plants that co-digest manure collected from several farms along with organic residues from industry and cities, and (ii) decentralized plants that co-digest manure with other agricultural wastes and increasingly with energy crops (Kadam et al., 2024). In our research, we decided to go against the grain of typical trends. Taking into account recent legislative changes (revision of the Wastewater Treatment Directive) (Sauthor1\$ et al., 2022), which talk about energy self-sufficiency of wastewater treatment plants, we decided to determine the effect of poultry manure on anaerobic digestion of sewage sludge. The identification of new potential co-substrate, so far not particularly considered, seems to be a very good solution, especially since changes in the law will result in stiffer competition in the raw material market. An additional argument in favour of this approach was the fact that this waste in Poland (which is one of the largest poultry producers in Europe) is found in large quantities, and its management is becoming increasingly problematic. Given these facts, co-digestion of sewage sludge with this waste stream seemed an attractive and sustainable approach. In addition, our research tested the methanogenic potential of locally generated waste, which could prompt its generators and stakeholders to manage such materials sustainably and economically. The scope of the work included performing mesophilic co-digestion of poultry manure and sewage sludge in a semi-continuous operation regime. In addition, a physicochemical analysis was made of the studied substrates, mixtures constituting the feedstock, and products of the process in the form of biogas and digestate. Potential greenhouse gas emissions were also estimated for various methods of poultry manure management.

2. Materials and methods

2.1. Substrates and inoculum

Poultry manure was obtained from a laying poultry farm in the Silesia region (Poland). The manure was produced by animals of adult age. During the experiment, poultry manure was collected twice, homogenized using an automatic mixer and stored at -20°C .

Sewage sludge, precisely a mixture of waste activated sludge and

primary sludge was collected at the municipal wastewater treatment plant located in the Silesia region (Poland). The WWTP produces annually approx. 3200 Mg dry mass of sewage sludge and treats about 90 000 m³/d of wastewater. Sludge was collected every two weeks and stored at 4°C prior to use.

As inoculum, digested sludge was used and collected from the mentioned plant. The addition of inoculum in the feedstock during the start-up of the digester was equal to 10 % (v/v).

The co-digestion mixtures consisting of poultry manure and sewage sludge were prepared every nine days and stored at 4°C prior to use.

Characteristics of the raw materials and prepared feedstock used in this study are presented in Table 1.

2.2. Experimental procedure

The process was performed in a semi-continuous mode under mesophilic conditions (37°C). The hydraulic retention time (HRT) was 20 days, which is a typical value on WWTP (Bolzonella et al., 2005).

The anaerobic digestion process was carried out in two continuously stirred glass reactors (CSTR) with a working volume=15 l. The reactors were equipped with thermostatic water jackets, mechanical stirrers with variable speed control (170 rpm), and sensors: temperature, redox. The test stands consisted of measuring cylinders (capacity 15 l) filled with saturated sodium chloride solution and equalization tanks (capacity 20 l).

At start-up, batch anaerobic digestion was carried out (Stage 1). Once steady state operation was reached, both reactors were fed once per day with sewage sludge. In the next stage, a co-digestion feedstock containing poultry manure and sewage sludge was added to the reactors, starting with a 2.5 % on volatile solids (VS) basis proportion of manure in the mixture. For the control trial, one of the reactors was fed with sewage sludge for the entire duration of the experiment. The proportion of poultry manure in each co-digestion mixture was systematically increased up to 60 % VS_{added} (Fig. 2). The gradual increase in the co-substrate content in the mixture was intended to provide the microorganisms with the opportunity to adapt to new environmental conditions (Grosser and Neczaj, 2018).

2.3. Sample analysis

The following parameters were measured during the experiment: total solids (TS), volatile solids (VS), pH value, volatile fatty acids (VFAs), alkalinity (A), ammonium nitrogen (N-NH₄₊), total carbon (TC) and total Kjeldahl nitrogen (TKN). The volatile fatty acids (VFAs), pH levels, alkalinity, ammonium nitrogen concentration were determined in the supernatants after centrifugation at 12100 relative centrifugal force (rcf) for a time span of 15 minutes, and then filtration through filter papers (3w). All analytical evaluations were carried out in triplicate to assure precision and reliability. All mentioned measurements were performed according to the standard method (APHA, 1999). Additionally, free ammonia (NH₃ or FA) was calculated based on pH value, temperature and ammonium nitrogen, using the formula as described by Ref (Yang et al., 2019; Grosser, 2017).]. Concentrations of

Table 1
Characteristics of substrates used in this study.

Substrate	PM (% basedon VS)	TS (%)	VS (%)	VS/TS	pH	VFAs (mg CH ₃ COOH/l)	VFAs/A (-)	N-NH ₄ (mg/l)	TC/TKN
Inoculum	-	2.52–2.56	1.44–1.46	0.57	7.80–7.82	480–514	0.21	537–543	7.51–7.57
SS	-	3.08–4.71	2.22–3.21	0.66–0.74	5.54–5.97	2131.42–2582.31	1.18–3.22	138.34–299.47	10.20–12.12
PM	-	27.44 ± 0.24	20.19 ± 0.81	0.74	5.41 ± 0.01	Nm	Nm	7000 ± 12.3	7.38 ± 0.51
Mixture 1	10	4.86 ± 0.01	2.81 ± 0.05	0.58	5.81 ± 0.01	2840 ± 9.9	2.48	294.93 ± 3.23	10.64 ± 0.04
Mixture 2	20	4.74 ± 0.05	3.13 ± 0.44	0.66	5.89 ± 0.01	3204 ± 36	3.01	396.06 ± 3.23	11.90 ± 1.41
Mixture 3	30	4.66 ± 0.02	3.37 ± 0.09	0.72	5.72 ± 0.01	2457.94 ± 36.69	2.21	461.61 ± 5.6	8.22 ± 0.77
Mixture 4	40	5.4 ± 0.04	3.92 ± 0.29	0.73	5.73 ± 0.01	2488.52 ± 22.23	2.18	512.40 ± 3.96	8.20 ± 1.00
Mixture 5	50	5.05 ± 0.05	3.85 ± 0.49	0.76	5.75 ± 0.01	2589.31 ± 31.35	2.33	579.13 ± 4.7	7.94 ± 1.30
Mixture 6	60	6.21 ± 0.11	4.66 ± 0.65	0.75	5.69 ± 0.01	2742 ± 57.68	2.17	625.48 ± 9.54	7.89 ± 0.27

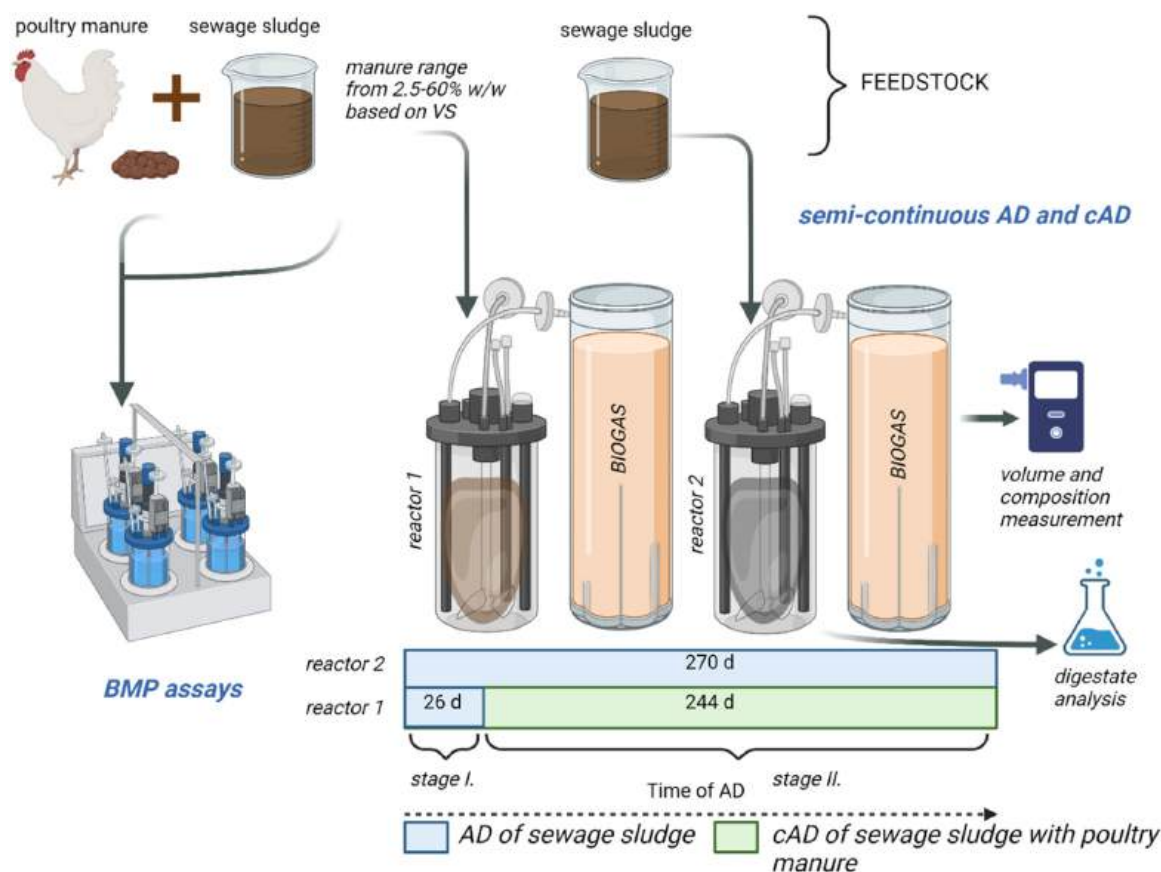


Fig. 2. Scheme of an experiment performed in this study.

heavy metals and nutrients were determined using an inductively coupled plasma optical emission spectrometer (ICP-OES). Prior to analysis, samples were extracted in reverse aqua regia, sonicated for one hour and digested in a digestion chamber (Milestone ultraWAVE). Daily biogas production was measured using the water displacement method, while biogas composition was determined using a portable gas analyser (NANOSENS DP-27 BIO+). The biogas volume and composition were monitored daily, while digested sludge and substrate were analysed once every seven days. The volume of biogas was converted to standard conditions (temperature = 273 K, pressure = 1 atm).

In addition, to determine whether synergistic effects occurred between the co-substrates, the co-digestion performance index (CPI) was estimated according to the formula presented by Ebner et al (Ebner et al., 2016a). In the numerator, the methane production obtained during the study was considered. In the denominator, on the other hand, the calculation considered the methane production coefficients estimated for each component using the automatic methane potential test system (AMPTS II) (own research not presented in the manuscript). For sewage sludge, the methane production coefficient was 0.310 l/g VS_{added} (In the manuscript, the average production for methane yield was 0.303 l/g VS_{added}), and for chicken manure, it was 0.28 l/g VS_{added}. The tests were conducted in mesophilic conditions with an I/S ratio of 1.

2.4. Calculation of emissions

Based on the data on the characteristics of the tested poultry manure, the potential for greenhouse gases (CH₄, CO₂, N₂O) emissions was estimated. Calculations were made based on equations proposed by Kreidenweis et al (Kreidenweis et al., 2021). for four scenarios: storage (1) and composting (2) of fresh manure and its anaerobic co-digestion with sewage sludge at PM = 60 % in the feedstock, taking into account storage of the digestate in a closed (3) and open storage tank (4).

The emission results obtained are presented in kg/t of poultry manure or co-digestion mixture.

2.5. Statistical analysis

The effect of poultry manure addition on the effectivity of anaerobic digestion was studied using a one-factor ANOVA analysis. The homogeneity of variances was checked using Levene's test. Data which failed the ANOVA assumptions were analyzed via the Kruskal-Wallis test. ANOVA was done with at least three replications for each combination of the nominal variables. The statistical analysis was carried out using STATISTICA software (STATISTICA 12 PL StatSoft, Inc.)

3. Results and discussion

3.1. Characterisation of raw materials and feedstock mixtures

Poultry manure had a relatively high TS content of about 27 %, but lower than the literature data indicate (for solid manure, the TS range is 33–79.4 %) (Ghirardini et al., 2020). It is worth noting that the dry matter content of manure depends on the form of storage and the presence of bedding material. From the point of view of the co-digestion process together with sewage sludge, whose hydration is high, the TS value of manure for wet digestion is satisfactory. However, both SS and PM had a high VS/TS ratio (<0.7), indicating their high biodegradability. As expected, with increasing the addition of PM in the feedstock mixture, the total solids and volatile solids contents increased.

A significant difference can also be seen in the C/N ratio of poultry manure was equal to 7.38, compared to references, where for manure the range of values for this parameter is 15–18 (Sadecka and Suchowska-Kisielewicz, 2016). The ammonium nitrogen content of the manure was high at 7000 mg/L. Mixing sewage sludge with poultry

manure led to an increase in C/N and a significant reduction in N-NH_4 concentration, which offset the risk of inhibition associated with ammonia production during the co-digestion process.

Both poultry manure and sewage sludge were characterized by low pH, while a high content of volatile fatty acids was reported for sewage sludge. The wide range of results obtained for the second mentioned material was due to the variability of the wastewater composition, which consequently translates into a lack of homogeneity in the chemical composition of the sludge.

3.2. Process stability

The stability of the process in both reactors was evaluated mainly by measuring the value of the VFAs/A ratio. During start-up, this ratio averaged for R1 = 0.17 and R2 = 0.46, respectively. The difference in value between the two reactors may have been due to the heterogeneous nature of the sludge. The stabilization process of the reactors was short, already on the 11. day the value of VFAs/A for R1 and R2 was reported below 0.2, which indicates good adaptation of the inoculum to the conditions. As the proportion of poultry manure in the co-digestion mixture increased, the ratio remained at a similar level, averaging 0.16–0.18. The highest VFAs/A ratio was reported for R1 on day 161, at 48 % proportion of poultry manure in the mixture, and it was 0.33. The literature data indicates the critical upper value of VFAs/A for proper anaerobic digestion as 0.3–0.4 (Grosser, 2017; Dąbrowska, 2015). Thus, the results obtained in both cases testify to the stable operation of the reactors, and single spikes in values above 0.3 did not disrupt the process. Fig. 3 shows the fluctuations in the values of VFAs and alkalinity in the R1 and R2 digestate during the experiment, as well as the quotient of these parameters.

At the initial stage of the experiment - anaerobic mono-digestion of sewage sludge, the content of volatile fatty acids in the digestate

averaged for R1 and R2, 662 and 818 mg $\text{CH}_3\text{COOH/l}$, respectively. With the introduction of co-digestion in R1, increasing the proportion of poultry manure in the mixture resulted in an increase in the concentration of VFAs, and the maximum value was 1823 mg $\text{CH}_3\text{COOH/l}$ at 60 % PM. An increase in the concentration of VFAs is observed with increasing FA content as methanogen activity decreases (Shi et al., 2017). Hence, the addition of manure, which is a substrate with high nitrogenous organic matters, resulted in higher production of VFAs. In the case of R2, the concentration of volatile fatty acids tended to decrease with the course of sludge digestion. As stated in the literature, the AD process is stable at values of 100–500 mg $\text{CH}_3\text{COOH/l}$, with alkalinity of not less than 500 mg CaCO_3/l (Magrel, 2002b), and inhibition can occur at concentrations of VFAs of 2500–4000 mg $\text{CH}_3\text{COOH/l}$ (Grosser, 2017). Co-digestion of sewage sludge and poultry manure significantly increased alkalinity, from an average of 3740 mg CaCO_3/l when co-substrate was introduced to 11080 mg CaCO_3/l at 60 % PM. During the sewage sludge mono-digestion process in R2, a decrease in CaCO_3 concentration to 2260 mg CaCO_3/l at day 260 was observed with the course of the experiment. Despite the relatively high values of VFAs reported during ACD, there was no interference with the process. Poultry manure has a high buffer capacity and high nitrogen content, thus showing the potential to raise the pH in the mixture. Many authors report the influence of a slightly acidic pH, close to 6, on improving the working conditions of hydrolytic-acid-forming bacteria and limiting the growth of methanogens (Ponsá et al., 2008). The stable pH of the ACD process, oscillating in the range of 7.70–8.30, prevented the transition of VFAs into dissociated forms detrimental to the process. In this case, the alkalinity of the manure is a neutralizing factor for the generated VFAs to offset the pH changes. Thus, the preference for VFA/A monitoring over pH is due to the fact that AD rarely returns to normal when pH drops dramatically. This makes pH less relevant, as Issah et al. (2021a) also point out in their study on co-digestion of palm nut paste

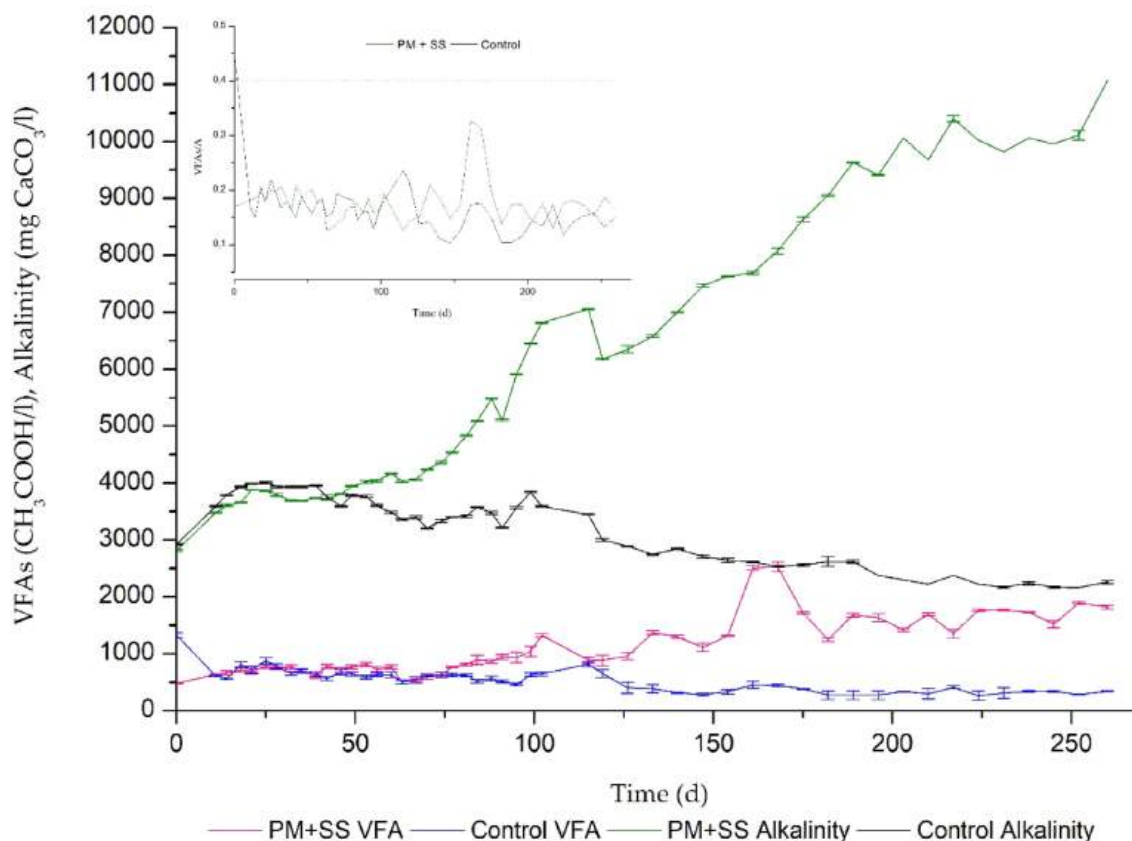


Fig. 3. Variations of volatile fatty acids, alkalinity and VFAs/A ratios during the experiment.

waste and anaerobic digested rumen waste (Issah and Kabera, 2021b). These researchers report that balancing high VFA production during anaerobic digestion requires maintaining alkalinity above 1.5 g CaCO_3/l .

The introduction of PM co-substrate into R1 also resulted in a significant increase in N-NH_4 concentration. While the analysis of digestate from R2 showed a decreasing trend in ammonium ions content with the course of the experiment, in R1, an accumulation of N-NH_4 was observed from 917 mg/l with 2.5 % PM (based on VS), to nearly 2750 mg/l with 60 % manure (based on VS). Borowski et al (Borowski and Weatherley, 2013b), in their study on the co-digestion of sewage sludge with poultry manure in a ratio of 30:70 % (w/w VS), reported ammonium ions concentrations of 2094 mg/l at HRT=20 days, with no process interference. The mechanism of ammonia inhibition during AD is still not sufficiently defined, hence a wide range of reported concentrations, from 1500 to 7000 mg/l (Jasińska et al., 2023a), can be found in the literature. However, it is known that the main factor of process interference is the presence of FAN (free ammonium nitrogen), the concentration of which depends on the process parameters - pH and temperature, as well as the adaptability of microorganisms. Cook et al (Cook et al., 2017), indicate the instability of AD at FAN concentrations above 200 mg/L. Altinbas et al (Altinbas and Cicek, 2019), in their study of co-digestion of cattle and poultry manure, found the maximum concentration of N-NH_3 tolerated by methanogens at 332 mg/l, but for a value of 253 mg/l, they already observed a 51 % reduction in methane production compared to the initial stage of the reactor operation only with cattle manure AD. The researchers also pointed out a trend of increasing free ammonia content with increasing the proportion of chicken manure in the co-digestion mixture. It is well known that this kind of manure contains more nitrogen than other manure because hens belong to monogastric animals (Borowski and Weatherley, 2013b).

The source of N-NH_4 in these wastes is nitrogen found in uric acid, excreted by the organisms along with urine. Fig. 4 illustrates the changes

in pH and N-NH_4 , and FAN content for the digestate in both reactors. However, the relatively high total concentration of ammonium ions and FAN in R1 did not lead to inhibition of the process, which means the good adaptation of the microorganisms to the process conditions, achieved, among other things, by the gradual introduction of the co-substrate in the form of a PM. In comparison to the studies of Sillero et al (Sillero et al., 2023a), slightly larger pH fluctuations were noted (7.70–8.30 - current studies vs 7.5–7.9). However, lower indicator fluctuations result from the fact that the process was conducted in a two-stage digestion system, and in addition to sewage sludge and poultry manure, a third co-substrate was dosed to the anaerobic chambers.

3.3. Process performance

The key parameters describing the efficiency of the anaerobic digestion process are the biogas/methane production ratio and the VS removal, the value of which largely depends on the ORL of the feedstock (Grosser et al., 2013). During the experiment, data on biogas production and volatile solids removal rate were reported for R1 and R2, as shown in Figs. 5, 6, 7 and 8. In the initial phase of anaerobic mono-digestion of sludge, irregular gas production was observed until the 11. day, which is related to the stabilization process of the reactors. From the 12. to the 25. day prior to the introduction of co-digestion, daily biogas and methane production averaged 7.7 and 5.2 l for R1 and 7.1 and 5.0 l for R2, respectively. For R2, where sludge mono-digestion was carried out, an average daily biogas production of 8.9 l and methane production of 5.64 l was reported from 26 to 260 days. In this case, the biogas and methane production yield, in this case, averaged 0.4 and 0.3 l/kg VS_{added} for R1 and R2, respectively. In the case of co-digestion, when the proportion of poultry manure in the mixture ranged from 10 % to 30 % (based on VS), there was no significant effect on increasing the efficiency of the process with respect to the control sample. In contrast, a

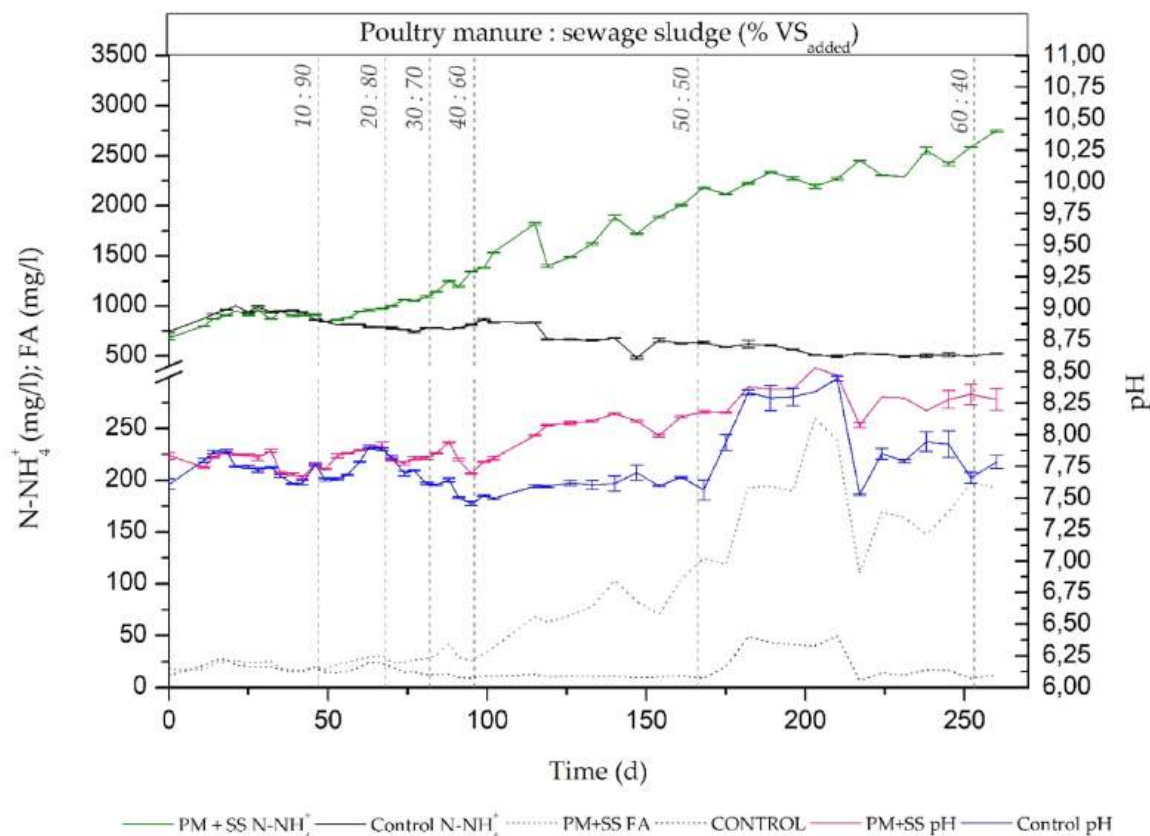


Fig. 4. Ammonium nitrogen, free ammonia concentrations and pH values for R1 and R2.

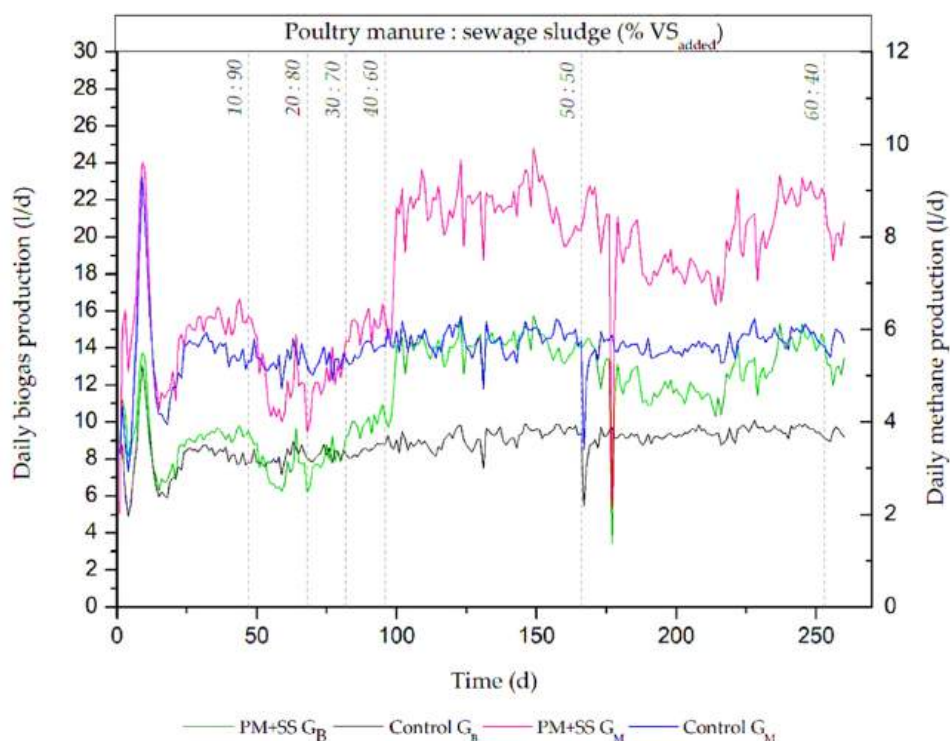


Fig. 5. Daily biogas and methane production reported during the experiment.

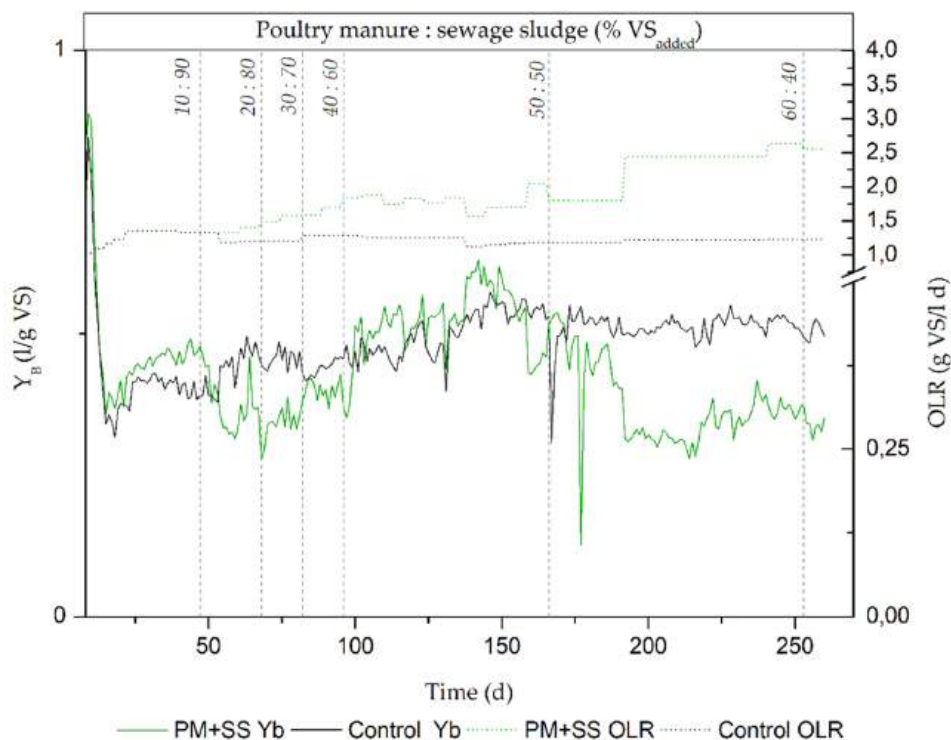


Fig. 6. Biogas production yield and organic loading rate of the anaerobic digestion processes.

significant increase in biogas production, by 35 % compared to the AD of sewage sludge, was obtained when the share of PM:SS co-substrates was 40:60 % w/w VS, respectively. Further increasing the proportion of PM in the mixture, up to 60 %, also resulted in a higher daily biogas gain ranging from 23 % to nearly 38 % with a PM:SS ratio of 42:58 % w/w VS.

The data on biogas and methane production yields, taking into account organic loading rates, were slightly different. During co-digestion at a share of PM up to 35 % in the mixture, fluctuations were observed in the values of biogas production yield in the range of 0.276–0.491 l/gVS_{added}, and methane production yield in the range of 0.168–0.334 l/gVS_{added}. When the share of PM was increased to 40 % in the input,

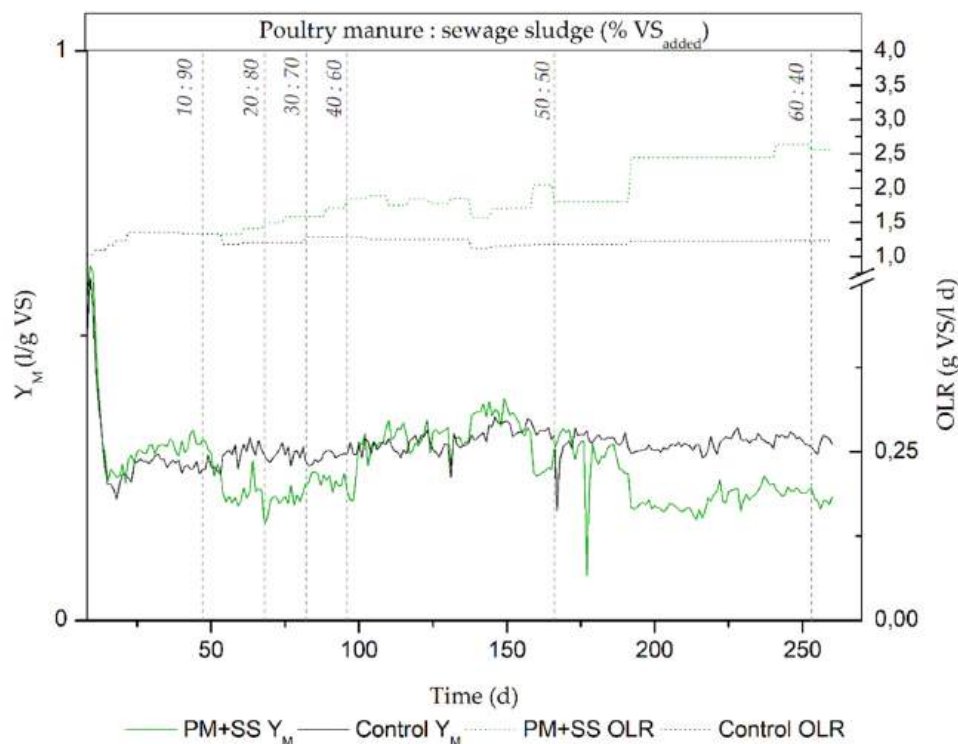


Fig. 7. Methane yield and organic loading rate of the anaerobic digestion processes.

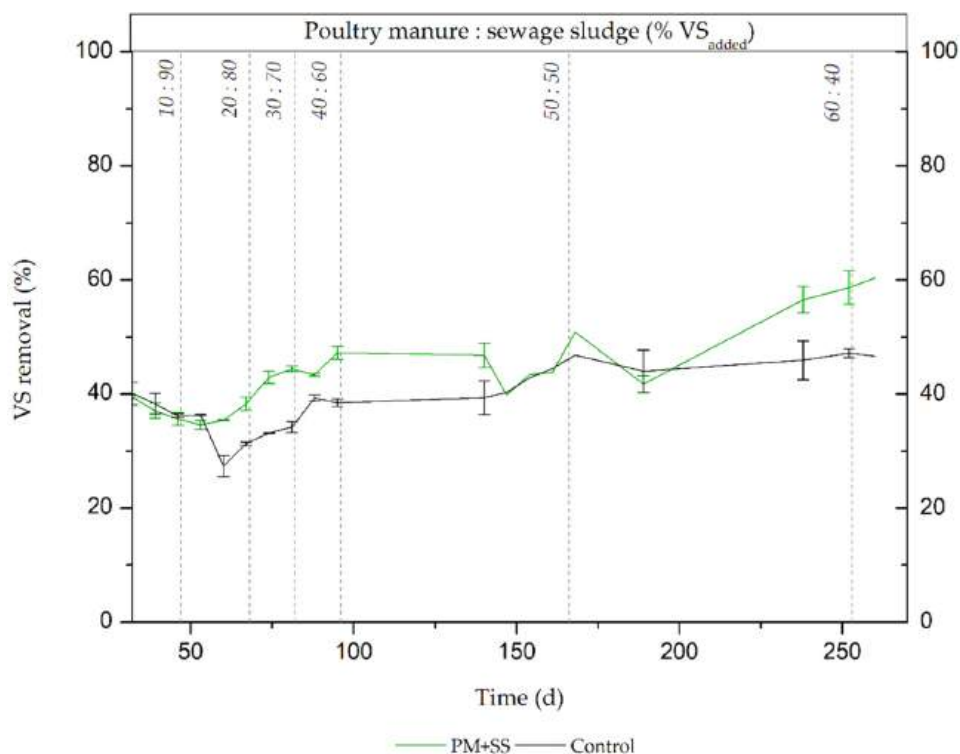


Fig. 8. Variations of volatile solids removal during the experiment.

higher values of these parameters were noted, averaging 0.503 l biogas/gVS_{added} and 0.309 l CH₄/gVS_{added}. The upward trend continued up to a PM share of 50 %, with the highest biogas production yield of 0.631 l/kgVS_{added} at PM:SS = 48:52 % w/w VS and a methane production yield of 0.389 l/gVS_{added} at PM:SS = 44:56 % w/w VS. Further increasing the

proportion of PM in the co-digestion mixture resulted in a decrease in the values of both parameters to an average of 0.340 l biogas/g VS_{added} and 0.213 l CH₄/g VS_{added}.

In the case of R2, where sewage sludge mono-digestion was carried out, the values of the biogas production factor oscillated in the range:

Table 2

Average composition of the biogas during experiment.

Reactor (% addition of PM in the feedstock)	CH ₄ (%)	CO ₂ (%)	O ₂ (%)	H ₂ S (p)	NH ₃ (p)	CO (p)
R1 (2.5–10 %)	66.67 ± 0.86	27.00 ± 0.45	0.00	0.95 ± 0.22	0.00	14.86 ± 6.02
R1 (10–20 %)	64.24 ± 2.28	27.48 ± 1.57	0.00	0.71 ± 0.46	0.00	14.48 ± 6.95
R1 (20–30 %)	61.6 ± 1.24	29.73 ± 0.59	0.00	1.00	0.00	16.73 ± 8.61
R1 (30–40 %)	61.5 ± 1.45	30.57 ± 0.51	0.00	0.71 ± 0.47	0.07 ± 0.27	9.14 ± 5.29
R1 (40–50 %)	61.55 ± 1.29	28.5 ± 1.6	0.00	0.07 ± 0.26	0.00	5.71 ± 0.95
R1 (50–60 %)	62.78 ± 1.30	29.42 ± 1.16	0.00	0.02 ± 0.21	0.00	38.19 ± 18.03
R1 (60 %)	62.21 ± 0.37	30.04 ± 0.26	0.00	0.38 ± 0.74	0.00	76.38 ± 21.88
R2 (0 %)	63.14 ± 2.64	29.55 ± 2.25	0.00	5.89 ± 7.83	1.71 ± 5.51	18.29 ± 16.84

0.308–0.573 l/gVS_{added}, and the methane production yield in the range: 0.193–0.357 l/gVS_{added}. A similar discrepancy in the results for daily methane production and the production yield taking into account the amount of VS_{added} was noted by Borowski et al (Borowski and Weatherley, 2013b). in their study on co-digestion of poultry manure and municipal sewage sludge at a ratio of 30:70 %VS_{added}. The lower gas yield per dry organic matter available in the reactor in the case of co-digestion may be due to the lower methanogenic potential of poultry manure relative to sewage sludge and the slight inhibition of the process due to ammonia release (Borowski and Weatherley, 2013b). Nevertheless, the author's research indicates an increase in the methane digestion efficiency of sewage sludge after introducing a 30 % addition of co-substrate in the form of poultry manure by about 50 %. There are few cases in the literature of joint digestion of the two wastes, but the available publications prove that co-digestion of sludge with poultry manure can increase the total biogas production volume and improve the sewage sludge treatment process (Borowski and Weatherley, 2013b; mahmoud et al., 2022; Mansour et al., 2023).

The observations were reflected in the estimated co-digestion performance index (CPI). Only for mixtures in which the proportion of poultry manure was between 40 % and 50 % based on VS, the value of the index was above 1, indicating a synergistic effect of co-digestion. In other co-digestion mixtures, CPI was lower than one, which indicates an antagonistic impact between substrates (Ebner et al., 2016b). The organic loading rate of the reactors for the R2 control trial ranged from 1.124 to 1.357 g/l·d. In the case of R1, where co-digestion was carried out, more considerable variations were observed in the value of this parameter, from 1.124 to 2.639 g/l·d, with a clear trend of increasing OLR with increasing the proportion of PM in the mixture. A similar relationship was observed in the case of the VS removal, where for the control sample, the average value was close to 40 %, while in the case of co-digestion, with the highest share of PM in the mixture, the value was 60 %.

When the proportion of PM in R1 did not exceed 15 %, no significant effect of co-digestion on more effective VS removal was observed, while at 20 % PM addition, a clear increase in this parameter was noted. At a co-substrate ratio of PM: SS=40:60 %, a decrease in the degree of digestion was observed, which may have been related to the higher concentration of ammonium nitrogen and thus the need for acclimatization of microorganisms. More effective VS removal was also observed by Sillero et al (Sillero et al., 2022). when poultry manure was added at 10 g/l to a 50:50 % mixture of sewage sludge and vine vinasses. These authors studied mono-digestion of sewage sludge, co-digestion of sewage sludge and vine vinasses in a 1:1 ratio, and three-component co-digestion: sewage sludge, vine vineasse and poultry manure in a 49.5:49.5:1 % ratio. They obtained the most satisfactory results for the last variant, where at different HRTs (20–6), the VS removal was in the range of 40–57 %, and the highest methane yield was obtained at HRT=13, with 261 mL CH₄/g VS added. Borowski et al (Borowski and Weatherley, 2013b). also indicate that co-digestion of poultry manure with sludge resulted in a higher VS removal efficiency (43.16–49.35 %) compared to the digestion of sludge alone (33.85–36.33 %). As shown by the researches (Sillero et al., 2023a, 2023b, 2024), the increase in methane production can be achieved by introducing a third co-substrate

and separating the acid phase from the methane phase in the processes of anaerobic co-digestion in the temperature phase (TPAD). However, the HRT for the individual phases should be selected experimentally. The mentioned authors, by reducing the length of the methane phase from 15 to 12 days, increased the methane yield from 320 (Sillero et al., 2023a) to 391 mLCH₄/gVS_{added} (Sillero et al., 2023b, 2024).

3.4. Quality of the anaerobic digestion products

Table 2. shows the approximate composition of biogas obtained during the various stages of the digestion process for the sludge alone and the mixture with poultry manure. Co-digestion did not significantly affect the quality of the gas, for both R1 and R2, the CH₄ content was more than 60 %, confirming the results obtained by Borowski et al (Borowski and Weatherley, 2013b). The highest percentage of methane was observed for PM addition at 2.5–10 % (VS_{added}), amounting to nearly 67 %. With sludge mono-digestion, relatively higher H₂S content was observed (5.89 ± 7.83 %) than co-digestion (0.02–1.00 %). Higher NH₃ concentrations were also observed for R2, at nearly 6 ppm, while for AcD, the value oscillated between 0.00 and 0.27 ppm. On the other hand, a marked increase in CO content in biogas was noted during co-digestion with PM addition above 50 % (VS_{added}), where its amount ranged from 38 to 76 ppm, while for AD of sewage sludge, it averaged 18 ppm.

It is well known that the anaerobic digestion process leads to the release of nutrients and trace elements from the substrates, causing them to accumulate more in the digestate (Borowski and Weatherley, 2013b; Czekala et al., 2020). Both poultry manure and sewage sludge belong to heterogeneous wastes, whose chemical composition varies depending on the diet and farming conditions in the case of manure and the source and technology of wastewater treatment. Agricultural management of these wastes, especially in the form of digestate, can positively affect crops and improve the physicochemical properties of soils, due to the presence of organic matter and nutrients, but it also involves the risk of secondary contamination with toxic substances (Jasińska et al., 2023b). Table 3. shows the elemental composition of the studied substrates and the digestate obtained from the anaerobic digestion and co-digestion processes. Among the processed wastes, poultry manure was characterized by a higher content of nutrients, such as Mg, Na, K and, in particular, calcium, which was determined at 82.014 mg/kg TS, which may be related to the presence of eggshell residues in the droppings of laying hens. On the other hand, sewage sludge was a better source of phosphorus and sulfur, which confirms literature reports that indicate high amounts of these elements in raw wastewater (Borowski and Weatherley, 2013b; Zerrouqi et al., 2020)]. Higher concentrations of metals such as Cr, Cu, Fe and Ni were also observed in the sewage sludge. Poultry manure contained more manganese and zinc, which is related to the high dietary requirements of animals for these elements, as they act as cofactors or activators for enzymes involved in the process of eggshell formation (Robert, 2004). For cadmium and lead, concentrations in both the raw waste and the digested sludge were below 1 mg/kg TS or below the detection levels. Cobalt was also not detected in the substrates, but in the digestate, its content was 13.31 mg/kg TS for R1 and 15.34 mg/kg TS for R2, which can be explained by the phenomenon

Table 3
Elemental content of the substrates and digestate (mg/kg TS).

Sample	Ca	Mg	Na	K	P	S	Al	Cd	Co	Cr	Cu	Fe	Mn	Ni	Pb	Zn
PM	82014 ±0.115	10018 ±0.149	3774 ±0.009	30094 ±0.071	22834 ±0.084	7541 ±0.018	1175 ±0.005	0.731 ±0.000	<dl	20.16 ±0.000	99.60 ±0.000	2437 ±0.011	566.6 ±0.002	23.00 ±0.000	<dl	584.2 ±0.001
SS	25158 ±0.097	3884 ±0.046	1936 ±0.044	3875 ±0.082	29091 ±0.216	10542 ±0.086	18442 ±0.332	0.947 ±0.000	<dl	351.4 ±0.005	216.0 ±0.002	23310 ±0.380	397.8 ±0.005	112.0 ±0.001	<dl	1897 ±0.021
D-AcD	59008 ±0.183	8213 ±0.327	15727 ±0.320	19899 ±0.413	36512 ±1.429	21236 ±0.898	8617 ±0.185	<dl	13.31 ±0.001	159.9 ±0.004	226.6 ±0.005	31827 ±1.097	786.9 ±0.033	117.7 ±0.003	<dl	1527 ±0.026
D-Con.	53429 ±0.460	10167 ±0.177	19214 ±0.451	14848 ±0.394	40163 ±1.090	21244 ±0.755	9773 ±0.301	<dl	15.34 ±0.000	204.4 ±0.006	236.6 ±0.007	39501 ±0.880	861.8 ±0.021	144.9 ±0.005	<dl	1672 ±0.053

PM – poultry manure; SS – sewage sludge; D-AcD – digestate from anaerobic co-digestion; D-Con. – digestate from anaerobic digestion of sewage sludge

of higher accumulation of metals in the AD product (Montusiewicz et al., 2020).

Undoubtedly, the products obtained from the anaerobic mono-digestion and co-digestion processes studied show potential for further use. The biogas obtained, both in the case of AD and AcD, was characterized by a high methane content, above 60 %, and a low percentage of pollutants. In practice, the treatment of digestion gas mainly focuses on the removal of H₂S (Angelidaki et al., 2018), which, in the case of the products obtained in this research, would only apply to biogas produced from sewage sludge mono-digestion. After eliminating H₂S, the biogas could be further upgraded to biomethane. The second product of the process, the digestate, could also find use in agriculture or as an additive for soil improvement due to the presence of organic matter and valuable elements. Therefore, this study carried out a physico-chemical study of the obtained digestate in terms of its fertiliser potential. In the case of poultry manure, the latest EU Fertilizer Regulation (Regulation, 2019) allows the use of this waste as a digestate, subject to subsequent thermal treatment in the case of mesophilic AD, such as through a pasteurization or composting process. However, the document excludes agricultural use of the product of AD of sludge, treating this material in the category of waste, the management of which is subject to other regulations.

In the European Union, the application of sewage sludge to soils is permitted, provided that the criteria contained in the Council Directive of June 12, 1986, on the protection of the environment, and in particular of the soil, when sewage sludge is used in agriculture (European Commission, 1986) are met. However, a new sewage directive is coming soon, which will include more extensive monitoring of the pollutants present in this waste. In Poland, however, the Regulation of the Minister of the Environment of February 6, 2015, on the use of municipal sewage sludge (Minister Środowiska, 2015) is in force. The main criterion determining the agricultural use of sewage sludge is its physicochemical and hygienic-sanitary properties. The resulting two types of digestate are characterized by trace element content below the upper limits of the value presented in the regulation, but the possibility of their use would require further testing for the presence of pathogens.

3.5. Greenhouse gases emissions

Management of bio-waste, including manure, is associated with the emission of gases responsible for the greenhouse effect. The most important of these include carbon dioxide, methane and nitrous oxide, with a global warming potential of 298 times higher for N₂O and 25 times for CH₄ compared to CO₂ (Pardo et al., 2015). Based on the results obtained from the substrate and methane production studies for anaerobic co-digestion, the emission potential of the above-mentioned gases was estimated for different poultry manure treatment scenarios. The calculations used equations proposed by Kreidenweis et al (Kreidenweis et al., 2021). in their study, who presented a new impact model that can assess the sensitivity of parameters and interdependencies between process emissions when comparing different broiler manure processing options. Fig. 8 shows the obtained results of CH₄, CO₂ and N₂O emissions calculations for poultry manure in the case of storage (1. scenario) and composting (2. scenario), and for anaerobic co-digestion of a mixture of poultry manure and sewage sludge, at a ratio of 60:40 % (VS_{added}), respectively, taking into account leakage when storing the digestate in an open (3. scenario) and closed (4. scenario) tank. First scenario assumes time-limited storage of manure in a closed concrete silo. Second scenario is a heap composting process on concrete slabs. For Scenarios 3. and 4., calculations were made for anaerobic co-digestion of a mixture of poultry manure and sewage sludge with the highest proportion of manure tested in the study, 60 %. This approach resulted from the limited practice of mono-digestion of manure, due to its properties and the associated risk of process inhibition (Kreidenweis et al., 2021). Fig. 9.

Analyzing the results, the highest methane emissions (0.4 kg/t of manure) were obtained for storage, and slightly lower levels of this gas

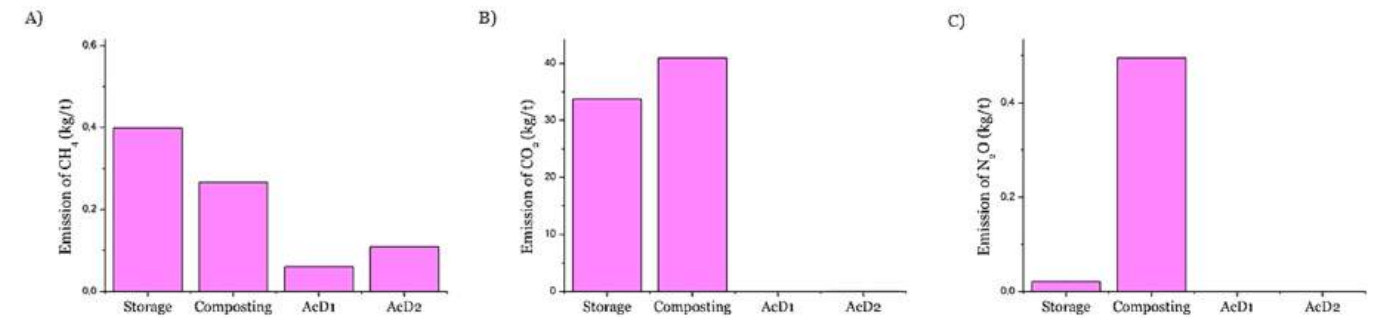


Fig. 9. Emissions of CH₄ (A), CO₂ (B) and N₂O (C) for four management options: storage of, composting of, anaerobic co-digestion of PM:SS = 60:40 % (VS_{added}) with closed digestate storage tank (AcD1), anaerobic co-digestion of PM:SS = 60:40 % (VS_{added}) with opened digestate storage tank (AcD2).

Table 4
Univariate Tests of Significance for VS removal.

Effect		F	p
Addition of	VS removal	36.92	0.00
	Methane yield	10.476	0.000000

were calculated for composting (0.27 kg/t of manure). In the case of co-digestion, the CH₄ emission values were lower, at 0.06 kg/t for AcD with a closed digestate storage tank and 0.11 kg/t for AcD with an open tank, with these emissions including potential leakage and not biogas production as the target product of digestion. For carbon dioxide, the highest emission value (40.99 kg/t) was calculated for the composting of manure and for storage the emission was slightly lower, amounted to 33.73 kg/t of manure. A significantly lower CO₂ level was calculated for anaerobic co-digestion, where for the AcD1 and AcD2 variants it was 0.04 and 0.07 kg/t of co-digestion mixture, respectively. Composting also produced the highest N₂O emissions, of 0.5 kg/t manure. For storage, the calculation result for this gas is 0.02 kg/t of manure, while no emissions were calculated for both AcD1 and AcD2.

The obtained results indicate the advantage of the process of anaerobic co-digestion of poultry manure over other methods of managing this waste in terms of GHG emissions, which confirms the conclusions of Kreidenweis et al (Kreidenweis et al., 2021). Nevertheless, in the case of calculations, it should be taken into account that for anaerobic co-digestion scenarios, the emission level refers to a ton of substrate

Table 6
Tukey HSD test; variable YM.

Addition of	YM	a	b	c	d	e
Mean						
D20	0.201251	****				
D52	0.209673	****				
D60	0.211221	****				
D12.5	0.211621	****				
D25	0.216216	****	****			
D54	0.226471	****	****	****		
D15	0.234088	****	****	****		
D35	0.242527	****	****	****		
D30	0.250249	****	****	****		
D48	0.260553	****	****	****	****	
D0	0.281197	****	****	****	****	****
D10	0.287415	****	****	****	****	****
D50	0.287796	****	****	****	****	****
D2.5	0.299701		****	****	****	****
D5.0	0.305331			****	****	****
D40	0.309666			****	****	****
D7.5	0.312901			****	****	****
D46	0.341680				****	****
D44	0.365810					****
D42	0.389050					****

in the form of a mixture of manure and sewage sludge, and not fresh manure under the assumptions for composting and storage. Therefore, these calculations are indicative, and a broader analysis should also take into account net GHG emissions from transport and further processing of

Table 5
Tukey HSD test; variable VS removal.

Addition of	VS removal	a	b	c	d	e	f	g	h
Mean									
D10	34.58301	****							
D12.5	35.41606	****	****						
D7.5	35.63270	****	****						
D5.0	37.07645	****	****	****					
D15	38.27354	****	****	****	****				
D2.5	39.46969	****	****	****	****				
D0	39.58947	****	****	****	****				
D42	39.78799	****	****	****	****				
D50	41.75263		****	****	****	****			
D20	42.93402			****	****	****			
D30	43.35962			****	****	****			
D44	43.48969			****	****	****			
D46	43.80062				****	****			
D25	44.39653				****	****	****		
D40	46.81155					****	****		
D35	47.22913					****	****		
D48	50.89729						****	****	
D52	56.55506							****	****
D54	58.67072								****
D60	60.33229								****

the products of the analyzed manure management methods, such as compost management or CHP units in the case of the anaerobic digestion process.

3.6. Statistical analysis

Results achieved for one-way ANOVA (F-values for selected parameters), confirm the previous observation that the addition of PM has the highest positive impact on VS removal and methane yield (Table 4). However, the final effect depended on the addition in the feedstock. In the case of VS removal, an increase in the parameter, compared to the control sample, was recorded when the co-substrate addition was higher than 15 % (VS_{added}). On the other hand, for YM, the highest values were obtained when the addition was greater than 40 % (VS_{added} (Tables 5 and 6).

4. Conclusions

The following conclusions can be drawn from this study:

- The anaerobic co-digestion of poultry manure and sewage sludge has a beneficial effect on the process performance and the quantity of the obtained products in the form of biogas and digestate.
- At 40–60 % (VS_{added}) share of manure in the co-digestion mixture, more than an average 40 % (VS_{added}) increase in daily biogas production was observed compared to the digestion of sewage sludge alone. Moreover, better VS removal (34.6–60.3 %) was reported for co-digestion than for sludge mono-digestion (27.4–55.2 %).
- No ammonia inhibition or other process interference was observed when the proportion of poultry manure was increased, in the feedstock.
- Co-digestion did not significantly affect the composition of the obtained biogas, in both cases, the methane content was over 60 %, with the gas from the manure mixture being cleaner from H₂S and NH₃, while containing more CO on average.
- The digestate from the waste mixture contained more Ca, K and NH₄⁺, while a higher amount of Mg, Na, P and Fe was found for the sludge digestate product. The concentrations of other elements were at similar levels for both samples, and the determined values met the criteria in the legal acts governing the possibility of agricultural use of the digestate.
- The GHG emission calculations results indicate the advantage of the process of anaerobic co-digestion of poultry manure over other methods of managing this waste.
- The treatment of multiple wastes, combined with an increase in the efficiency of anaerobic digestion (AD), is in line with the new EU policy supporting an increase in the share of renewable energy in the overall energy balance, as well as sustainable waste management and the rational use of natural resources.
- Nonetheless, implementing this solution within the wastewater treatment facility demands further investigations, including determining optimal anaerobic digestion parameters (e.g. HRT, OLR) and comprehensive solutions about wastewater treatment plant infrastructure (e.g., regarding pumping co-substrate into digestion chambers). In addition, in further research it is worth considering, among others: 1) the issue of the possibility of inhibiting the process not so much as a result of the accumulation of ammonia nitrogen but rather the impact on the efficiency of the process of antibiotics present in poultry manure (determination of inhibiting levels); 2) the evaluation of the ecological influence of the suggested resolution employing LCA instruments; 3) reduction of high concentrations of ammonia nitrogen in the supernatant, 4) changes in the population of microorganisms during the process.

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CRediT authorship contribution statement

Erik Meers: Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition. **Ana Robles Aguilar:** Writing – review & editing, Methodology, Investigation. **Anna Jasińska:** Writing – review & editing, Writing – original draft, Visualization, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Anna Grosser:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

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Data Availability

Data will be made available on request.

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D.5

Anna Jasińska, Erik Meers, Anna Grosser (2025)

*Sustainable digestate management as a supplement
to the casing layer in mushroom cultivation*

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Sustainable digestate management as a supplement to the casing layer in mushroom cultivation

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Abstract

Digestate, a by-product of anaerobic digestion, is increasingly considered a valuable resource for sustainable agriculture. While its use in soils and mushroom substrates has been studied, little is known about its potential as a casing amendment in *Agaricus bisporus* cultivation. This study evaluated the effects of two types of digestates, derived from sewage sludge anaerobic mono-digestion (Ss) and sewage sludge anaerobic co-digestion with poultry manure (PM), applied at 3%, 5%, and 10% (w/w) of casing mass. Mushroom yield, biological efficiency, cap diameter, and chemical composition (TS, VS, TKN, macro- and micronutrients, heavy metals) were assessed over two flushes, while casing and substrates were analyzed before and after cultivation. Results showed that low digestate doses (3%) maintained yields comparable to the control, whereas higher levels (10%) reduced biological efficiency and cap size. Correlation analysis highlighted strong positive associations between yield parameters and K, Mg, and P contents in mushrooms. ANOVA confirmed significant effects of both digestate type and dose on yield and mineral composition, with notable interactions. These findings indicate that digestate can serve as a partial casing amendment, enabling nutrient recycling, but its effectiveness depends strongly on dose and origin.

Keywords: digestate, mushroom cultivation, *Agaricus bisporus*, poultry manure, sewage sludge, casing, circular economy, anaerobic co-digestion.

Abbreviations

AD, anaerobic digestion; BE, biological efficiency; cAD, anaerobic co-digestion; DM, dry mass; EC, electrical conductivity; IQR, interquartile range; N-NH₄⁺ ammonium nitrogen; PCA, principal component analysis; PM, digestate obtained from anaerobic co-digestion of sewage sludge with poultry manure; Ss, digestate obtained from anaerobic digestion of sewage sludge; TC, total carbon; TKN, total Kjeldahl nitrogen; TS, total solids; VS, volatile solids.

1. Introduction

In the face of increasing social awareness of climate change and the limited availability of natural resources, actions towards broadly understood sustainable development are gaining importance. The concept of the circular economy is being implemented by policymakers, businesses, and consumers alike. Current environmental challenges concern the use of alternative energy sources, rational waste and resource management, as well as sustainable agriculture and food production, including meat and livestock farming [1,2]. The beginning of the 21st century marked a clear shift towards the development of renewable energy sector, supported both by the market and by legislation, with the aim of achieving climate neutrality and resilience of the energy system in the European Union [1,3]. The revised RED III Directive, in force since November 2023, sets a binding target of a 42.5% share of renewable energy sources (including wind, solar, hydropower, and biomass) by 2030 [4]. Biomass plays a particularly important role, linking energy issues with waste management[3]. Agriculture generates considerable amounts of by-products, many of which remain underutilized. These residues are usually stored or incinerated, which poses a potential threat to the environment [1]. Agricultural waste includes animal manure, which is produced in large quantities as a result of intensive meat, dairy, and egg production. Poland is one of the leaders in poultry farming in Europe, which means also facing the challenge of managing the waste generated in this sector [5,6]. Due to the high fertilizing potential of manure, it has been common practice to apply it directly to the soil. However, this method of management carries the risk of contamination with pathogens, nitrogen compounds, greenhouse gas emissions resulting from uncontrolled decomposition and is subject to strict legal regulations [7]. In 2023, the Polish government implemented the Nitrate Action Programme [8], which regulates, among other things, the use of raw manure as fertilizer and sets out guidelines for good agricultural practices. The above-mentioned regulation completely prohibits the storage of poultry manure directly on the field, while other types of manure are not subject to such restrictions.

One of the promising methods for managing organic waste that addresses the challenges described above is anaerobic digestion. This technology is well established and applied on a large scale in several European countries [9]. This process enables the recovery of valuable nutrients and organic matter present in biowaste, as well as the production of an alternative energy source in the form of biogas [3]. Anaerobic digestion is based on a sequence of processes involving the decomposition of biodegradable materials by microorganisms without the presence of oxygen. The gaseous product of the reaction is a mixture consisting mainly of methane and carbon dioxide. This process also generates a digestate containing water, minerals and organic compounds, that are not decomposed by microorganisms [2,3]. The final properties of the digestion product depend on the substrates used and the process conditions [10]. In addition to agricultural waste, other streams of biowaste can be used for biogas production. Due to the intensive development of wastewater treatment infrastructure and technology in recent years, sewage sludge is constantly generated in large quantities. Its heterogeneous composition and the presence of

contaminants pose a challenge in selecting the appropriate method of its management [11,12]. Furthermore, with the adoption of The revised Urban Wastewater Treatment Directive [13], which entered into force on January 1, 2025 by EU member states, efforts in this sector must be strengthened. The latest update to the directive lists new pollutants that will be monitored in wastewater. In addition, the document regulates the issue of energy self-sufficiency of wastewater treatment plants. By 2045, some facilities will be required to provide 100% of energy from their own sources, primarily from biogas production. Mono-digestion of sewage sludge may not be effective enough to meet the target set by EU decision-makers. Therefore, many authors have conducted research on methods to increase the efficiency of anaerobic digestion. One of the strategies used for this purpose is the simultaneous processing of several types of waste, i.e., co-digestion. This approach is very promising due to the possibility of managing more than one type of waste, and its simplicity also offers economic benefits [14,15]. The factors determining the efficiency of the co-digestion process are the appropriate selection of raw materials and process conditions optimal for microorganisms [16]. The proper balance of feedstock components enables the efficient production of biogas and digestate with high fertilizing potential.

Digestate is primarily used in agriculture as a component of cultivation substrates or as a soil conditioner [17]. This material contains both valuable macro- and micronutrients as well as organic matter, and therefore competes strongly with mineral fertilizers [3]. Following the outbreak of the pandemic and the war in Ukraine, a raw material crisis significantly reduced the conventional production of fertilizers through chemical synthesis. Disrupted supply chains and difficulties in accessing deposits of natural gas and potash salts have led to greater interest in the valorization of organic waste into fertilizers [18,19]. However, the application of digestate to agricultural land is strictly regulated by law, due to the risk of secondary soil contamination [1]. In addition to relatively well-known pollutants such as heavy metals, new compounds such as pharmaceuticals, microplastics, and household and industrial chemicals are increasingly being identified in biowaste [2,20]. While the use of digestate from processed agricultural residues does not usually cause much controversy, the attitude towards the aforementioned manure and sewage sludge is different [3]. An important document that harmonized regulations concerning the use of bio-based fertilizers and their introduction to the EU market is Regulation (EU) 2019/1009 of the European Parliament and of the Council of June 5, 2019 [21]. This gives the green light for fertilizing land with products from agricultural biogas plants or manure after appropriate treatment, but excludes the use of sewage sludge, which is regulated by separate regulations. However, it should be considered that with the adoption of significant amendments to the Urban Wastewater Treatment Directive [13] many installations have been required to introduce third and fourth stage of wastewater treatment. Therefore, sewage sludge of better quality, free of many contaminants, may be obtained, which may influence its future valorization into fertilizer.

In line with the goals of the Green Deal, cooperation between the waste management and food production sectors is extremely important in order to keep nutrients in circulation. Consumer awareness is increasing with regard to the nutritional value of products, their origin and

sustainable production with respect to the environment. With the growing global population, the demand for food will increase. Paradoxically, large amounts of food are still wasted, ending up in landfills or incinerated [1,15]. It is estimated that by 2050 global meat production will rise to 465 million tons, compared to 330 million tons in 2017 [22]. It should be noted that GHG emissions from this sector account for about 18% of total global emissions [23]. Research on future diets suggests reducing meat consumption in favor of vegetables and other protein substitutes [24–26]. In this context, mushrooms have gained importance as foods rich in protein and valuable elements, with additional medicinal properties, and their cultivation is less resource-intensive in terms of energy, production time, and land use [1,27,28]. Mushrooms can also be used as feed additives, pharmaceuticals, nutraceuticals, and even in fashion and architecture [29,30]. Their production generates significant economic income in many regions of the world, and the most popular species cultivated is *Agaricus bisporus* [31]. Poland is the main producer and exporter of white mushrooms in Europe, where mushroom consumption is very high [32–34]. This is related not only to the availability of agricultural substrates, but also to relatively low labor costs compared to other European countries, as well as the use of the national currency [34].

Agaricus bisporus is a heterotrophic organism that naturally grows on partially degraded organic matter [35]. In commercial (controlled) cultivation, the substrate consists of digested plant biomass, e.g., straw and poultry or horse manure, depending on the availability of substrates [36]. Due to their metabolism, mushrooms have a high potential for degrading difficult-to-decompose lignocellulosic materials such as agricultural waste [29,37]. However, the heterogeneous mixture of substrates for mushroom growth must first be partially depolymerized by other microorganisms during the composting process. In subsequent stages of mushroom cultivation, the remaining microorganisms interact directly with the mycelium, enabling further development and fruiting body formation [31]. Cultivation is based on composted mixtures of plant biomass and manure [35,36], in which microbial activity prepares the substrate for colonization and fruiting [31,37]. The appropriate selection and preparation of the substrate is thus crucial for yield and mushroom quality. Importantly, the chemical composition of mushroom fruiting bodies is strongly influenced by the growth medium, including macro- and micronutrient availability. Variations in nitrogen, phosphorus, potassium, and trace elements in substrates have been shown to affect protein content, mineral profiles, and even antioxidant properties of mushrooms [38–40]. This highlights the importance of both substrate composition and potential new nutrient sources such as digestate. In addition to the substrate, the casing layer plays a pivotal role in *A. bisporus* cultivation. Unlike the compost substrate, which provides nutrients, the casing is primarily responsible for maintaining moisture, regulating gas exchange, and supporting the microbial community that stimulates fruiting initiation (pinning) [41,42]. It is intentionally poor in readily available nutrients to avoid excessive vegetative mycelial growth (“overlay”), which can delay or reduce fruiting [43,44]. Instead, the casing functions mainly as a water reservoir and physical barrier, with only limited transfer of soluble ions to fruiting bodies [42]. Studies have demonstrated that modifications in casing composition, such as replacing peat with soil, coir, or compost can significantly affect fruiting dynamics, disease incidence, and yield quality [45].

To date, scientific literature contains very few reports on the use of digestate in casing layers. Most studies have focused on its use in substrates, where digestate can partially or fully replace animal manure without yield reduction [46,47]. A limited number of studies have explored composted sewage sludge as a casing material, with results indicating possible uptake of heavy metals by mushrooms [48]. However, direct application of digestate from anaerobic mono- or co-digestion of sewage sludge and poultry manure in casing has not been systematically studied. This represents a significant research gap.

From a valorization perspective, the addition of digestate to the casing layer may hold promise. The casing is the primary water reservoir and ecological niche in *A. bisporus* cultivation, and its physicochemical properties and associated microbiome strongly regulate primordia initiation and subsequent yield. Modifications in casing composition that enhance water-holding capacity or shift microbial communities have been repeatedly linked to earlier pinning, improved fruiting dynamics, and higher productivity [45]. By analogy, carefully dosed digestate additives could improve water retention, enrich microbial activity, and potentially influence the nutritional composition of fruiting bodies. Evidence from substrate studies further supports this hypothesis, as digestate-based composts have successfully substituted manure without yield loss, indicating agronomic compatibility within mushroom production systems [46,47]. Nonetheless, caution is warranted: studies involving sewage-derived compost in casing materials have documented elevated transfer of heavy metals to fruiting bodies, emphasizing the need for rigorous monitoring of pH, EC, ammonium, and trace metals when using sewage sludge-based digestate [48].

Therefore, this study investigated the effect of adding digestate from sewage sludge anaerobic digestion and from sewage sludge co-digestion with poultry manure to the casing layer at inclusion rates of 3%, 5%, and 10% of the total casing mass. The primary objective was to evaluate the potential of digestate as a casing amendment in mushroom cultivation, focusing on its role in improving physical properties, enhancing water retention and providing an additional source of nutrients and trace elements. In addition, the study aimed to assess its impact on mushroom yield and fruiting body composition, with particular attention to the possible migration of trace elements from the casing into the mushrooms.

2. Materials and Methods

2.1. Digestate and commercial cultivation kit for *Agaricus bisporus*

In this study, two types of digestate were applied: (Ss) derived from anaerobic digestion of sewage sludge, and (PM) obtained from anaerobic co-digestion of sewage sludge with poultry manure. The sewage sludge was collected from a municipal wastewater treatment plant located in the Silesia region of Poland, while poultry manure was sourced from a laying poultry farm in the same region. The manure originated from adult animals. Detailed descriptions of the substrates, anaerobic digestion process conditions, and digestate characteristics were reported in previous research [49]. Digestates were collected directly after anaerobic digestion as whole (non-separated) material and homogenized prior to experimental use.

For mushroom cultivation, commercially available compost-based substrates (phase III), already colonized with *Agaricus bisporus* mycelium (Netherlands), were used. The compost was supplied with a standard peat-based casing soil, designated as the control treatment. In experimental variants, the peat casing was supplemented with digestate (Ss or PM) at inclusion rates of 3%, 5%, and 10% of the total casing mass.

Characteristics of the materials used in this study are presented in Table 1.

2.2. Analytical methods

Physicochemical analyses included the determination of total solids (TS), volatile solids (VS), pH, electrical conductivity (EC), ammonium nitrogen (N-NH_4^+), total carbon (TC), and total Kjeldahl nitrogen (TKN). All measurements were performed according to APHA Standard Methods. For compost and casing soil, pH, EC and N-NH_4^+ concentrations were measured in aqueous extracts prepared by mixing 10 g of fresh material with 25 mL of distilled water (1:2.5 w/v), followed by shaking and filtration. For digestate samples, pH, EC and N-NH_4^+ were determined in the liquid fraction, after centrifugation and subsequent filtration.

TS and VS were determined gravimetrically by drying samples at 105 °C and igniting residues at 550 °C in a muffle furnace. TKN and TC were analyzed using a CN analyzer.

Nutrients and heavy metals concentrations were determined by inductively coupled plasma optical emission spectrometry (ICP-OES, Thermo iCAP 7400). Prior to analysis, compost, casing soil, and digestate samples were digested in reverse aqua regia using a microwave digestion system (Milestone UltraWAVE). For mushroom fruiting bodies, ICP-OES analysis was performed after digestion in 10% HNO_3 under the same conditions.

All analyses were carried out in triplicate.

2.3. Mushroom cultivation and yield evaluation

The cultivation experiment was conducted in 21 boxes (volume ~7.5 L), divided into seven treatments with three replicates each: control (peat casing), 3% SS, 5% SS, 10% SS, 3% PM, 5% PM, and 10% PM (percentage values refer to the proportion of digestate in the total casing mass).

Mushrooms were irrigated with tap water every 3–4 days to maintain casing moisture. After the first flush, ~200 mL of water was applied to each box to replenish losses. Fruiting bodies were harvested before cap opening (no sporulation). A total of two flushes were obtained during the experimental period.

All harvested fruiting bodies were counted and weighed. Yield was expressed as kilograms of fresh mushrooms per kilogram of substrate. Biological efficiency (BE%) was calculated as the ratio of fresh mushroom weight to dry substrate weight $\times 100$. The average cap diameter was measured for each treatment using calipers. Dried mushroom samples were further analyzed for nutrient and heavy metal concentrations as described in Section 2.2.

Table 1. Basic parameters of digestate, substrate and casing layers

Sample	TS (%)	VS (%)	pH	EC (mS/cm)	N-NH4 (mg/l)	TKN (mg/kg)	TC/TKN
<i>Ss</i>	1.43 ± 0.02	0.80	7.27	8.62	788.7	1060	3.36
<i>PM</i>	2.63 ± 0.42	1.53	7.33 – 7.53	12.47 – 12.48	1241 - 1274	1410 – 1630	2.11
<i>Substrate</i>	36.20 ± 1.35	22.15	5.98	13.06	351.3 ± 0.68	21140 - 21350	13.91
<i>Control</i>	22.46 ± 0.19	15.88	7.46	0.38	5.90 ± 0.13	10920 ± 981	14.61
<i>Ss 3%</i>	22.45 ± 1.90	14.82	7.46	0.40	5.10 ± 0.08	11480 ± 280	15.76
<i>Ss 5%</i>	20.86 ± 0.54	15.96	7.46	0.45	3.90 ± 0.14	10640 ± 840	12.30
<i>Ss 10%</i>	20.22 ± 0.30	15.49	7.47	0.45	3.10 ± 0.15	11200 ± 741	11.79
<i>PM 3%</i>	21.19 ± 0.46	15.57	7.44	0.41	8.90 ± 0.03	11013 ± 162	12.22
<i>PM 5%</i>	21.28 ± 0.40	15.54	7.53	0.41	7.70 ± 0.04	10733 ± 162	13.89
<i>PM 10%</i>	20.10 ± 0.22	15.83	7.65	0.51	5.70 ± 0.11	10827 ± 900	11.77

Table 2 presents nutrient composition: Na, Mg, K, Ca, S, P (mg/kg) in digestate, substrate, and casing.

The content of trace elements: Al, Cr, Mn, Fe, Co, Ni, Cu, Zn, Cd, Pb determined in the materials used for the experiment is presented in Table 3.

Table 2. Nutrients content of the digestate, substrate and casing [mg/kg]

<i>Sample</i>	Na	Mg	K	Ca	P	S
<i>Ss</i>	19213 ± 0.45	10167 ± 0.18	14848 ± 0.39	53429 ± 0.46	40163 ± 1.09	21244 ± 0.76
<i>PM</i>	15380 ± 0.32	9049 ± 0.33	19282 ± 0.41	58606 ± 0.28	37197 ± 1.43	21125 ± 0.90
<i>Substrate</i>	3080 ± 0.22	4072 ± 0.15	34079 ± 2.13	31525 ± 3.81	5910 ± 0.01	23558 ± 0.65
<i>Control</i>	252.5 ± 0.02	5852 ± 0.13	710.5 ± 0.01	35360 ± 0.63	245.6 ± 0.02	1864 ± 0.12
<i>Ss 3%</i>	300.7 ± 0.04	4779 ± 0.71	662.2 ± 0.03	30061 ± 3.29	277.7 ± 0.02	1636 ± 0.09
<i>Ss 5%</i>	409.6 ± 0.02	5310 ± 0.42	766.1 ± 0.05	33200 ± 1.92	341.2 ± 0.01	1713 ± 0.07
<i>Ss 10%</i>	477.4 ± 0.04	5213 ± 0.23	823.4 ± 0.03	33308 ± 1.44	479.2 ± 0.03	1789 ± 0.06
<i>PM 3%</i>	333.2 ± 0.02	5294 ± 0.15	749.3 ± 0.03	32759 ± 0.14	316.3 ± 0.02	1708 ± 0.03
<i>PM 5%</i>	348.3 ± 0.01	5776 ± 0.07	796.9 ± 0.04	35492 ± 0.66	382.6 ± 0.01	1783 ± 0.10
<i>PM 10%</i>	552.1 ± 0.03	5571 ± 0.26	950.4 ± 0.01	34333 ± 0.52	564.4 ± 0.04	1862 ± 0.07

Table 3. Trace elements content of the digestate, substrate and casing [mg/kg]; <dl – under detection level

<i>Sample</i>	Al	Cr	Mn	Fe	Co	Ni	Cu	Zn	Cd	Pb
<i>Ss</i>	9773 ± 0.30	204.4 ± 0.01	861.8 ± 0.03	39501 ± 0.88	15.34 ± 0.00	144.9 ± 0.00	236.6 ± 0.01	1672 ± 0.05	<dl	<dl
<i>PM</i>	8740 ± 0.19	161.0 ± 0.00	818.5 ± 0.02	32511 ± 1.22	13.31 ± 0.00	119.4 ± 0.01	227.0 ± 0.00	1543 ± 0.03	<dl	<dl
<i>Substrate</i>	1517 ± 0.05	6.06 ± 0.00	223.4 ± 0.01	1401 ± 0.08	<dl	2.93 ± 0.00	32.78 ± 0.00	152.12 ± 0.01	<dl	<dl
<i>Control</i>	3187 ± 0.08	6.82 ± 0.00	145.9 ± 0.00	3792 ± 0.38	<dl	4.61 ± 0.00	<dl	9.07 ± 0.00	<dl	<dl
<i>Ss 3%</i>	2832 ± 0.19	6.70 ± 0.00	151.6 ± 0.02	3793 ± 0.78	<dl	3.93 ± 0.00	<dl	10.17 ± 0.00	<dl	<dl
<i>Ss 5%</i>	2991 ± 0.03	7.13 ± 0.00	148.7 ± 0.01	3990 ± 0.80	<dl	4.47 ± 0.00	3.50 ± 0.00	15.59 ± 0.00	<dl	<dl
<i>Ss 10%</i>	3080 ± 0.10	7.97 ± 0.00	145.7 ± 0.01	4165 ± 0.66	<dl	5.14 ± 0.00	4.01 ± 0.00	20.26 ± 0.00	<dl	<dl
<i>PM 3%</i>	2948 ± 0.11	11.00 ± 0.01	138.6 ± 0.00	3367 ± 0.13	<dl	4.29 ±	<dl	13.31 ± 0.00	<dl	<dl
<i>PM 5%</i>	3004 ± 0.15	6.69 ± 0.00	154.8 ± 0.01	3891 ± 0.52	<dl	4.45 ± 0.00	<dl	15.34 ± 0.00	<dl	<dl
<i>PM 10%</i>	3138 ± 0.15	7.31 ± 0.00	155.6 ± 0.01	3775 ± 0.21	<dl	5.07 ± 0.00	4.99 ± 0.00	24.24 ± 0.00	<dl	<dl

2.4. Statistical analysis

The statistical analyses were carried out to evaluate the effects of digestate type (sewage sludge – SS, and sewage sludge co-digested with poultry manure – PM) and dose (3%, 5%, and 10% of casing mass) on mushroom yield, morphological parameters, and elemental composition, as well as on the physicochemical properties of the casing material before and after cultivation.

The distribution and variability of the experimental data were assessed using descriptive statistics and box plots, which visualized data dispersion, central tendency, and the presence of potential outliers. The box represents the interquartile range ($IQR = Q3 - Q1$), encompassing the middle 50% of observations; the horizontal line indicates the median; whiskers extend to $1.5 \times IQR$; dots denote outliers; and the mean is marked by a circle.

To explore relationships among quantitative variables, correlation analyses were performed using two complementary approaches. Pearson's correlation coefficient (r) was applied to evaluate linear associations between variables under the assumption of an approximately normal distribution, while Spearman's correlation coefficient (ρ) served as a non-parametric measure of monotonic relationships, less sensitive to outliers and deviations from normality. These analyses were used to examine potential interdependencies among mushroom yield, quality traits, nutrient content and composition of the casing materials.

Subsequently, a two-way analysis of variance (ANOVA) was conducted to simultaneously assess the main effects of digestate type and dose, as well as their interaction, on yield, morphological traits, nutrient composition, and selected physicochemical properties of the casing before and after cultivation. Statistical significance was established at a level of $\alpha = 0.05$. The effect size was estimated using the η^2 (eta squared) coefficient, which quantifies the proportion of total variance explained by each factor. When significant effects were detected, Tukey's HSD post hoc test was applied to identify homogeneous groups of means not differing significantly from each other. The results are presented in both tabular and graphical form, with homogeneous groups indicated by letters above mean values.

To assess the impact of various additives and doses on the yield parameters and quality of mushroom fruiting bodies, a PCA analysis was performed, taking into account all quantitative variables (weight, diameter, yield, biological efficiency, macro- and microelement content, TS and VS parameters).

All statistical analyses were performed using standard statistical software. Statistical significance and multivariate outputs were interpreted in the context of experimental variability and biological relevance.

3. Results and discussion

3.1. Characterisation of digestate, substrate and casing

The physicochemical characteristics of the digestates, compost-based substrate and casing mixtures used in this study are presented in Tables 1–3. Both digestates differed in their basic composition, reflecting the distinct feedstocks and anaerobic digestion conditions. The digestate derived from sewage sludge (Ss) exhibited lower total solids (TS = 1.43%) and volatile solids (VS = 0.80%) compared to the digestate from sewage sludge co-digested with poultry manure (PM; TS = 2.63%, VS = 1.53%). These values fall within the typical range for liquid digestates (1–10% TS) reported in the literature [50,51]. The pH of both digestates was slightly alkaline (7.27–7.53), which is common for stabilized digestates after methanogenesis [52]. Electrical conductivity (EC) was markedly higher in the PM digestate (≈ 12.5 mS/cm) than in the Ss digestate (8.6 mS/cm), indicating a higher ionic load, particularly due to elevated ammonium nitrogen ($\text{N-NH}_4^+ = 1241\text{--}1274$ mg/L) and total Kjeldahl nitrogen (TKN = $1410\text{--}1630$ mg/kg). These differences result from the nitrogen-rich nature of manure feedstock [53]. The carbon-to-nitrogen (C/N) ratio was lower in PM (2.1) compared to Ss (3.4), consistent with previous findings that co-digestion with poultry manure produces nutrient-dense digestates with a more mineralized nitrogen fraction.

The mushroom substrate showed high total solids (36.2%) and organic matter content (VS = 22.2%), with a slightly acidic pH (5.98) and high EC (13.06 mS/cm). Such properties are typical for mature phase III compost used for *Agaricus bisporus* cultivation [54,55]. Ammonium nitrogen concentration in the substrate (351 mg/L) and total nitrogen content (≈ 21 g/kg) confirm that compost provides sufficient nutrients for mushroom development, whereas its high C/N ratio (~ 14) indicates moderate organic stability.

The control casing (peat-based) was characterized by neutral pH (7.46), very low EC (0.38 mS/cm), and minimal available ammonium nitrogen (5.9 mg/L), reflecting its role as a biologically active but nutrient-poor layer designed to support fruiting rather than vegetative growth [43]. Addition of digestate to the casing layer caused only minor fluctuations in pH (7.44–7.65) and slightly increased EC, most notably at a 10% additive dose (up to 0.51 mS/cm in PM 10%). This indicates a dose-dependent rise in soluble ions. The increase in N-NH_4^+ concentration was more pronounced for PM-enriched casings (up to 8.9 mg/L at 3%), suggesting a greater availability of mineral nitrogen forms than in Ss variants (3.1–5.1 mg/L). However, TKN values in all treatments remained within a narrow range ($\approx 10.6\text{--}11.5$ g/kg), confirming that digestate additions mainly altered the readily available rather than total nitrogen pool.

Nutrient analysis (Table 2) revealed that both digestates contained substantial amounts of macronutrients such as Ca, P, and K, with higher Ca concentrations in PM (58.6 g/kg) and higher P in Ss (40.2 g/kg). These levels exceed those found in the substrate and especially in the peat-based control casing, which was nutrient-poor as expected (Ca = 35.4 g/kg; P = 245 mg/kg). The incorporation of digestate increased nutrient levels in the casing, particularly Na, K, and P, in a dose-dependent manner. For instance, the concentration of K rose from 710

mg/kg in the control to 950 mg/kg in PM 10%, while P increased more than twofold (from 246 to 564 mg/kg).

Trace element analysis (Table 3) showed that the digestates, especially Ss, contained markedly higher Fe, Al, and Zn concentrations compared to the substrate and casing. The addition of digestate slightly increased trace element concentrations in the casing mixtures, but the overall levels of potentially toxic metals (Cd, Pb, Ni, Cr) remained well below regulatory limits for agricultural use (EU Regulation No. 2019/1009). The increase in Fe and Zn was most evident at 10% Ss and PM doses, consistent with the metal profiles of the respective digestates. These findings indicate that controlled inclusion rates up to 10% do not pose risk of heavy metal contamination and can enhance the micronutrient content of the casing layer without compromising safety.

3.2. Mushroom production and composition of fruiting bodies

The first flush of mushrooms began on day 29 after the application of the casing layer and lasted for 11 days, with fruiting bodies appearing at different intervals across the experimental variants. Irregular pinning of the casing surface was observed, particularly at the highest digestate inclusion rates. In the control treatment, the casing surface was evenly covered with fruiting bodies of uniform size and color. Moderate additions of PM digestate (3–5%) resulted in satisfactory fruiting, while the highest dose (10%) led to reduced density and slightly darker caps. In contrast, the use of SS digestate produced less uniform pinning and visibly fewer mushrooms, especially at the 10% level. The second flush occurred on day 52 after casing application and lasted approximately one week. Selected images taken during the experiment are presented in Figure 1.

The above observations were consistent with the yield results ($\text{g}\cdot\text{kg}^{-1}$ fresh substrate) obtained during the two flushes. During the first flush, the highest yield ($468.29 \text{ g}\cdot\text{kg}^{-1}$) was recorded for the treatment with 5% SS addition, while the control and the variants with PM 3%, PM 5%, and SS 3% showed comparable yields ranging from 432.78 to $462.07 \text{ g}\cdot\text{kg}^{-1}$. Lower production was observed for the 10% additions of SS and PM, where yields reached 370.81 and $381.40 \text{ g}\cdot\text{kg}^{-1}$, respectively.

In the second flush, the mushroom production was substantially lower, ranging from $30.10 \text{ g}\cdot\text{kg}^{-1}$ for 10% PM to a maximum of $141.19 \text{ g}\cdot\text{kg}^{-1}$ for 3% SS. The highest total yield from both flushes was obtained for the 3% PM variant, reaching $603.26 \text{ g}\cdot\text{kg}^{-1}$, while the control treatment produced a similar result ($593.55 \text{ g}\cdot\text{kg}^{-1}$). The remaining 3% and 5% additions resulted in intermediate yields ranging between 534.89 and $563.66 \text{ g}\cdot\text{kg}^{-1}$. The lowest overall yields were observed for the 10% additions of SS and PM, with values of 434.59 and $411.51 \text{ g}\cdot\text{kg}^{-1}$, respectively.

The distribution of the total yield from both flushes is shown in the box plot (Figure 2). Each box represents the middle 50% of the data (interquartile range, IQR), with the horizontal line marking the median and whiskers extending to $1.5 \times \text{IQR}$. Mean values are shown as dots, illustrating the variability among experimental treatments.

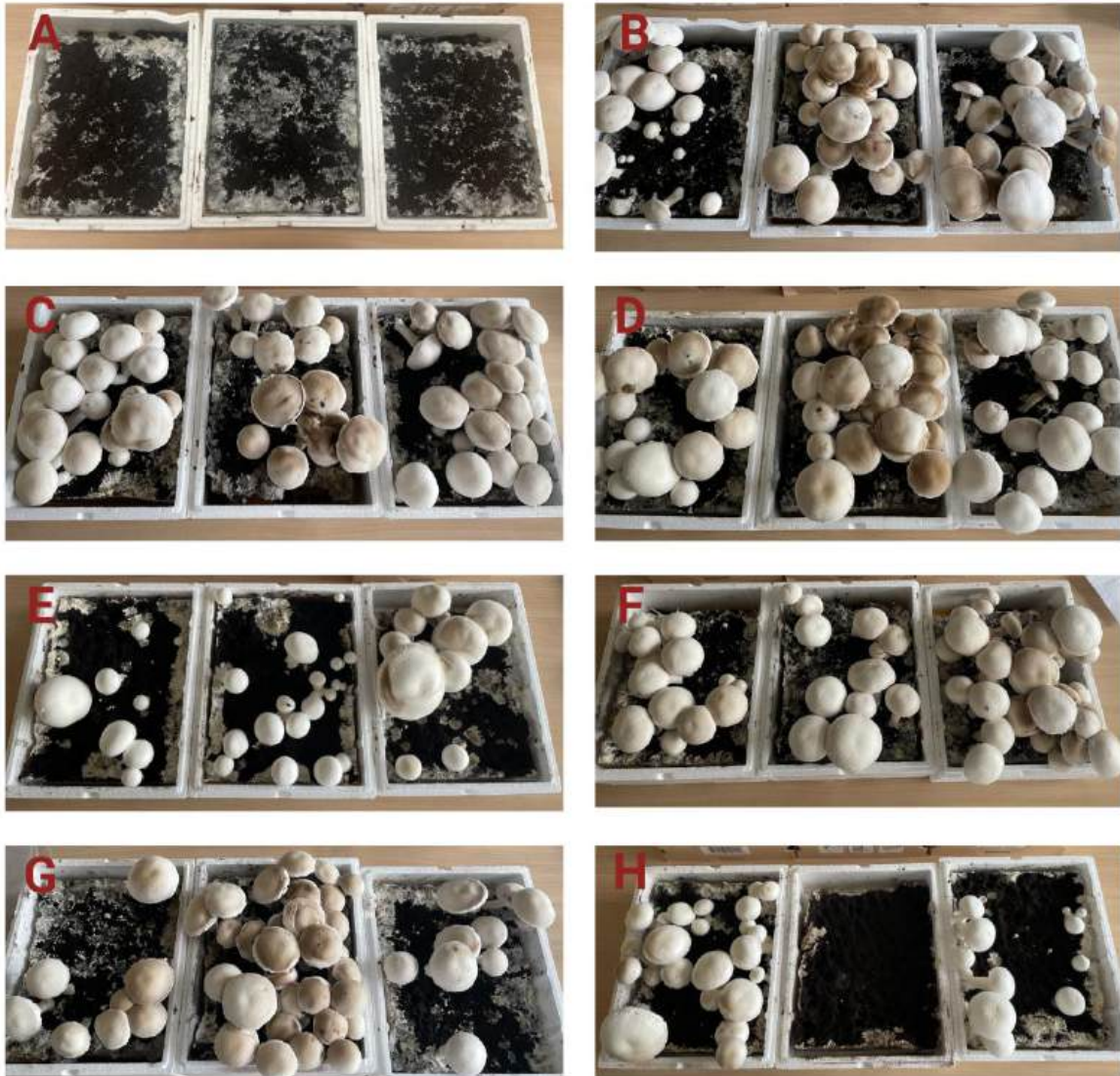


Figure 1. Selected images presenting *Agaricus bisporus* cultivation: (A) primordia formation on substrate covered with casing - control, various digestate treatments during first flush: (B) Control (0% of digestate) (C) Ss 3%, (D) Ss 5%, (E) Ss 10%, (F) PM 3%, (G) PM 5%, (H) PM 10%.

According to the two-way ANOVA results (Figure 3), digestate type had no significant effect on yield ($F = 0.143$, $p = 0.7106$), while the effect of dose was statistically significant ($F = 9.322$, $p = 0.0008$). The interaction between the two factors was not significant ($F = 0.29$, $p = 0.8319$). A gradual decrease in yield was observed with increasing digestate dose, regardless of the digestate type.

The highest total yields were obtained in the control and 3% PM variants (593.55 and $603.26 \text{ g} \cdot \text{kg}^{-1}$, respectively), while the lowest yields were recorded for the 10% SS and PM additions (411.51 and $434.59 \text{ g} \cdot \text{kg}^{-1}$, respectively). These findings confirm that increasing the digestate concentration in the casing layer negatively affected mushroom productivity.

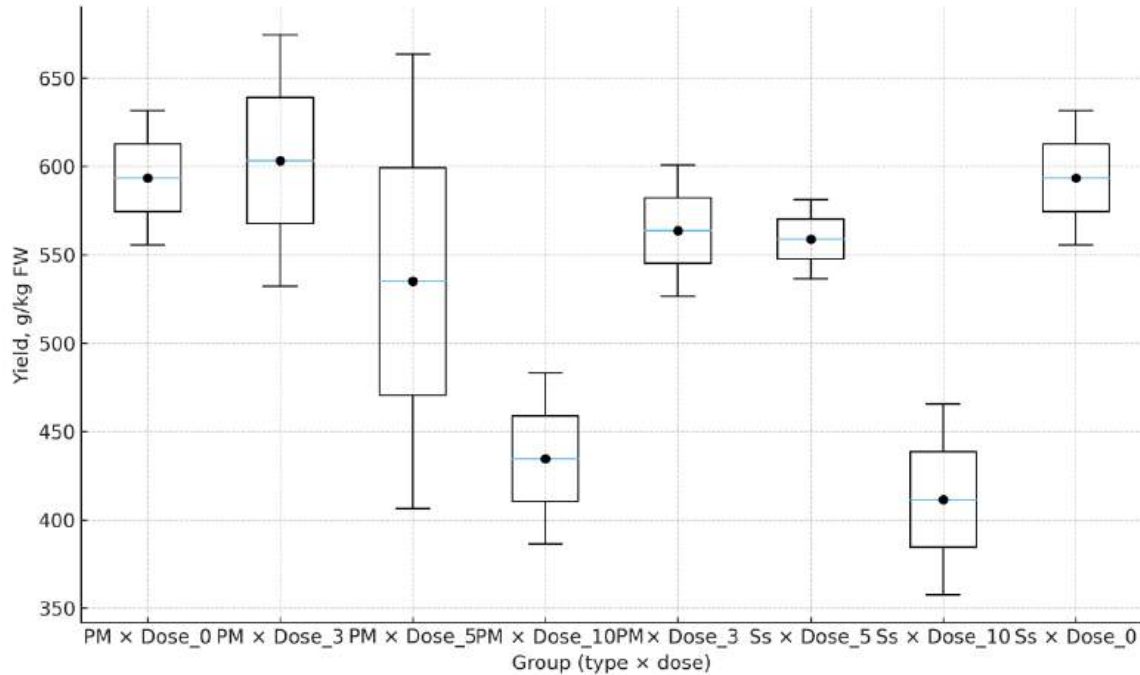
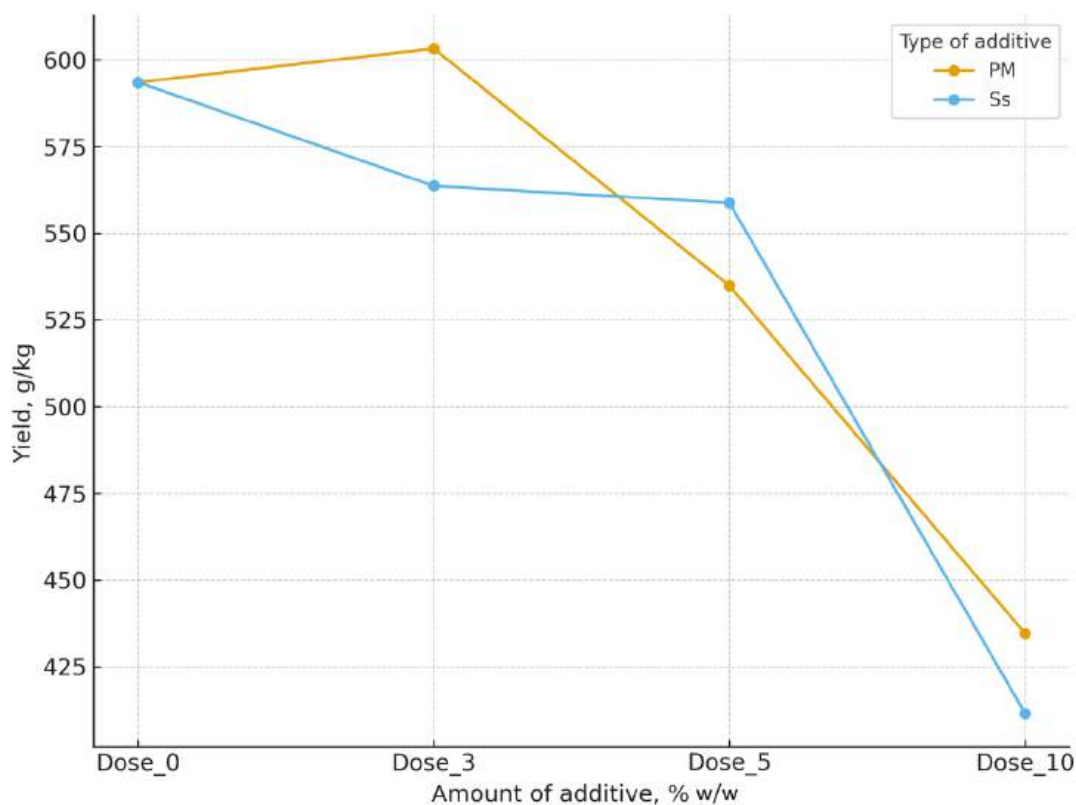


Figure 2. Distribution of mushroom total yield from two flushes ($\text{g}\cdot\text{kg}^{-1}$ fresh weight) depending on digestate type (SS, PM) and dose (0%, 3%, 5%, 10% w/w). The box represents the interquartile range (IQR, $Q3-Q1$), the horizontal line indicates the median, whiskers extend to $1.5 \times \text{IQR}$, and dots represent mean values ($n = 3$).

Post hoc comparisons using Tukey's HSD test revealed statistically significant differences between the control/low-dose variants and the treatments with the highest digestate additions (Table 4). The 10% SS and 10% PM treatments formed a separate homogeneous group (b) with the lowest yields (411.51 and $434.59 \text{ g}\cdot\text{kg}^{-1}$, respectively), significantly lower than those of the control and 3% PM variants, which belonged to the highest-yielding group (a) with mean values of 593.55 and $603.26 \text{ g}\cdot\text{kg}^{-1}$. Intermediate yields were recorded for the 3% and 5% SS and 5% PM treatments (534.90 – $563.67 \text{ g}\cdot\text{kg}^{-1}$), which did not differ significantly from either of the two groups. These results confirm that the application of high digestate doses (10%) had a negative impact on yield, regardless of digestate type.

Reported yields of *Agaricus bisporus* obtained under conventional and commercial cultivation systems typically range around $400 \text{ g}\cdot\text{kg}^{-1}$ of substrate for the total harvest period. These values have been documented for peat-based casing layers and standard compost formulations, representing the productivity achievable under optimized growth conditions [46,56,57]. The total yields obtained in the present study are therefore comparable to those reported in the literature, indicating that the applied cultivation conditions and the use of digestate amendments did not markedly deviate from the performance observed in conventional peat-based systems.



ANOVA results:
 Type of additive: $F=0.143$, $p=0.7106$
 Amount of additive: $F=9.322$, $p=0.0008$
 Type of additive x Amount of additive: $F=0.29$, $p=0.8319$

Figure 3. Effect of digestate type (Ss, PM) and dose (0%, 3%, 5%, 10% w/w) on mushroom yield ($\text{g} \cdot \text{kg}^{-1}$ substrate). Data represent mean values $\pm$ standard deviation ($n = 3$).

Table 4. Results of Tukey's HSD post hoc test ($\alpha = 0.05$) for mushroom yield depending on digestate type (SS, PM) and dose (% w/w). Means followed by the same letter within a column do not differ significantly according to Tukey's HSD test.

Type of additive	Amount of additive (% w/w)	Yield (g/kg) Mean	a	b
<i>Ss</i>	10	411.51	****	
<i>PM</i>	10	434.59	****	****
<i>PM</i>	5	534.90	****	****
<i>Ss</i>	5	558.78	****	****
<i>Ss</i>	3	563.67	****	****
<i>Ss</i>	0	593.55		****
<i>PM</i>	0	593.55		****
<i>PM</i>	3	603.26		****

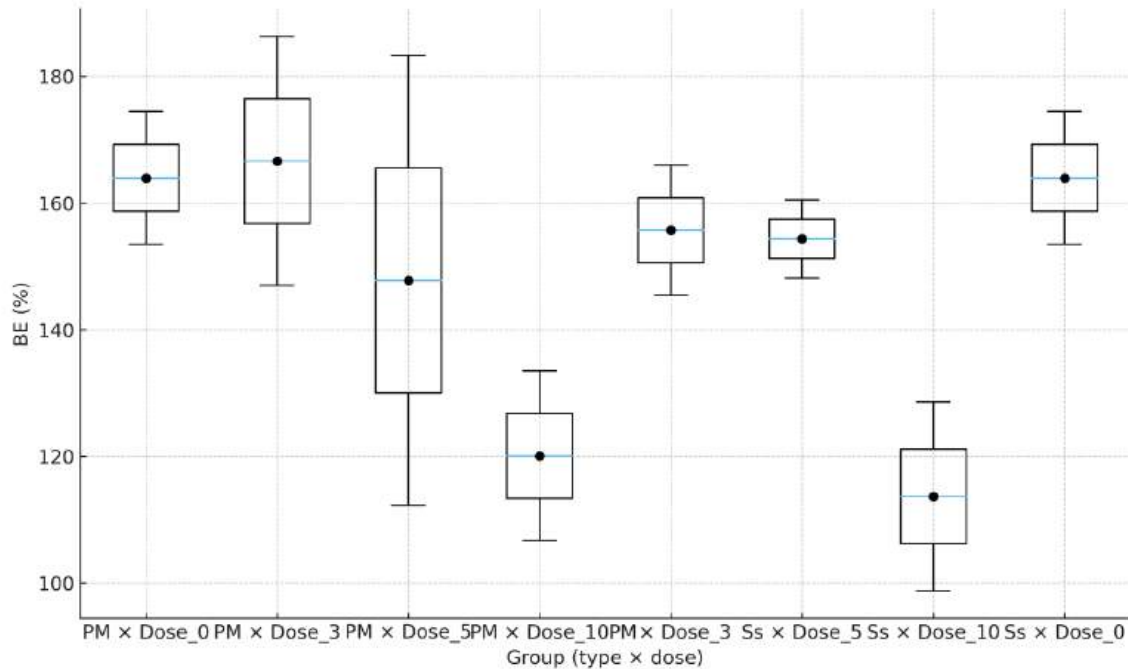
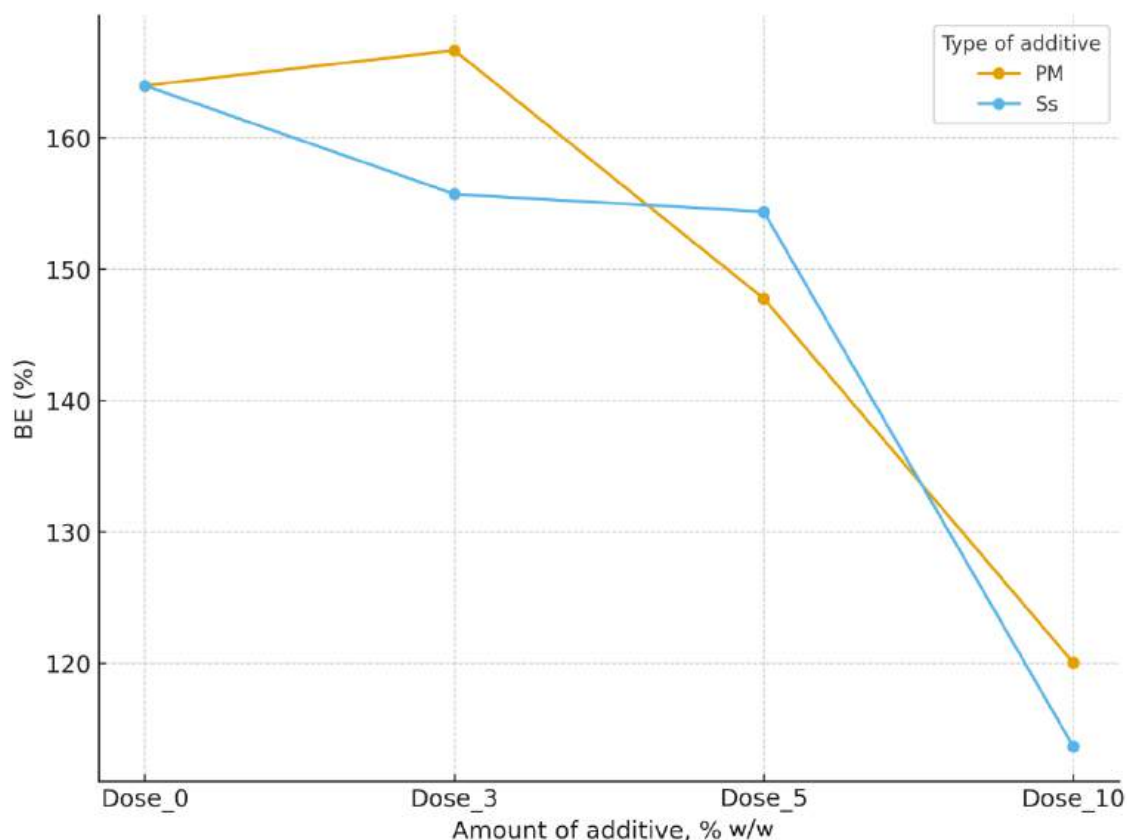


Figure 5. Variation of biological efficiency (BE, %) depending on digestate type (SS, PM) and dose (0%, 3%, 5%, 10% w/w).

The biological efficiency (BE) of *Agaricus bisporus* ranged from 113.68% to 166.65%, with the highest values recorded for the control and the 3% digestate additions (PM = 166.65%; SS = 155.71%). A gradual decrease was observed with increasing digestate dose, and the lowest BE occurred at 10% additions (PM = 120.05%; SS = 113.68%).

The variability and distribution of BE across treatments are illustrated in the box plot (Figure 5), while the ANOVA results (Figure 6) confirmed that the dose of additive had a significant effect ($p = 0.0008$), whereas the type of additive and their interaction were not significant. The Tukey HSD post hoc test (Table 5) further indicated that the 3% digestate treatments did not differ significantly from the control, but both performed significantly better than the 10% variants.

The obtained BE values are consistent with literature data for conventional *Agaricus bisporus* cultivation, where biological efficiency typically ranges from 140% to 150% under optimal peat-based conditions[45,46,58]. The similarity between the control and the 3–5% digestate treatments suggests that moderate digestate inclusion did not impair mushroom productivity, whereas higher doses negatively affected casing properties and reduced fruiting performance.



ANOVA results:
 Type of additive: F=0.143, p=0.7106
 Amount of additive: F=9.322, p=0.0008
 Type of additive x Amount of additive: F=0.29, p=0.8319

Figure 6. Biological efficiency (BE, %) of *Agaricus bisporus* as affected by digestate type and dose. Means $\pm$ SD (n = 3).

Table 5. Tukey's HSD test results ($\alpha = 0.05$) for the effect of digestate type and dose on biological efficiency (BE, %)

Type of additive	Amount of additive (% w/w)	BE (%) Mean	a	b
<i>Ss</i>	10	113.68	****	
<i>PM</i>	10	120.05	****	****
<i>PM</i>	5	147.76	****	****
<i>Ss</i>	5	154.36	****	****
<i>Ss</i>	3	155.71	****	****
<i>Ss</i>	0	163.97		****
<i>PM</i>	0	163.97		****
<i>PM</i>	3	166.65		****

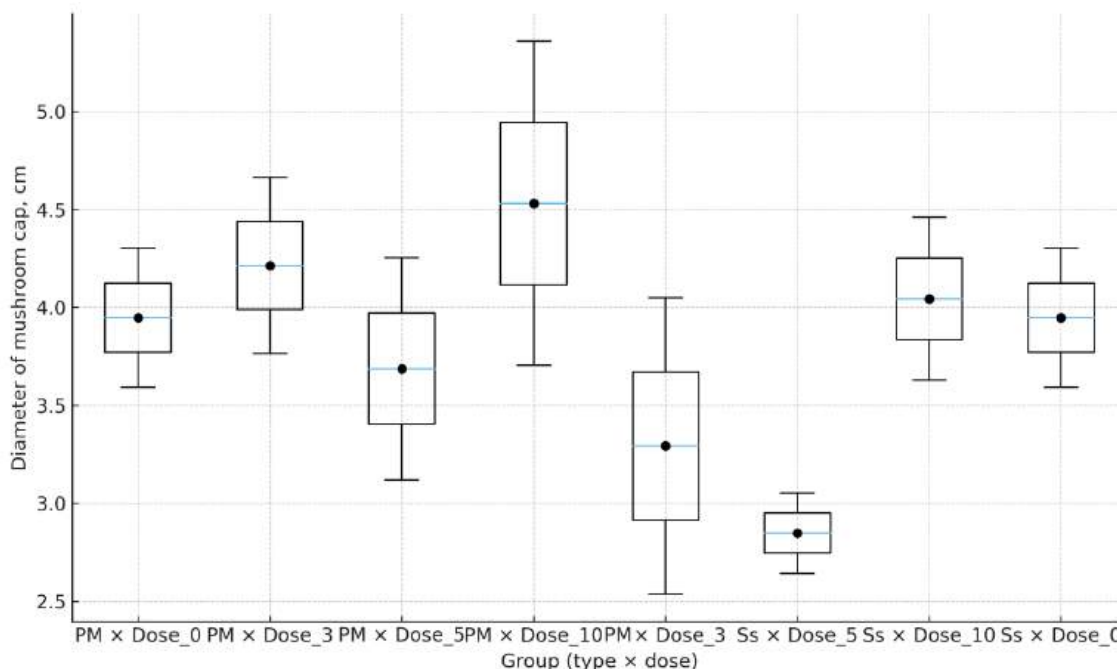
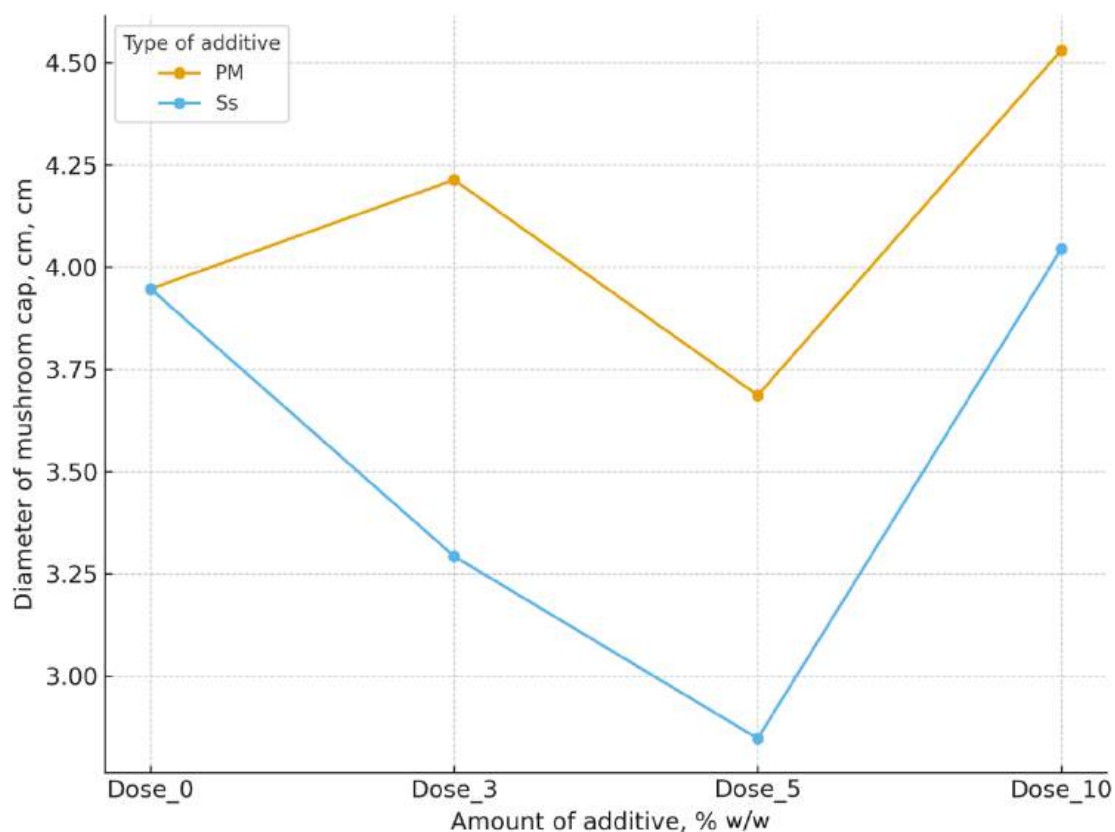


Figure 7. Variation in mushroom cap diameter (cm) across treatments differing in digestate type and dose

The obtained BE values are consistent with literature data for conventional *Agaricus bisporus* cultivation, where biological efficiency typically ranges from 140% to 150% under optimal peat-based conditions [Stoknes et al., 2013; Carrasco et al., 2018; Wang et al., 2018; Cruz et al., 2022]. The similarity between the control and the 3–5% digestate treatments suggests that moderate digestate inclusion did not impair mushroom productivity, whereas higher doses negatively affected casing properties and reduced fruiting performance.

In the present study, the mean cap diameters (Figure 7) were slightly lower than those reported in the literature [59,60], but still within the expected range for standard cultivation systems. The diameter of fruiting body caps varied significantly depending on the type and amount of digestate incorporated into the casing layer. ANOVA (Figure 8) revealed significant effects of both the additive type ($F = 6.73$, $p = 0.0196$) and dose ($F = 3.878$, $p = 0.0293$), while the interaction between factors was not significant ($p = 0.4455$). According to the Tukey HSD test (Table 6), the largest caps were recorded for PM 10% (4.53 cm), which differed significantly from Ss 5% (2.85 cm), the treatment with the smallest caps. The control (PM 0%, Ss 0%) and the intermediate doses (PM 3–5%, Ss 3–10%) formed a statistically homogeneous group, with no significant differences between them or compared to the extremes.

These results suggest that the co-digestion-derived digestate (PM), particularly at 10%, tended to promote the formation of larger fruiting bodies, while sewage sludge digestate (Ss) at low doses (5%) limited cap expansion. Overall, the observed cap diameters fall within the expected morphological range for commercial *A. bisporus* cultivation and indicate that moderate digestate inclusion does not impair the physical quality of fruiting bodies.



ANOVA results:
 Type of additive: F=6.73, p=0.0196
 Amount of additive: F=3.878, p=0.0293
 Type of additive x Amount of additive: F=0.938, p=0.4455

Figure 8. Effect of digestate type and dose on mushroom cap diameter (cm)

Table 6. Tukey's HSD test results ($\alpha = 0.05$) for the effect of digestate type and dose on mushroom cap diameter (cm)

Type of additive	Amount of additive (% w/w)	Diameter of mushroom cap (cm) Mean	a	b
<i>Ss</i>	5	2.85	****	
<i>Ss</i>	3	3.29	****	****
<i>PM</i>	5	3.69	****	****
<i>Ss</i>	0	3.95	****	****
<i>PM</i>	0	3.95	****	****
<i>Ss</i>	10	4.05	****	****
<i>PM</i>	3	4.21	****	****
<i>PM</i>	10	4.53		****

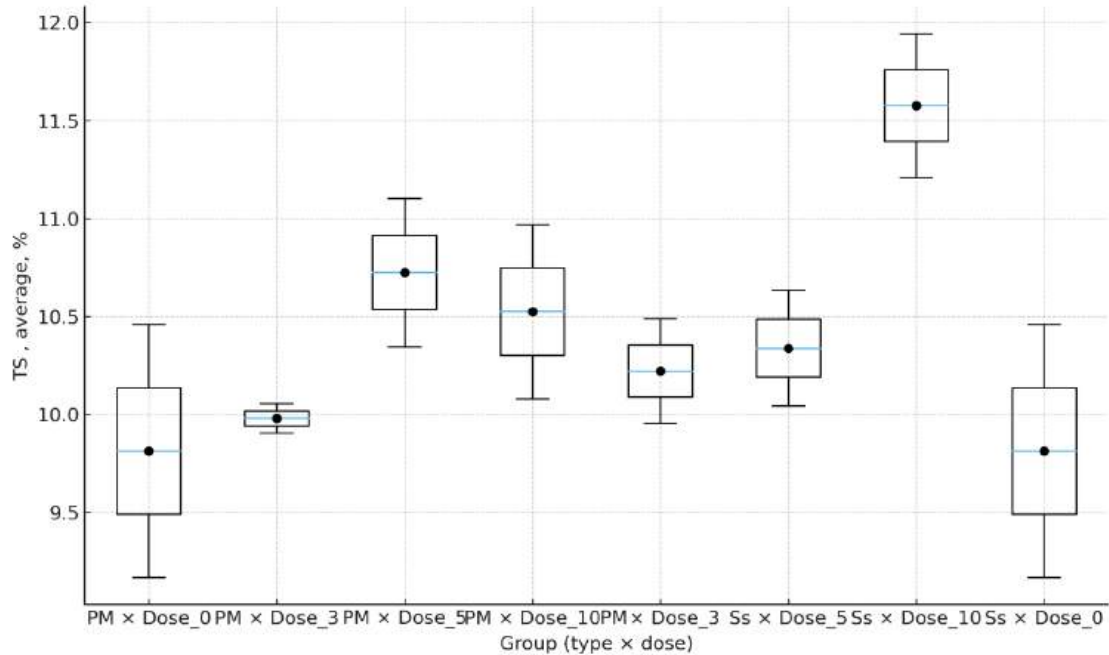
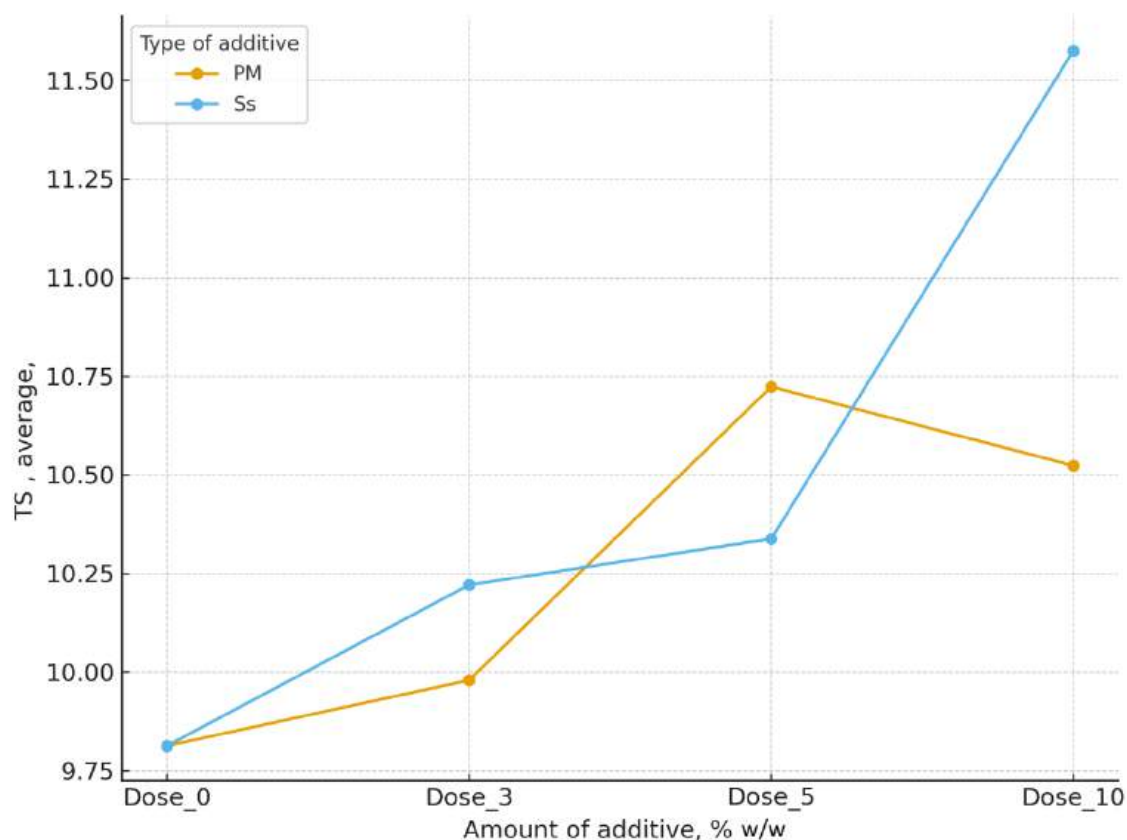


Figure 9. Average total solids values from two flushes of mushrooms for different casing variants

The total solids (TS) content of the fruiting bodies ranged from 9.81 to 11.58% of fresh weight showing a clear increasing trend with the dose of digestate added to the casing layer (Figure 9). The highest TS values were observed in the 10% Ss and PM variants (11.58% and 10.52%, respectively), whereas the lowest were recorded in the control and 3% treatments (~9.8–10.2%). ANOVA results (Figure 10) confirmed a significant effect of digestate dose ($F = 9.45$, $p = 0.0008$), while neither the type of additive nor the interaction between factors was statistically significant. Tukey's HSD post hoc test further indicated that the 10% Ss treatment formed a distinct homogeneous group, significantly differing from the control and lower-dose variants. This suggests that the increase in digestate concentration enhanced the proportion of solid matter in mushroom tissues, possibly reflecting higher dry matter accumulation or reduced water content in fruiting bodies. The obtained TS values are consistent with those reported for *Agaricus bisporus* in the literature, where dry matter typically ranges from 7–12% FW [15,61,62].

Although the observed fluctuations in dry matter content may seem negligible, they have practical implications for the nutritional composition of the mushrooms. Increased moisture content dilutes the solid fraction, leading to a reduced concentration of nutrients per unit of fresh weight.



ANOVA results:
 Type of additive: F=1.677, p=0.2137
 Amount of additive: F=9.445, p=0.0008
 Type of additive x Amount of additive: F=3.005, p=0.0613

Figure 10. Effect of different digestate treatment on total solids of mushroom (%)

Table 7. Tukey's HSD test results ($\alpha = 0.05$) for the effect of digestate type and dose on average TS (%) of *Agaricus bisporus* from two flushes

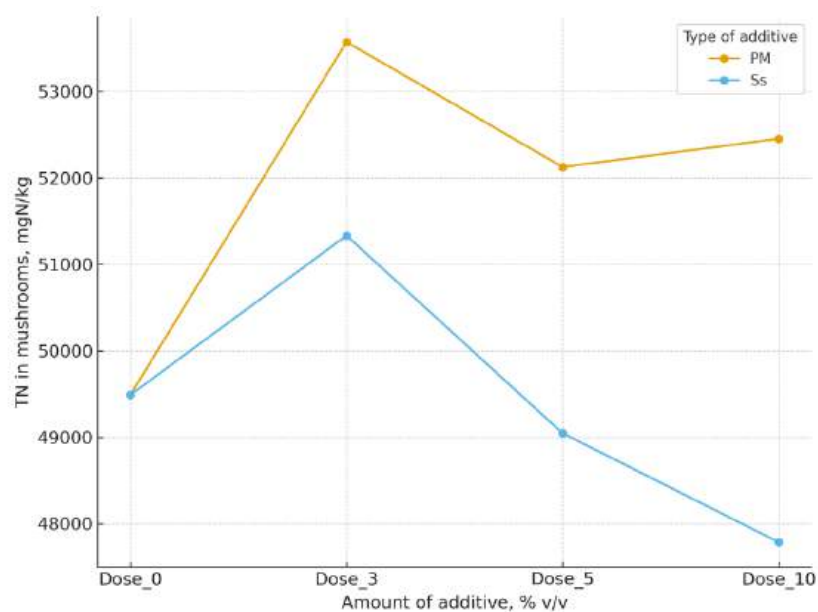
Type of additive	Amount of additive (% w/w)	TS (%) Mean	a	b
<i>Ss</i>	0	9.81	****	
<i>PM</i>	0	9.81	****	
<i>PM</i>	3	9.98	****	
<i>Ss</i>	3	10.22	****	
<i>Ss</i>	5	10.34	****	
<i>PM</i>	10	10.52	****	****
<i>PM</i>	5	10.72	****	****
<i>Ss</i>	10	11.58		****

3.3. Nutrients and heavy metals profile

The mineral composition of *Agaricus bisporus* fruiting bodies reflected the influence of digestate-amended casing layers, although the mushrooms maintained a relatively stable elemental balance through internal nutrient homeostasis. Differences in mineral uptake among treatments corresponded to the nutrient profiles of the applied digestates, but nutrient availability in the casing material was not the only determinant of the final composition of the fruiting bodies.

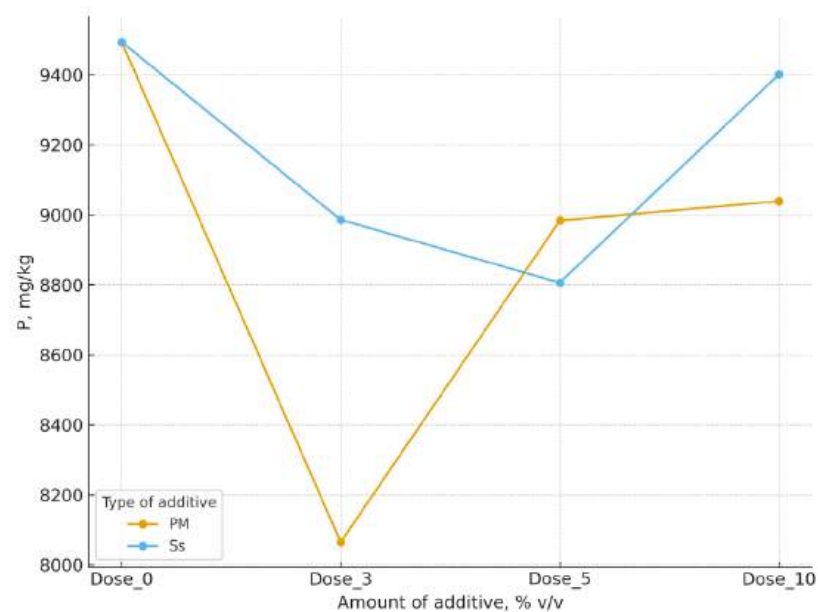
The total nitrogen (TN) content in *Agaricus bisporus* fruiting bodies (Figure 11) ranged from approximately 47,800 to 53,400 mg N mg/kg, with the highest mean value observed in the PM 3% treatment. No statistically significant effect of digestate type or dose was detected ($p > 0.05$). Despite the higher total Kjeldahl nitrogen (TKN) and ammonium nitrogen ($N-NH_4$) contents in PM digestate compared with Ss digestate, these differences did not translate into significant variation in the nitrogen content of the mushrooms. The stable TN concentrations across treatments suggest that *A. bisporus* effectively buffers external variations in nitrogen availability. This ability is supported by the presence of key enzymes involved in nitrogen assimilation, which enable the fungus to regulate uptake and conversion of ammonium and organic nitrogen forms according to metabolic needs. Previous studies have shown that *A. bisporus* can modulate these pathways in response to different nitrogen sources in the substrate or casing layer [63]. Such adaptive regulation may explain the consistent TN levels observed in the present study, regardless of digestate type or dose.

In accordance with published data, potassium (K) was the predominant macronutrient in *A. bisporus*, followed by phosphorus (P), while calcium (Ca), magnesium (Mg), and sodium (Na) occurred at lower concentrations [64,65]. The relative proportions of these elements are known to vary slightly depending on strain, substrate, and cultivation conditions, but K and P consistently dominate the mineral profile of cultivated mushrooms. In the present study, Ca ranged from approximately 780 to 1320 mg/kg, showing the highest variability among macronutrients ($p < 0.001$). Its content increased with higher doses of 3-10% PM digestate, whereas a clear decline was observed at 10% Ss. These differences likely reflect variations in Ca solubility and exchangeable fractions between the two digestate types, since cAD-delivered digestate generally contains more readily available Ca. The obtained values were within the range reported for cultivated mushrooms [38], confirming that digestate application did not lead to excessive Ca accumulation. Potassium concentrations, ranging from 24,000 to 27,000 mg/kg were the highest among all detected elements, whereas phosphorus ranged between 6,500 and 7,300 mg/kg. Both elements remained unaffected by digestate type or dose ($p > 0.05$), indicating strong physiological control of their uptake. In contrast, Na exhibited a marked and statistically significant increase ($p < 0.001$), from 460 to 920 mg/kg, with the highest levels recorded in PM 10%, consistent with the elevated Na content of PM digestate. The recorded Na concentrations were within or slightly above the typical range reported for *A. bisporus* [66,67]. The concentrations of Mg (920–1120 mg/kg) and S (580–640 mg/kg) did not differ significantly between treatments ($p > 0.05$), remaining within the expected limits for cultivated mushrooms [38,68].



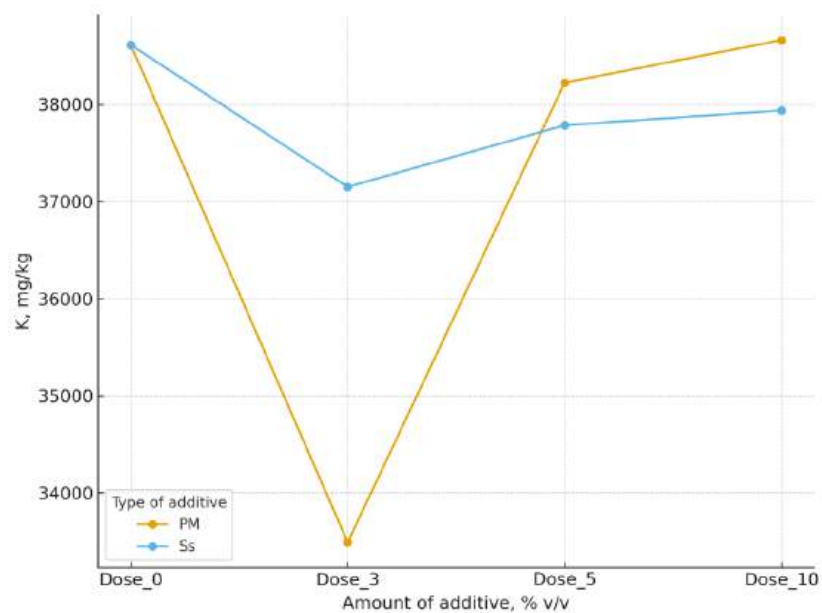
ANOVA results:
 Type of additive: $F=0.575$, $p=0.4593$
 Amount of additive: $F=0.15$, $p=0.9281$
 Type of additive x Amount of additive: $F=0.087$, $p=0.966$

Figure 11. Total nitrogen (TN) levels in *Agaricus bisporus* fruiting bodies grown with different types and doses of digestate in the casing layer.



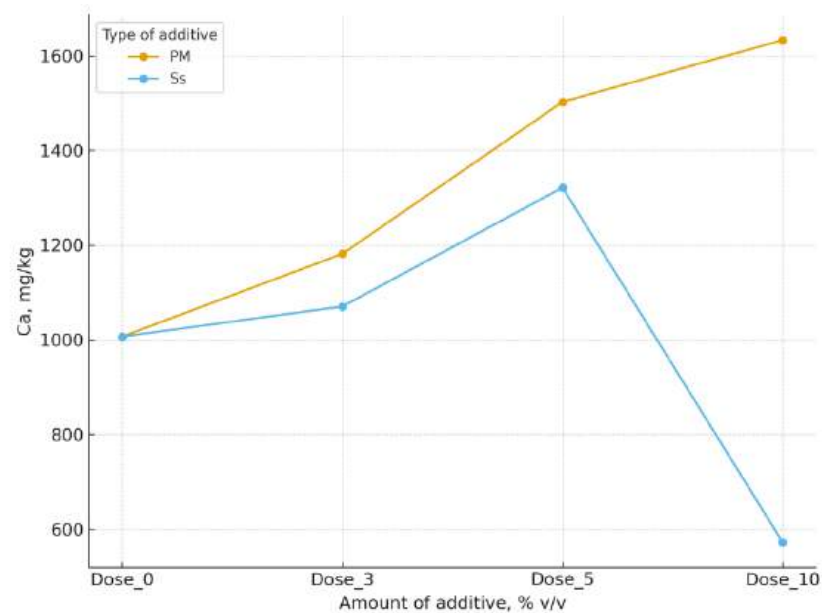
ANOVA results:
 Type of additive: $F=0.691$, $p=0.418$
 Amount of additive: $F=1.582$, $p=0.2328$
 Type of additive x Amount of additive: $F=0.532$, $p=0.6668$

Figure 12. Effect of digestate addition on phosphorus (P) accumulation in *Agaricus bisporus* fruiting bodies.



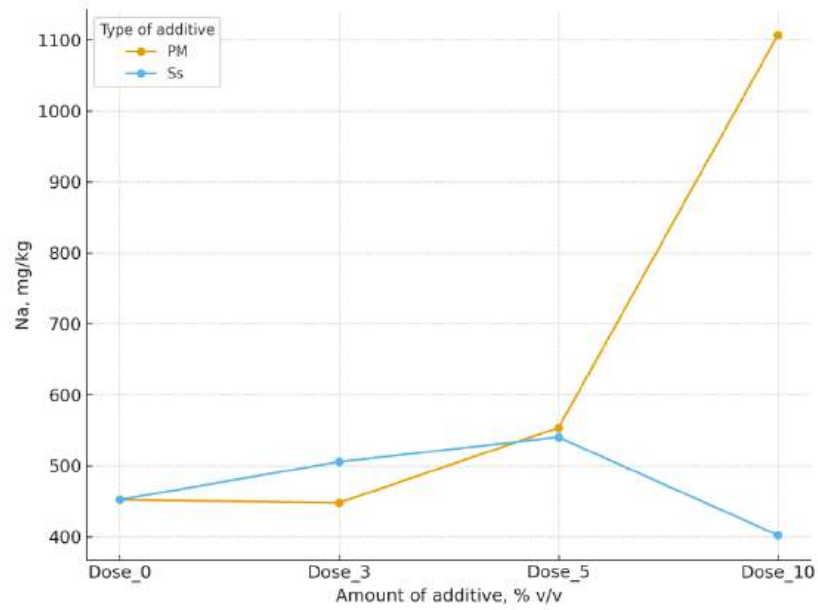
ANOVA results:
 Type of additive: $F=0.204$, $p=0.6572$
 Amount of additive: $F=1.201$, $p=0.3412$
 Type of additive x Amount of additive: $F=0.549$, $p=0.6562$

Figure 13. Potassium (K) concentrations in mushrooms as influenced by the type and proportion of digestate used.



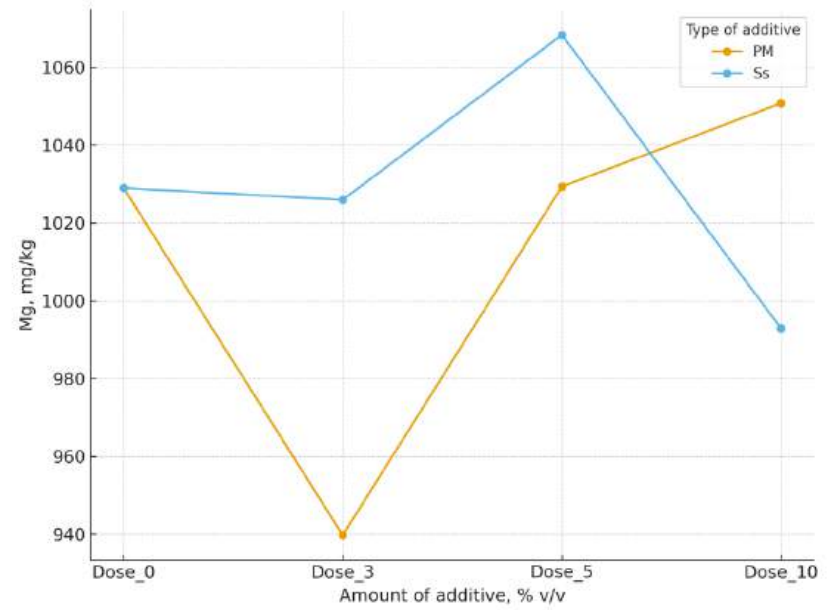
ANOVA results:
 Type of additive: $F=58.635$, $p=0.0$
 Amount of additive: $F=15.615$, $p=0.0001$
 Type of additive x Amount of additive: $F=30.443$, $p=0.0$

Figure 14. Changes in calcium (Ca) content of *A. bisporus* in response to increasing digestate doses.



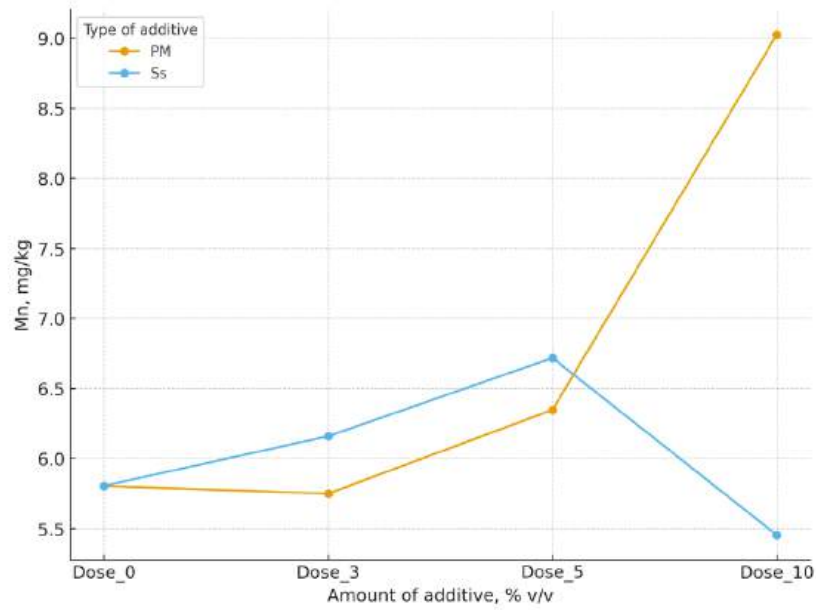
ANOVA results:
 Type of additive: $F=56.689$, $p=0.0$
 Amount of additive: $F=39.223$, $p=0.0$
 Type of additive x Amount of additive: $F=67.774$, $p=0.0$

Figure 15. Sodium (Na) levels in fruiting bodies depending on digestate origin and application rate.



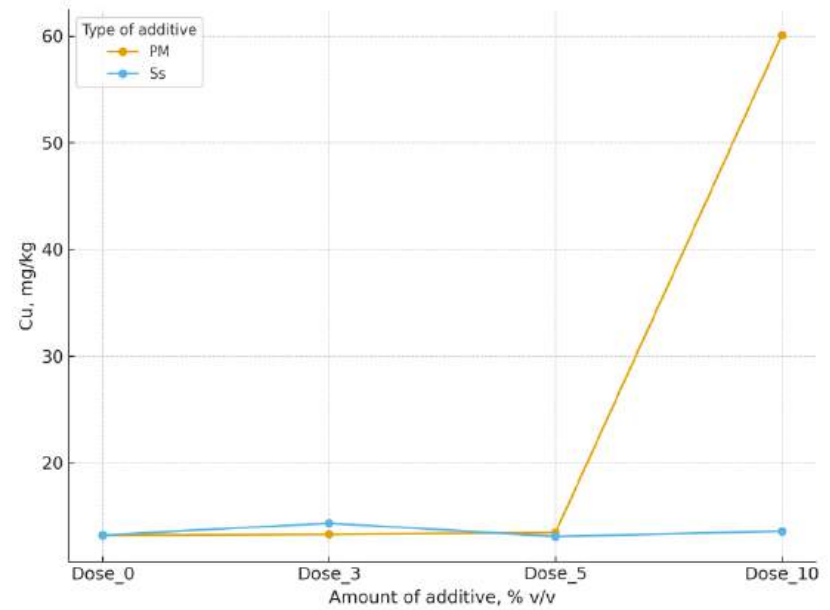
ANOVA results:
 Type of additive: $F=0.202$, $p=0.6595$
 Amount of additive: $F=0.543$, $p=0.6597$
 Type of additive x Amount of additive: $F=0.66$, $p=0.5883$

Figure 16. Variations in magnesium (Mg) content across treatments with digestate.



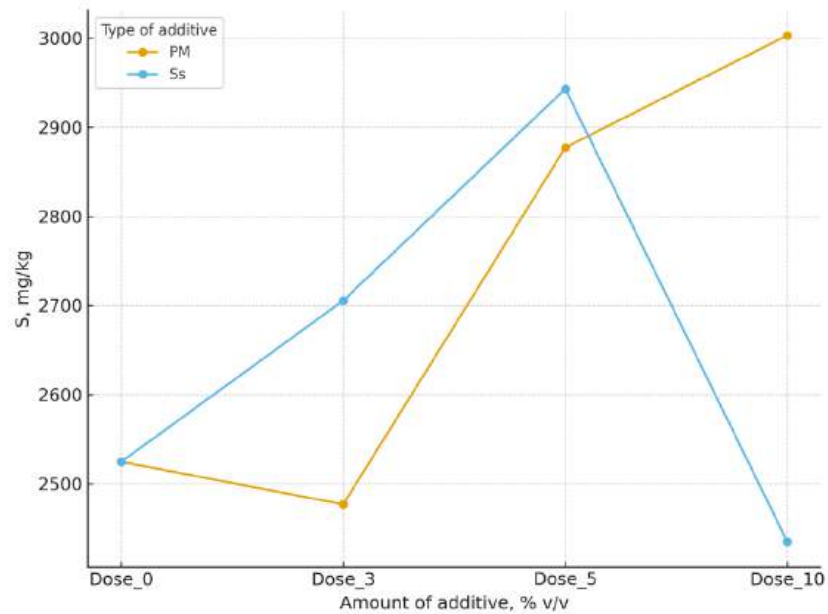
ANOVA results:
 Type of additive: $F=8.616$, $p=0.0097$
 Amount of additive: $F=7.516$, $p=0.0023$
 Type of additive x Amount of additive: $F=16.389$, $p=0.0$

Figure 17. Manganese (Mn) concentrations in *A. bisporus* fruiting bodies under different digestate amendment scenarios.



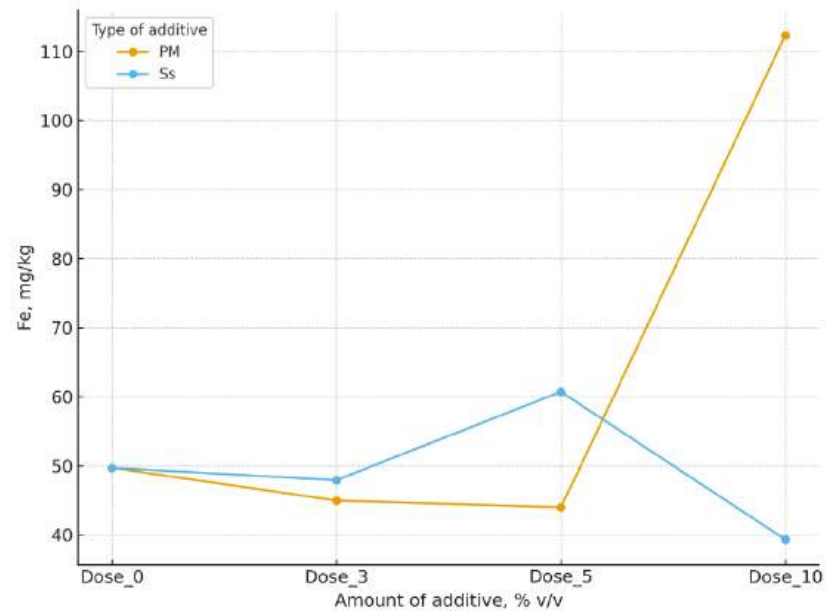
ANOVA results:
 Type of additive: $F=159.333$, $p=0.0$
 Amount of additive: $F=166.43$, $p=0.0$
 Type of additive x Amount of additive: $F=165.854$, $p=0.0$

Figure 18. Influence of digestate enrichment on copper (Cu) accumulation in mushroom fruiting bodies.



ANOVA results:
 Type of additive: $F=0.477$, $p=0.4996$
 Amount of additive: $F=2.933$, $p=0.0653$
 Type of additive x Amount of additive: $F=3.064$, $p=0.0582$

Figure 19. Sulfur (S) content in *A. bisporus* as affected by digestate addition to the casing layer.



ANOVA results:
 Type of additive: $F=36.254$, $p=0.0$
 Amount of additive: $F=36.529$, $p=0.0$
 Type of additive x Amount of additive: $F=83.432$, $p=0.0$

Figure 20. Iron (Fe) concentrations in mushrooms cultivated with increasing levels of digestate.

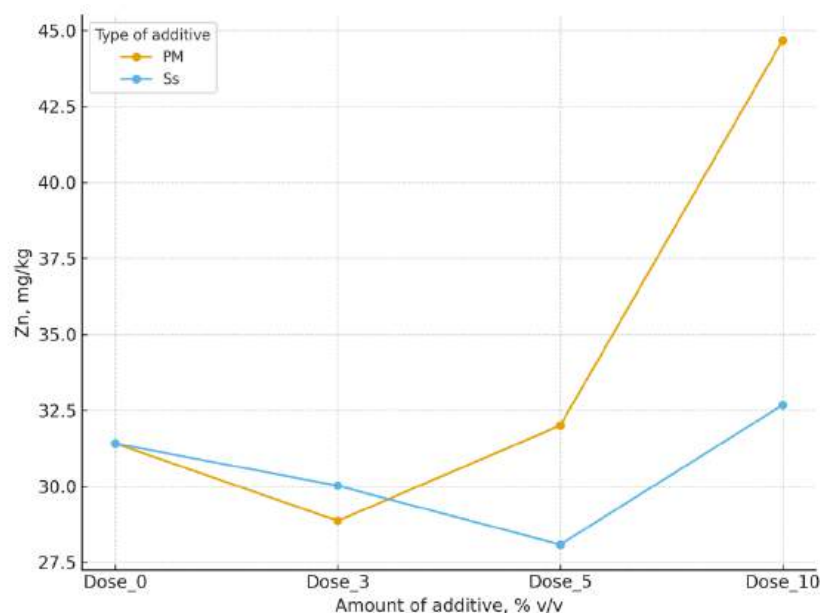


Figure 21. Zinc (Zn) content in *Agaricus bisporus* fruiting bodies depending on the type and dose of digestate applied to the casing layer.

The concentration of trace elements in *Agaricus bisporus* fruiting bodies varied depending on digestate type and dose, with statistically significant differences ($p < 0.05$) observed for several metals. Overall, Fe, Zn, Cu, and Mn were among the key trace elements analyzed, consistent with comprehensive elemental profiling studies of *A. bisporus* [69]. In general, Fe and Zn tend to dominate among trace metals, whereas Mn occurs at the lowest concentrations, as reported for cultivated mushrooms by Vetter [68] and Kalač [38]. In the present study, Fe concentrations ranged from approximately 85 to 165 mg/kg, showing a significant increase in the PM 10% treatment and a noticeable decrease in Ss 10% ($p < 0.001$). Such variations likely reflect differences in Fe availability between the two digestates and the ability of *A. bisporus* to regulate Fe uptake to prevent excessive accumulation. Mn contents were comparatively low (3.8–8.6 mg/kg), but increased significantly with rising PM additions ($p < 0.01$), consistent with the higher Mn levels found in PM digestate compared with Ss digestate. Cu concentrations varied between 10 and 57 mg/kg showing a significant interaction between digestate type and dose ($p < 0.001$). The highest Cu contents were observed in the PM 10% treatment, suggesting increased Cu bioavailability from poultry-manure digestate. Zn concentrations ranged from 28 to 45 mg/kg, increasing gradually with higher PM doses ($p = 0.0057$). The observed trend indicates that Zn from digestate was bioavailable to mushrooms but did not result in excessive accumulation.

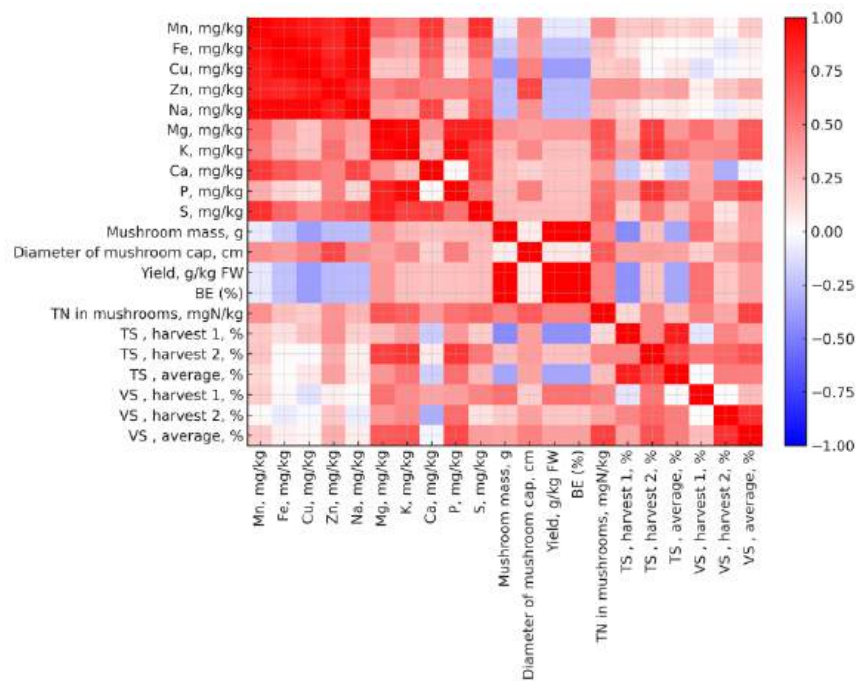


Figure 22. Pearson's correlation heatmap showing relationships among elemental composition, production parameters, and physicochemical properties of *Agaricus bisporus* fruiting bodies.

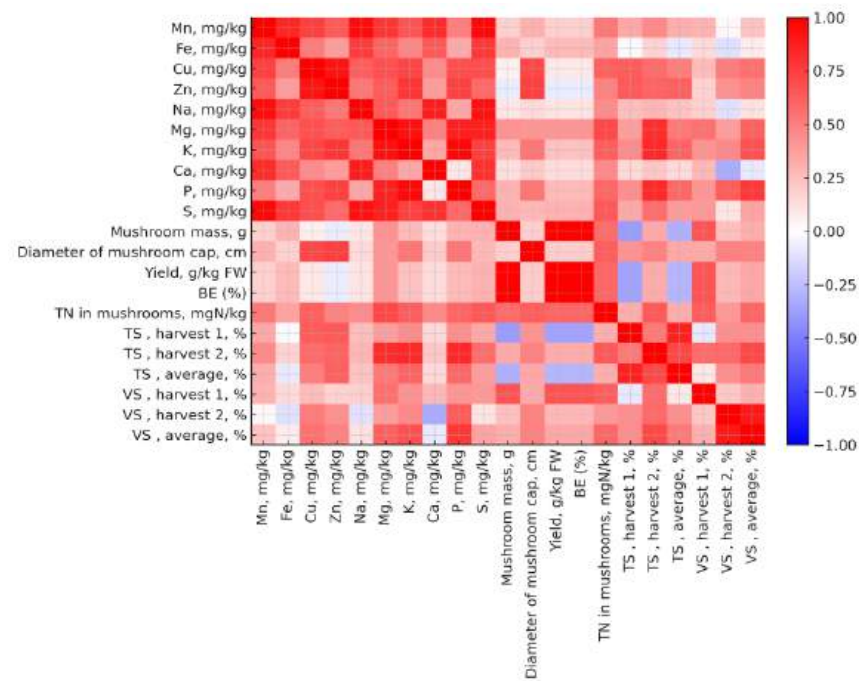


Figure 23. Spearman's rank correlation heatmap illustrating monotonic relationships between elemental contents and productivity indicators in *Agaricus bisporus*.

To explore potential relationships among all analysed parameters — including the elemental composition of *Agaricus bisporus* fruiting bodies, their physicochemical properties and production performance — Pearson's and Spearman's correlation analyses were conducted. These analyses aimed to identify how variations in the concentrations of individual elements corresponded both with each other and with key cultivation indicators such as yield, biological efficiency (BE), and dry and organic matter contents (TS, VS).

The Pearson and Spearman correlation analyses (Fig. 1–2) revealed a series of significant relationships between the elemental composition of *Agaricus bisporus* fruiting bodies and the main production parameters. Both correlation types showed strong positive associations among the principal macronutrients (K, P, S, Mg, and Ca), reflecting their co-occurrence and shared roles in the metabolic pathways involved in mushroom nutrition. The highest correlation coefficients ($r > 0.8$) were observed between potassium, phosphorus, and sulphur, suggesting that their accumulation proceeds synchronously during fruit body development. A positive association was also noted between these elements and yield as well as biological efficiency (BE), implying that higher macronutrient contents support more intensive growth and greater productivity. In contrast, several micronutrients (e.g., Mn, Fe, and Zn) exhibited negative or weak correlations with yield-related parameters, possibly indicating competitive uptake or limited mobility within the fungal tissue during fruiting. Total nitrogen (TN) content in mushrooms was positively correlated with BE and with the levels of phosphorus and potassium, suggesting that enhanced nitrogen assimilation accompanies the accumulation of key structural nutrients under favourable growth conditions. Parameters related to dry matter (TS) and organic matter (VS) content showed moderate positive relationships with most macronutrients, particularly during the second flush, reflecting progressive concentration of mineral constituents in later stages of production. When comparing both correlation methods, it was evident that the Spearman coefficients provided a more sensitive representation of the monotonic relationships among the analysed variables, particularly where the associations were non-linear in nature. This approach appears more suitable for complex biological systems such as mushroom cultivation, where interactions between nutrient accumulation and production parameters are often governed by multifactorial and dynamic physiological processes rather than strictly linear dependencies.

Principal Component Analysis (PCA) was conducted to integrate all quantitative variables describing mushroom productivity (yield, biological efficiency, and cap diameter) and the elemental composition of fruiting bodies. The first two components explained 66.3% of the total variance (PC1 – 40.5%; PC2 – 25.8%). PC1 was positively correlated with Ca, P, K, Mg, S, total solids (TS), and cap diameter, and thus represented the elemental and nutritional value gradient of the mushrooms. Treatments with higher PC1 scores were characterized by increased mineral accumulation and a greater proportion of dry matter in fruiting bodies. In contrast, PC2 was positively associated with Cu, Na, and Fe but negatively related to yield and biological efficiency, reflecting a microelement enrichment–yield trade-off, where excessive trace element accumulation coincided with reduced productivity.

The PCA biplot (Figure 24) distinctly separated treatments according to the type and dose of digestate. Digestate from mono-digestion of sewage sludge (Ss) treatments clustered on the central and left-hand side of the plot (negative PC1), corresponding to lower mineralization and moderate microelement levels. Increasing the Ss dose shifted samples upward along PC2, indicating rising Cu, Na, and Fe contents accompanied by yield decline. Digestate derived co-digestion of poultry manure with sewage sludge (PM) occupied the right-hand section (positive PC1), characterized by higher concentrations of Ca, K, and P in mushrooms. The 10% PM treatment, positioned in the upper right quadrant, exhibited the highest mineralization but the lowest productivity, confirming the negative effect of excessive digestate addition. The control (peat casing) was located in the lower left quadrant, showing the lowest mineral content and the highest yield and biological efficiency.

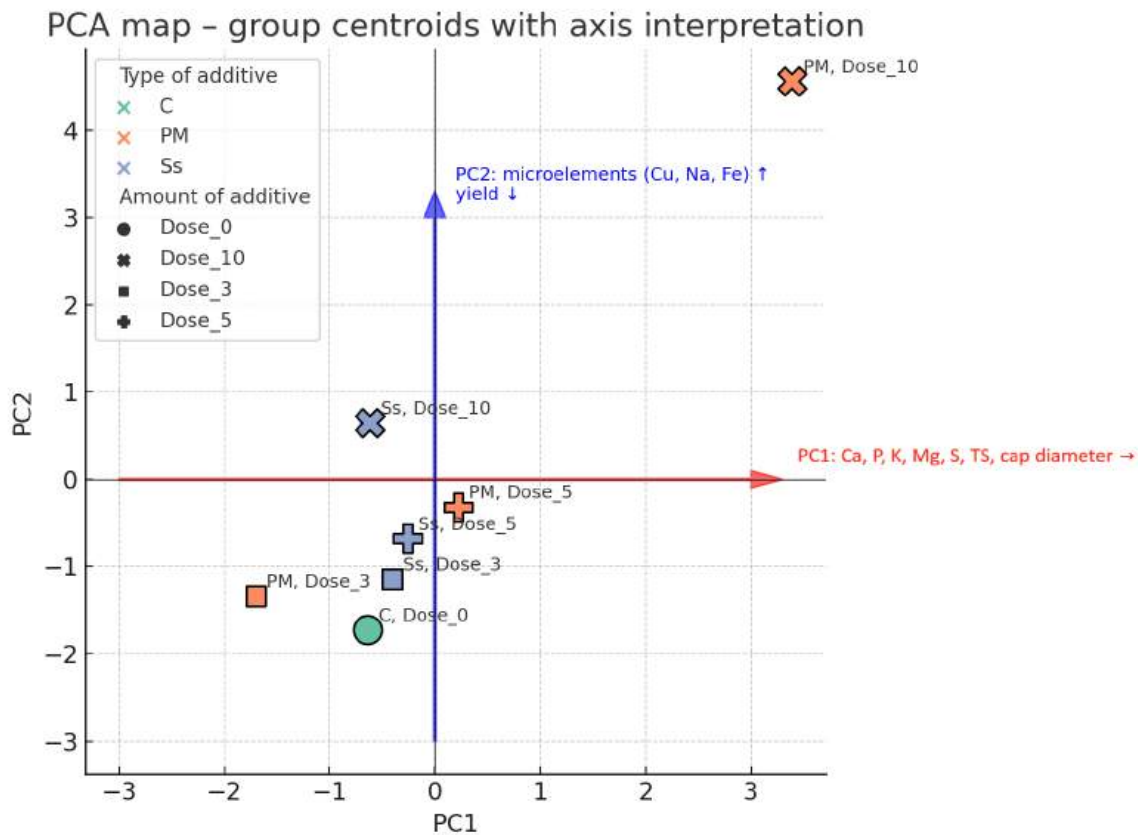


Figure 24. Principal Component Analysis (PCA) biplot showing the distribution of treatments according to the first two principal components (PC1 and PC2). PC1 – elemental and nutritional value gradient; PC2 – microelement enrichment negatively correlated with yield.

Overall, PCA results demonstrate that the type and inclusion rate of digestate were the primary factors influencing mushroom responses. PC1 represented differences in the nutritional composition of fruiting bodies, while PC2 captured the inverse relationship between microelement accumulation and yield. Moderate digestate additions (3–5%), especially PM 3%, ensured a favourable balance between productivity and mineral enrichment, whereas higher doses (10%) caused excessive mineralization and yield

reduction. These findings confirm that the effectiveness of digestate as a casing amendment depends on both its origin and the applied proportion.

3.4. Changes in nutrient and elemental composition among casing treatments with digestate supplementation

After the cultivation of *Agaricus bisporus*, marked changes were observed in the mineral composition of the casing layers, reflecting the dynamic exchange of nutrients between the compost, casing, and fruiting bodies. Table 7, 8 shows elemental profile of different variant of casing after mushroom cultivation. Although the casing layer does not serve as the main nutrient source, it undergoes continuous physicochemical transformations during the cropping cycle, which influence water balance, ion exchange, and mushroom productivity [45,70].

The most pronounced post-harvest differences were found for macronutrients, particularly potassium (K), phosphorus (P), sulphur (S), and sodium (Na). Potassium content in the control layer increased from approximately 711 to 14273 mg/kg, while in digestate-amended variants it reached up to 21191 mg/kg (Ss 10%). A similar trend was recorded for phosphorus (rising from about 246 to 878 mg/kg in the control and 1726 mg/kg in Ss 10%), as well as for sulphur and sodium. Such enrichment likely results from the high mobility of these elements in moist casings and their upward diffusion from the compost layer. However, part of these nutrients was also absorbed by the fruiting bodies. Previous studies have shown that *A. bisporus* accumulates significant amounts of K and P in the mushroom tissue, whereas S and Na tend to remain within the casing system [71].

The total nitrogen (TN) content remained close to the initial range (10640–11480 mg/kg) across all treatments, showing no clear relationship with the type or rate of digestate addition. This suggests a balanced nitrogen turnover, where mineralization and nitrogen inflow from the compost compensate each other. Calcium (Ca) and magnesium (Mg) also exhibited only minor variations: Ca slightly decreased (from 35360 to 34989 mg/kg), and Mg remained within 6,100–6,500 mg/kg, confirming their limited mobility in the peat-digestate matrix.

Among trace elements, increases were noted in iron (Fe), zinc (Zn), and copper (Cu), particularly in variants with higher digestate doses, while manganese (Mn) remained relatively stable. These trends may reflect both the partial release of metals from the digestate and their redistribution within the casing, alongside selective uptake of some elements (notably Zn and Fe) by the mushrooms. Concentrations of cadmium (Cd) and lead (Pb) remained below the detection limit (<dl) in all samples, confirming the chemical safety of the materials used and the absence of heavy-metal accumulation during cultivation.

Most macro- and micronutrients increased in the casing layer after the cropping cycle, whereas TN, Ca, and Mg remained stable. The observed trends likely result from mineral migration from the compost and selective nutrient uptake by fruiting bodies, which are known to accumulate K, P, and Zn. These findings align with existing literature indicating that

Table 8. Nutrient concentrations (mg/kg DM) in casing treatments after mushroom cultivation

Sample	TN	Na	Mg	K	Ca	P	S
<i>Control</i>	10920 ± 11.18	1410 ± 0.27	6189 ± 0.10	14273 ± 2.47	34989 ± 1.11	877,9 ± 0.16	6034 ± 1.30
<i>Ss 3%</i>	11480 ± 2.44	2023 ± 0.24	6345 ± 0.38	16605 ± 2.47	35216 ± 1.93	1257 ± 0.10	7416 ± 0.90
<i>Ss 5%</i>	10640 ± 7.90	2058 ± 0.27	6527 ± 0.29	19627 ± 1.73	34598 ± 1.14	1483 ± 0.05	7591 ± 0.77
<i>Ss 10%</i>	11200 ± 6.61	2188 ± 0.26	6310 ± 0.12	21191 ± 1.86	33342 ± 2.09	1726 ± 0.30	9083 ± 0.57
<i>PM 3%</i>	11013 ± 1.47	1603 ± 0.21	6103 ± 0.48	15781 ± 1.56	33629 ± 1.16	1158 ± 0.04	6589 ± 0.91
<i>PM 5%</i>	10733 ± 1.51	1577 ± 0.25	6119 ± 0.04	15494 ± 2.03	33254 ± 0.76	1303 ± 0.14	6806 ± 0.66
<i>PM 10%</i>	10827 ± 8.31	1994 ± 0.20	6341 ± 0.40	19168 ± 1.47	33475 ± 0.70	1583 ± 0.24	7994 ± 0.31

Table 9. Concentrations of trace elements and heavy metals (mg/kg DM) in casing materials amended with digestates after cultivation; <dl – under detection level

Sample	Al	Cr	Mn	Fe	Co	Ni	Cu	Zn	Cd	Pb
<i>Control</i>	2796 ± 0.01	6.09 ± 0.00	155.7 ± 0.00	3211 ± 0.01	<dl	3.34 ± 0.00	3.92 ± 0.00	10.51 ± 0.00	<dl	<dl
<i>Ss 3%</i>	2967 ± 0.08	6.97 ± 0.00	152.0 ± 0.01	3435 ± 0.15	<dl	4.46 ± 0.00	4.78 ± 0.00	24.12 ± 0.00	<dl	<dl
<i>Ss 5%</i>	3229 ± 0.48	8.62 ± 0.00	160.0 ± 0.01	4049 ± 0.31	<dl	4.46 ± 0.00	5.69 ± 0.00	24.39 ± 0.00	<dl	<dl
<i>Ss 10%</i>	3088 ± 0.41	8.01 ± 0.00	147.7 ± 0.02	3881 ± 0.35	<dl	4.61 ± 0.00	6.29 ± 0.00	29.48 ± 0.01	<dl	<dl
<i>PM 3%</i>	2864 ± 0.10	6.37 ± 0.00	147.8 ± 0.01	3390 ± 0.10	<dl	3.75 ± 0.00	4.94 ± 0.00	16.27 ± 0.01	<dl	<dl
<i>PM 5%</i>	2786 ± 0.02	7.11 ± 0.00	153.9 ± 0.01	3512 ± 0.18	<dl	4.07 ± 0.00	4.99 ± 0.00	20.20 ± 0.00	<dl	<dl
<i>PM 10%</i>	2784 ± 0.21	7.68 ± 0.00	148.2 ± 0.02	3416 ± 0.10	<dl	4.45 ± 0.00	6.48 ± 0.00	26.36 ± 0.00	<dl	<dl



Figure 25. Heatmap illustrating Pearson's correlation coefficients between elemental contents in casing layers after *Agaricus bisporus* cultivation.

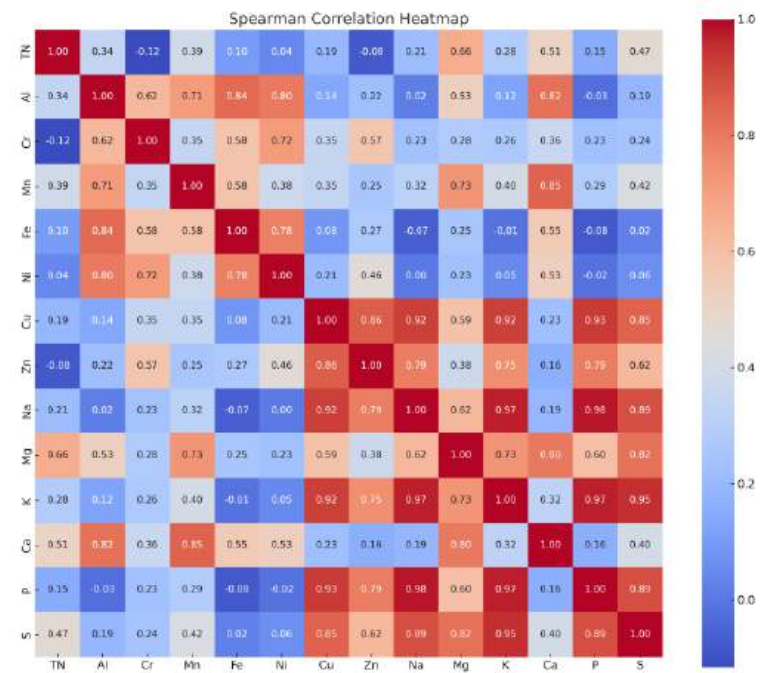


Figure 26. Heatmap presenting Spearman's correlation coefficients for elemental relationships in different casing treatments following mushroom cultivation.

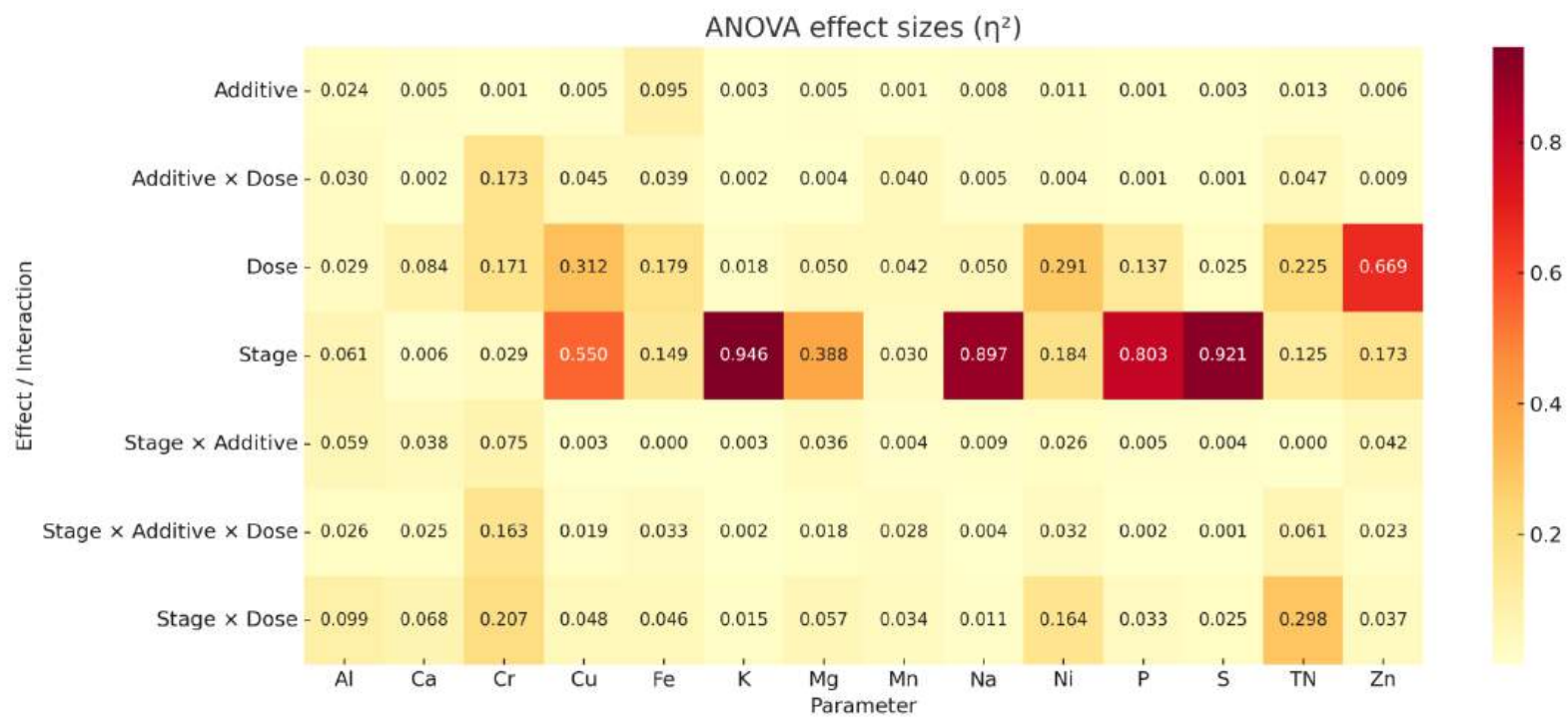


Figure 27. Heatmap showing η^2 (eta-squared) effect sizes from two-way ANOVA assessing the influence of digestate type, dose, and sampling stage on the elemental composition of the casing materials; additive - type (Ss or PM), dose - 3, 5 or 10%, stage - before/after cultivation.

the enrichment of casing layers with organic materials such as digestates mainly affects ion redistribution and the physicochemical transformations occurring during *A. bisporus* cultivation [45,70,71].

Correlation analyses using Pearson's and Spearman's coefficients (Figure 25, Figure 26) revealed clear relationships among the elemental contents in the casing layers after *Agaricus bisporus* cultivation. The strongest positive associations were found for Na, K, P, and S ($r > 0.9$), indicating that these elements co-occur in the aqueous phase of the casing and are subject to similar migration processes from the compost substrate. Strong correlations of Mg with these same elements suggest a shared redistribution of cations and anions, whereas weaker correlations of Ca confirm its limited mobility in the peat–digestate matrix.

Among trace elements, strong relationships were observed between Fe, Al, and Mn, likely reflecting their comparable geochemical behaviour, while Cu and Zn exhibited very strong mutual dependence, suggesting similar mechanisms of mobility and retention in the casing material. Total nitrogen (TN) showed only moderate correlations with other parameters, indicating that its transformations in the casing were largely independent of ionic balance and primarily driven by microbial mineralization rather than ionic exchange.

When comparing the two correlation approaches, the Spearman rank correlation revealed clearer and more consistent relationships between the analysed elements than the linear Pearson correlation. This pattern was especially evident for elements such as K, Na, P, S, and Mg, which displayed stronger and more coherent associations in the Spearman matrix. Such results indicate that the relationships among mineral components in the casing were predominantly monotonic rather than strictly linear, reflecting complex microbiological, sorption, and redistribution processes. Therefore, the Spearman method provides a more appropriate representation of dependencies in environmental and physicochemical data of this type.

The ANOVA results (Figure 27) included data obtained both before and after cultivation, allowing the evaluation of the effects of cultivation stage, digestate type, and dose on the elemental composition of the casing. The most pronounced influence was attributed to the *Stage* factor (sampling period), confirming that the observed mineral composition changes were primarily driven by cultivation processes. The highest η^2 values were recorded for K (0.946), S (0.921), and P (0.803), indicating that these elements underwent the greatest transformation during the growth cycle. The effects of digestate type and dose were weaker and significant mainly for Zn ($\eta^2 = 0.669$), whereas most trace elements displayed low η^2 values (< 0.2).

Comparison of the correlation and ANOVA results indicates that elemental redistribution in the casing was largely governed by the cultivation stage rather than by the type or amount of digestate added. The most mobile elements, including K, Na, P, and S, showed the highest variability, while Ca, Mg, and TN remained relatively stable. This confirms that the changes in mineral composition resulted mainly from ion migration and selective uptake by fruiting bodies, with a limited effect of organic amendments.

4. Conclusions

1. Digestates obtained from anaerobic digestion of sewage sludge (Ss) and from anaerobic co-digestion of sewage sludge with poultry manure (PM) can be used as alternative additives to the casing layer in *Agaricus bisporus* cultivation. Their influence on crop performance depends on both the digestate type and the applied dose..
2. The addition of digestate at levels of 3–5% of the casing mass maintained yield and biological efficiency (BE) comparable to the control, while higher doses (10%) caused a significant reduction in production parameters and led to uneven pinning and irregular fruiting patterns..
3. Digestate incorporation increased the concentration of mineral nutrients (K, P, S, Na) in the casing layer without exceeding the permissible content of heavy metals (Cd, Pb, Ni, Cr), confirming the chemical safety of the material for horticultural use.
4. The elemental composition of *A. bisporus* fruiting bodies reflected the properties of the applied digestates. Increased PM share enhanced the accumulation of Fe, Zn, Cu, and Mn, while the contents of major macronutrients (K, P, Mg, TN) remained stable, which indicates the ability of the mycelium to regulate nutrient uptake and maintain homeostasis under varying mineral conditions.
5. Correlation analyses demonstrated strong positive relationships between yield, BE, and the content of macronutrients (K, P, S, Mg, Ca), suggesting their synergistic role in productivity. In contrast, the accumulation of micronutrients (Fe, Zn, Mn, Cu) was negatively or weakly correlated with yield-related parameters, indicating possible competitive uptake mechanisms.
6. Principal Component Analysis (PCA) provided an integrated view of the interactions between chemical and production parameters. The results confirmed that both the digestate origin and dose were the main factors differentiating treatments. Moderate digestate additions (3–5%), especially PM 3%, ensured a favourable balance between productivity and mineral enrichment, whereas higher doses (10%) promoted excessive microelement accumulation (Cu, Na, Fe) accompanied by decreased yield and BE.
7. The application of digestate up to 5% of the casing mass can be recommended as a safe and environmentally sustainable solution for partial peat substitution. This approach supports nutrient recycling, reduces the environmental burden associated with peat extraction, and aligns with circular bioeconomy principles.
8. Future studies should focus on long-term assessments of digestate use in casing materials, including its influence on microbial community structure, physicochemical stability, and trace element dynamics during successive cultivation cycles, to further optimize sustainable practices in mushroom production.

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D.6

**Anna Jasińska, Anna Grosser, Erik Meers,
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*Stimulating Methane Production from Poultry
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Article

Stimulating Methane Production from Poultry Manure Digest with Sewage Sludge and Organic Waste by Thermal Pretreatment and Adding Iron or Sodium Hydroxide

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Abstract: The European Union's energy policy favors increasing the share of renewable energy in total energy production. In this context, the co-digestion of various waste streams seems an interesting option. This study aimed to determine the effect of selected pretreatment methods on the efficiency and kinetics of the co-digestion process of poultry manure with sewage sludge and organic waste. This research was carried out in four stages: (1) the selection of the third component of the co-digestion mixture; (2) the determination of the most favorable inoculum-to-substrate ratio for the co-digestion mixture; (3) the selection of the most favorable pretreatment parameters based on changes in volatile fatty acids, ammonium nitrogen, extracellular polymers substances (EPS) and non-purgeable organic carbon (NPOC); and (4) the evaluation of anaerobic co-digestion based on the result of the BMP tests and kinetic studies. All the pretreatment methods increased the degree of organic matter liquefaction as measured by the NPOC changes. Waste with a high fat content showed the highest methane potential. The addition of grease trap sludge to feedstock increased methane yield from 320 mL/g VS_{add} to 340 mL/g VS_{add}. An optimal inoculum-to-substrate ratio was 2. The pretreatment methods, especially the thermochemical one with NaOH, increased the liquefaction of organic matter and the methane yield, which increased from 340 mL/g VS_{add} to 501 mL/g VS_{add} (trial with 4.5 g/L NaOH).

Keywords: poultry manure; sewage sludge; grease trap sludge; co-digestion; pretreatment



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1. Introduction

One of the primary challenges currently confronting the energy sector is achieving autonomy from diminishing reserves of fossil fuels. For this reason, a pivotal course of action for the advancement of this industry involves shifting towards eco-friendly energy systems with reduced emissions. New renewable energy sources are being sought to pursue this objective, and existing solutions are being developed. Waste generated in rural and urban areas seems to be a valuable raw material for energy production. Among these resources are animal waste, sewage sludge, the organic fraction of municipal waste, by-products from the food industry such as fat waste, whey, or brewery grain, and energy crops like corn and forest biomass [1–3].

Poultry manure's high organic matter content and highly biodegradable substances make it a promising material for anaerobic digestion (AD). However, to address the high nitrogen levels which could impede the AD process, it is crucial to modify the C/N ratio for this waste [4,5]. For this reason, various methods are used, e.g., dilution, so that the feedstock sent to the digestion chambers contains about 3–6% total solids (TSs). This approach helps mitigate ammonia inhibition issues but often results in an insufficient

methane content in the produced biogas, thereby impacting the economic viability of the process. Moreover, the large volume of waste to be processed resulting from dilution makes this method economically unattractive [4]. An alternative strategy to improve biogas production efficiency is adding co-substrates into the digestion broth [6,7]. Poultry manure has been co-digested with a wide range of waste types, including lignocellulosic biomass [8], kitchen waste [9], sugar mill press mud [10], wine vinasse [11], sewage sludge [12–14], water hyacinth [15], sheep manure [16], etc. Co-digestion (AcD) not only boosts biogas output but also facilitates the dilution of harmful substances, reduces the operational expenses, and enhances the C/N balance, thereby increasing the appeal of this approach [17–19]. An interesting option is their joint anaerobic digestion with sewage sludge [4]. Compared to the AD of sewage sludge alone, after introducing poultry manure into the AD chambers, an increase of up to 40% in the daily biogas production has been observed [20].

The simultaneous processing of many waste types and improving AD efficiency align with the current European Union policy, supporting the increase in the share of renewable energy in the overall energy balance. This strategic focus suggests that co-digestion may play a more significant role in energy production in the future than the current dominant mono-digestion practice. However, the production of biogas from diverse waste materials might be constrained by factors such as the slow rate of biodegradation of the feedstock, the low efficiency of the AD process, and the low methane production [21,22]. Implementing pretreatment methods for feedstock (co-digestion mixture) appears promising in addressing these challenges. Such a pretreatment, an additional stage carried out before the AD/AcD process, makes it possible to increase the degree of liquefaction of the feedstock, improve its biodegradation, and, consequently, improve the efficiency of AD/AcD [23–25].

The selection of appropriate pretreatment techniques is crucial. This decision should be based on the substrate's physical and chemical properties and address specific critical requirements because pretreatment techniques have to meet certain criteria, including leading to a reduced substrate size, enhancing porosity, and increasing the feedstock's degradability and solubility. Additionally, eliminating inhibitory by-products (which may be produced following conditioning) and a low energy input are essential to ensure the method's cost-effectiveness [23]. Pretreatment methods can generally be categorized into three main groups: physical, chemical, and biological pretreatments. Sometimes, at least two pretreatment methods may be employed simultaneously to enhance the degradability of waste materials and eliminate toxic compounds [25,26]. Opinions on the effectiveness of combining AcD and pretreatments are divided. For this reason, further research focusing on integrating these two processes is required, as it allows for a better understanding of the interactions between the properties of substrates and determining their impact on the kinetics and efficiency of the process.

In this context, this research aimed to (1) select the third component of the co-digestion mixture (together with sewage sludge and poultry manure), (2) determine the most favorable inoculum-to-substrate ratio for the co-digestion mixture, and (3) determine the impact of the selected pretreatment methods on the efficiency and kinetics of the co-digestion process of sewage sludge and organic waste.

The novelty of this research is the selection of local co-substrates and the determination of the impact of pretreatment on the efficiency of the process. Of all types of manure, poultry manure is the least described in terms of the possibility of recovering methane from it. Most often, these publications concern its co-digestion with other waste generated on the farm. Taking into account the recent legislative changes (revision of the wastewater treatment directive), which provide for the energy self-sufficiency of wastewater treatment plants (WWTPs), we decided to determine the impact of poultry manure on the anaerobic digestion of sewage sludge. In order to further improve the profitability of the solution, the selection of the third component of the co-digestion mixture was made. To the best of our knowledge, no one has so far investigated the potential for the joint AD of sewage sludge with poultry manure and another organic waste type. In our research, we focused on the selection of the third component among local waste types, which could further direct their

generators to manage these products in a sustainable and, at the same time, economical way. The added value of this work is its focus on changes in the EPS structure that are not often found in the literature in the context of co-digestion processes.

2. Materials and Methods

2.1. Raw Materials

The substrate in the research was the following:

- The co-digestion mixture consisted of poultry manure and a mixture of primary and excess sludge. Sewage sludge (Ss) was collected from a municipal wastewater treatment plant located in the Silesian Voivodeship, while poultry manure (PM) was sourced from a laying poultry farm in the Silesia region of Poland. The manure was homogenized before the preparation of the co-digestion mixture, and the share of PM in the co-digestion mixture was 40% based on volatile solids (VSs);
- Inoculum—digested sludge collected from heat exchangers at a municipal wastewater treatment plant located in the Silesian Voivodeship (I). The WWTP produces annually approx. 3200 Mg dry mass of sewage sludge and treats about 90,000 m³/d of wastewater. In the mentioned WWTP, a mixture of waste-activated sludge and primary sludge was digested under mesophilic conditions, and the HRT of AD was equal to 30 d;
- Selected organic waste listed in Table 1.

Table 1. Place of collection of organic waste considered as the third component of the co-digestion mixture.

Substrate	Collection Site
By-product of the refining of vegetable fats (waste soap—WS)	Refining technological line
Fats contained in wastewater from the production of dairy products—flotation-thickened sludge (F)	Grease trap sludge
Grease trap sludge from meat-processing plant (GTS)	Grease trap sludge
Fruit waste (cherry processing period) (FW)	Waste storage yard

The characteristics of the substrates are shown in Table 2.

Table 2. Characteristics of raw materials.

Substrate	TS (%)		VS (% TS)		VS (%)	VS/TS	pH (–)	
	Average	SD	Average	SD	Average	–	Average	SD
F	11.38	0.12	77.22	0.11	8.79	0.77	6.18	0.01
GTS	14.62	0.02	89.05	0.56	13.02	0.89	5.44	0.01
WS	17.01	0.10	91.85	0.06	15.63	0.92	6.02	0.01
FW	12.87	0.07	87.88	0.29	11.31	0.88	4.21	0.01
PM	27.44	0.24	73.58	0.81	20.19	0.74	5.41	0.01
Ss	3.21–3.43		77.77–80.67		2.59–2.66	0.77–0.81	5.69–5.81	
I	2.46–2.47		62.85–63.32		1.55–1.56	0.62–0.63	8.47–8.51	

2.2. Experimental Procedure

This research was carried out in the following stages (Figure 1):

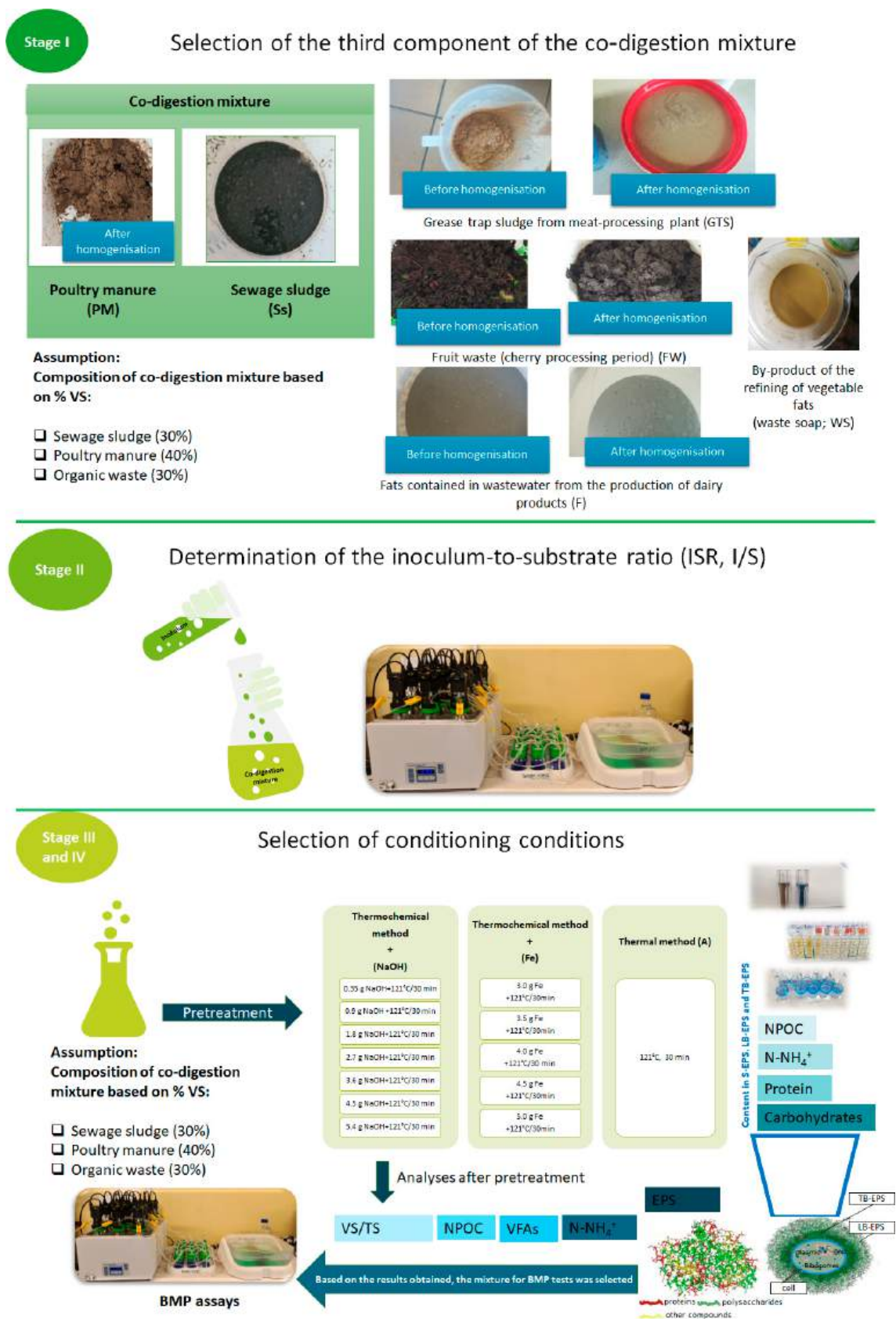


Figure 1. Scheme of this experiment.

1. Selecting, based on the results of the biomethane potential (BMP) tests, the third component of the co-digestion mixture (the methane potential of the waste itself was assessed, and then, for the selected waste, their joint anaerobic co-digestion with sewage sludge and poultry manure was carried out); based on our previously conducted research, it was assumed that the third component would constitute 30% of the co-digestion mixture based on the VS [27–29];
2. Determination of the most favorable inoculum-to-substrate ratio (I/S) for the co-digestion mixture. This research determined the impact of I/S ratios of 0.5, 1.0, 1.5, and 2 on the methane yield factor;
3. Selection of the most favorable parameters for the pretreatment of the feedstock based on changes in volatile fatty acids (VFAs), ammonium nitrogen, TS, VS, and non-purgeable organic carbon (NPOC), as well as the content of extracellular polymers substances (EPS) in individual fractions broken down by their concentration of carbohydrates, ammonium nitrogen, protein, and NPOC. The three-component co-digestion mixture and sewage sludge were treated with the following pretreatment methods (Figure 1): (a) the thermochemical method—thermal method using various doses of NaOH [g/L], temperature 121 °C, conditioning time 30 min (NaOH); (b) thermochemical method—thermal method using various doses of metallic iron, [g/L], temperature 121 °C, conditioning time 30 min (Fe); and (c) the thermal method—temperature 121 °C, conditioning time 30 min (A);
4. Determining the impact of the pretreatment methods of the feedstock (three-component co-digestion mixture) selected in stage 3 on the effectiveness of the anaerobic co-digestion process measured by the methane yield, the VS removal, and the kinetic parameter.

Anaerobic digestion (BMP tests) was carried out using the AMPTS II (Automatic Methane Potential Test System) from Bioprocess control. The AMPTS II device consists of three units connected. The first unit (unit A) consists of a water bath and a set of 15 bioreactors whose contents are mixed using mechanical mixers. In the second unit (unit B) (Duran bottles filled with sodium hydroxide), acidic biogas components such as CO₂ and H₂S are adsorbed. Adding thymolphthalein as a pH indicator to each of the vessels enables a simple, optical assessment of the ability of the NaOH solution to bind CO₂. Only methane flows through this unit and goes to the third unit (unit C) of the gas quantity measurement system.

BMP assays were performed in triplicate for each substrate–inoculum combination. The process was monitored daily for 21 days and carried out in mesophilic conditions (37 °C). The collected data were the basis for estimating the kinetic parameters. The mentioned parameters were determined using the models listed in Table 3.

Table 3. Kinetic models.

Model	Equal
Modified Gompertz equation (MG)	$y = P \times \exp\left\{-\exp\left[\frac{R_m \times e}{P} \times (\lambda - 1) + 1\right]\right\}$
Logistic function model (LF)	$y = \frac{P}{1 + \exp\left(\frac{4R_m \times (\lambda - t)}{P} + 2\right)}$
Transference function (TF)	$y = P \times \left(1 - \exp\left(-\frac{R_m \times (\lambda - 1)}{P}\right)\right)$

y—the cumulative of specific methane production, mL CH₄/g VS. P—potential methane production, mL CH₄/g VS. R_m—maximum rate of methane production, mL CH₄/(g VS d). t—measured time, d. λ—lag time phase, d. and, e—base of the natural logarithms, namely, 2.718282.

In turn, sample pretreatment was carried out using the ELMI ESS 207 industrial autoclave.

2.3. Physicochemical and Physical Analyses

During this study the following analyses were carried out: (a) volatile fatty acids (VFAs) by indirect distillation with steam, with a Büchi K-355 distiller in accordance with [30]; (b) ammonium nitrogen (N-NH_4^+) by the distillation method, in accordance with the APHA [30], and, in the case of the EPS fraction, spectrophotometrically, using the phenol-hypochlorite method described in Schillak [31]; (c) non-removable organic carbon (NPOC), determined after dry combustion using the Multi N/C H1300 carbon and nitrogen analyzer from Analytikjena; (d) total solids and volatile solids, residue after ignition, losses on ignition, dry organic matter, by the weight method, in accordance with [30]; (e) pH, via the potentiometric method in accordance with [30]; (f) extracellular polymers substances (EPS), for which individual fractions were extracted according to the methodology described by Yang et al. [32]; (g) carbohydrates, measured spectrophotometrically according to the procedure described by Nielsen [33]; and (h) proteins, via the Bradford method according to the Thermo Scientific™ procedure, using the Coomassie (Bradford) Protein Assay Kit number 23200. Equations (1)–(3) show how the VS and TS contents were calculated.

$$\%TS = \frac{(A - B) \times 100}{C - B} \quad (1)$$

$$VS (\%TS) = \frac{(A - D) \times 100}{A - B} \quad (2)$$

$$\%VS = \%TS \times VS \times \%TS \quad (3)$$

A—weight of dried residue + dish, g

B—weight of dish, g

C—weight of wet sample + dish, g

D—weight of residue + dish after ignition, g

Additionally, to determine whether there was a synergistic effect between the components of the mixture, the co-digestion performance index (CPI) often used in AcD studies was estimated according to the formula presented in Ebner et al. [34].

2.4. Statistical Analyses

The results were treated for statistical analysis. For this purpose, a one-way analysis of variance was used. For data for which significant statistical differences were found, further tests were performed, namely post hoc, more precisely Tukey's test. The analyzes were performed in the Statistica 10 software by Statistica.

3. Results and Discussion

3.1. Selection of the Third Component of the Co-Digestion Mixture

Among the organic waste types tested as part of this research, the highest methane yield was observed in waste with a high fat content, for which this parameter ranged from 630 to 750 mL/g VS_{add} (Table 4). Therefore, this type of waste was within the typical range indicated in the literature (430–990 mL/g VS_{add}) (Table 5). The methane yield in the case of the other tested waste types was much lower and most often below 300 mL/g VS_{add} . An exception to this rule was noted only for sewage sludge and a two-component co-digestion mixture consisting of poultry manure and sewage sludge. Similarly to waste with a high fat content, the obtained methane yield did not differ from the values reported in the literature (Table 5).

The BMP tests in the next phase for the three-component co-digestion mixture confirmed the assumption that the highest methane production was achieved for waste with a high fat content. Based on the results obtained, it was decided to conduct further research using a co-digestion mixture consisting of poultry manure, sewage sludge, and grease trap sludge from meat-processing plants (Table 6). Due to its highest cumulative production of methane, GTS was selected for further research as the third co-substrate of the co-digestion mixture.

Table 4. Methane yield (Y_M) of the tested organic waste.

Feedstock	Y_M (mL/g VS _{add})	SD
By-product of the refining of vegetable fats (waste soap—WS)	630	50
Fats contained in wastewater from the production of dairy products—flotation-thickened sludge (F)	750	70
Grease trap sludge from meat-processing plant (GTS)	740	60
Fruit waste (cherry processing period) (FW)	250	30
Poultry manure (PM)	270	20
Sewage sludge (Ss)	307	40
Ss + PM	320	30

Table 5. Summary of the methane yield for selected group waste [27,35].

Feedstock	Y_M (mL/g VS)
Sewage sludge	143–460
Fruit and vegetable waste	420
Fruit waste	180–732
Fats from flotation	600–700
Grease trap sludge	700 (1000)
Waste from grease trap sludge	845–928
Poultry manure	150–410

Table 6. Cumulative methane production for a three-component co-digestion mixture.

Feedstock	Cumulative Methane Production (mL)	SD
Ss + PM + F	1061.8	7.933
Ss + PM + GTS	1297.4	31.8
Ss + PM + WS	1110.27	28.5
Ss + PM + FW	696.7	4.8

3.2. Determination of the Inoculum-to-Substrate Ratio (ISR, I/S)

One of the most important parameters in batch tests, but often ignored in the research, is the proportion of inoculum to substrate. In the literature, this ratio is often marked as ISR or I/S (inoculum-to-substrate ratio), and sometimes an inverse relationship is given. Then, it is described with the symbol S/X (waste-to-inoculum ratio) or SIR (substrate-to-inoculum ratio). The I/S ratio may be expressed in terms of TS, VS, or chemical oxygen demand, and the second method is most often used for the above. The influence of the discussed ratio on the BMP test result is unclear. Theoretically, it should not affect the value of the methane/biogas yield but only the kinetics of the process, including the length of the lag phase (it shortens as the I/S value increases) [36–38]. Numerous literature reports, however, demonstrate the significance of the I/S ratio in preventing the process inhibition caused by the accumulation of inhibitors like VFAs, while also impacting the efficacy of the anaerobic digestion process [39]. Each substrate has an optimal characteristic value of the I/S ratio, and its value is a specific compromise between the endogenous production of inoculum biogas, the acceptable lag phase length, and the buffer capacity. For instance, Agrawal et al. [40] recorded the highest biogas production at an I/S of 0.3. Above the indicated value, they observed a decrease in biogas production, which they associated with the imbalance of nutrients in the feedstock (fruit and vegetable waste). In turn, Al-Iraqi et al. [39], for a feedstock composed of simulated food waste and common reeds, recommend an I/S ratio of 1. Other studies have shown that, for easily biodegradable substrates like household waste, I/S ratios ranging from 5 to 15 are suitable for batch assays without volatile fatty acid accumulation [41]. Meanwhile, in Elsayed et al. [42], the highest methane yield was noted at an I/S ratio of 3 during the anaerobic co-digestion of sludge, straw, and buckwheat

husk. Most often, the value of the I/S quotient given in the literature ranges from 1 to 4 [43]. Therefore, this research determined the impact of the following I/S ratios on the co-digestion of poultry manure, sewage sludge, and selected organic waste: 0.5, 1.0, 1.5, and 2 (Table 7).

Table 7. The influence of I/S on the methane yield of a mixture of poultry manure and sewage sludge.

I/S	Cumulative Methane Production (mL)	SD	Y _M (l/kg VS)	SD
0.5	2137.57	9.15	372.05	1.34
1.0	1356.93	19.98	370.51	4.57
1.5	1028.57	14.15	380.43	4.41
2.0	826.77	6.35	385.50	2.50

A one-way ANOVA showed statistically significant differences between the obtained methane production coefficients and the I/S ratio ($F = 12.44$, $p = 0.002$) (Table 8). Based on these results, it was decided to carry out further research for an I/S of 2.0.

Table 8. Post hoc test results.

I/S	Y _M (l/kg VS)	a	b	c
I/S_1.0	372.05	****		
I/S_0.5	370.51	****	****	
I/S_1.5	380.43		****	****
I/S_2.0	385.50			****

****—average values included in the same statistical group.

3.3. Selection of Pretreatment Conditions

In accordance with “Regulation (EC) No. 1774/2002 of the European Parliament and of the Council of 3 October 2002 laying down health rules concerning animal by-products not intended for human consumption” [44], animal manure is classified in the second category. For this reason, sterilization was used to pretreat the feedstock in this study. Additionally, the thermal method was combined with a chemical pretreatment to examine how the addition of iron or sodium hydroxide would affect the efficiency of the process.

The use of an alkaline pretreatment involves the application of chemicals that are mostly environmentally friendly and not prone to causing corrosion, such as different forms of ammonia (aqueous, liquid, and gaseous), sodium hydroxide, sodium carbonate, and calcium hydroxide (commonly known as lime). Compared to acid pretreatment, alkaline pretreatment can be conducted under less-severe conditions, making it a more feasible option for many applications [45]. In this study, sodium hydroxide was used for this purpose, with a dose not exceeding the inhibitory level reported in the literature [46]. In turn, iron’s impact on anaerobic digestion (AD) can be significant because it acts as a cofactor for enzymes involved in various metabolic pathways and serves as a catalyst for certain reactions (e.g., stimulation of direct interspecies electron transfer). Furthermore, iron is a crucial micronutrient for many microbial species, and its availability can affect their growth rates and metabolic activities and create a more favorable environment for them by reducing the ORP level. The source of iron in studies on its influence on AD included, among others, iron(III) chloride, nZVI (nano-zero-valent iron), iron(III) hydroxide, amorphous ferric, oxyhydroxide, ferrihydrite, lepidocrocite, hematite, magnetite, and akaganeite [47,48].

Regardless of the pretreatment method used, there was no significant increase in the concentration of volatile fatty acids in the supernatant. The highest VFA content was recorded in the pre-conditioned sample using the thermochemical method with the lowest dose of sodium hydroxide. However, it was less than eight percent higher than the untreated mixture. Increasing the base dose gradually decreased the indicator concentration (Figure 2a, Supplementary Materials, Table S1).

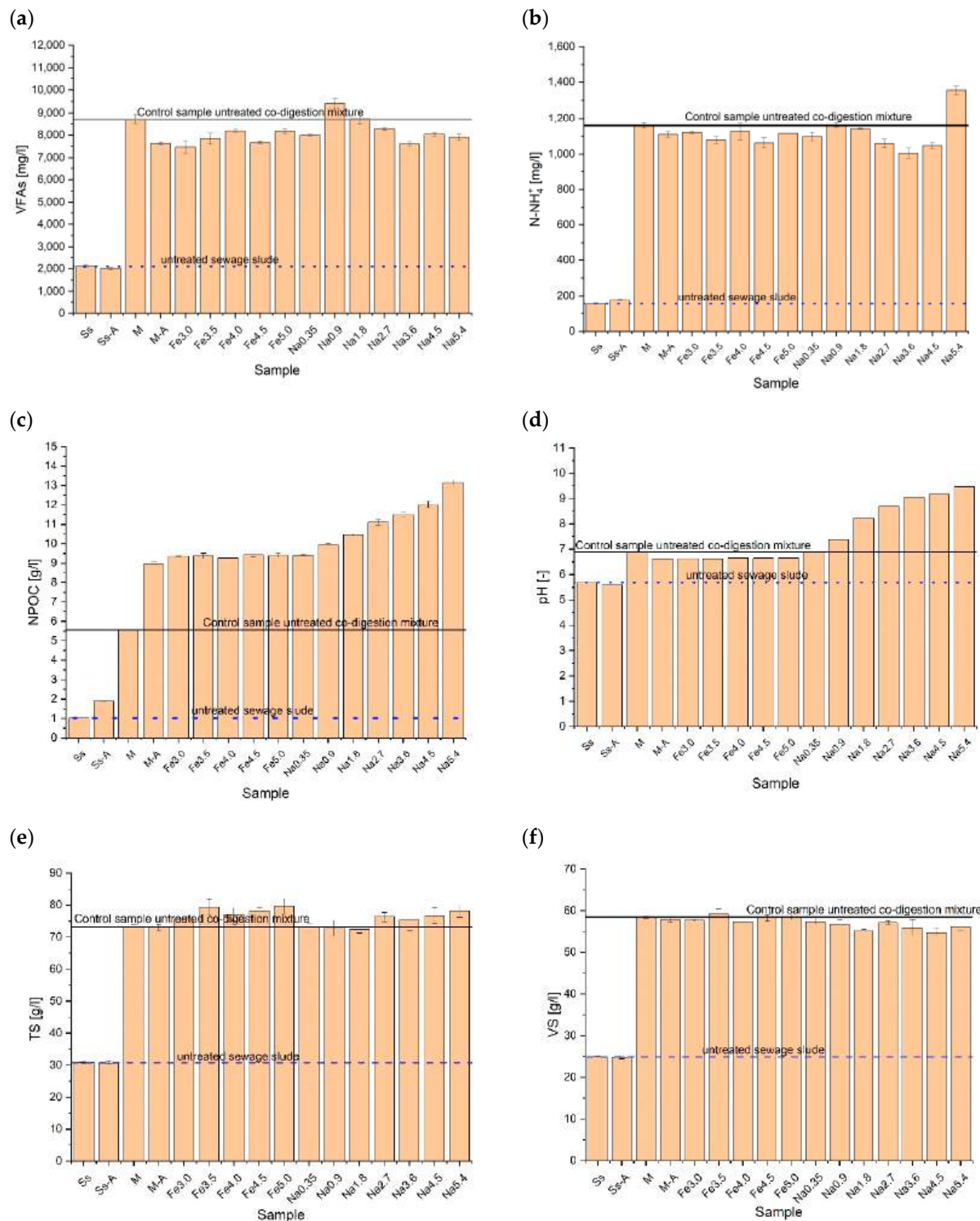


Figure 2. The influence of the pretreatment methods on selected indicators: (a) VFAs; (b) N-NH₄⁺; (c) NPOC; (d) pH; (e) TS; and (f) VS. Ss—untreated sewage sludge; Ss-A—sewage sludge treated with a thermal method; M—untreated co-digestion mixture of sewage sludge, poultry manure, and grease trap sludge (control sample); M-A—co-digestion mixture treated with the thermal method; Fe3, Fe3.5, Fe4, Fe4.5, and Fe5—co-digestion mixture treated with the thermochemical methods with iron addition, where the digit is the amount of iron used in the study, i.e., respectively, 3, 3.5, 4, 4.5, and 5 g/L; Na0.35, Na0.9, Na1.8, Na2.7, Na3.6, Na4.5, and Na5.4—co-digestion mixture treated with the thermochemical methods with NaOH addition, where the digit is the amount of NaOH used in the study, i.e., respectively, 0.35, 0.9, 1.8, 2.7, 3.6, 4.5, and 5.4 g/L.

A one-way ANOVA showed statistically significant differences between the ammonium nitrogen content in the samples (Figure 2b, Table S2). The highest ammonium nitrogen content was noted in the thermochemically conditioned sample 1, specifically in the mixture in which NaOH had been dosed at 5.4 g/L. The concentration of ammonium nitrogen was 1355.2 mg/L. Compared to the control sample, it was 196 mg/L higher and eight times higher than that observed in unconditioned and pretreated sewage sludge. The ammonium nitrogen content was 154 and 176.4 mg/L, respectively (Table S2). Dosing iron into the mixture resulted in a lower concentration of ammonium nitrogen in the overlying liquid compared to the control sample. A similar trend was noted in the research by Yang et al. [32], who hypothesized that iron could effectively inhibit the transformation of organic nitrogen into ammonium nitrogen.

Pretreatment of the co-digestion mixture positively affected the degree of liquefaction of organic matter measured by changes in the non-removable organic carbon (NPOC). However, the iron dose did not statistically affect the NPOC changes (Table S3, Figure 2c). The opposite trend was observed for the first of the tested thermochemical methods. Moreover, the NPOC concentration increased with the increase in the NaOH dose. For the highest dose of NaOH, the NPOC content was 2.4 times higher than for the control sample (increase from 5.55 to 13.14 mg/L). The lowest NPOC value was obtained for sewage sludge only (1.02 g/L), and it was slightly higher for conditioned sewage sludge (1.90 g/L). A much more significant increase in the decomposition and liquefaction of organic matter is reported in the literature. For instance, Liu and others [49] reported an almost six-fold increase in SCOD after the alkaline pretreatment of polyacrylamide flocculant waste-activated sludge. In turn, they noted a 7-fold increase for the thermal method, and the combination of both approaches resulted, in their study, in an almost 12-fold increase in SCOD.

Because one of its components was poultry manure, the co-digestion mixture had a much higher pH than the sewage sludge. According to what had been expected, introducing hydroxide into the mixture increased the pH. The pH increased with the hydroxide dosed into the co-digestion mixture (Figure 2d, Table S4). The dose of iron did not significantly impact the changes in the described indicator. For all the tested doses, the pH of the mixture after pretreatment was lower than that of the control sample (<6.89). This observation is inconsistent with the data presented in the work by Yang et al. [32]. The research substrate in the mentioned publication was sludge from an industrial sewage treatment plant located at a coking plant. The authors attributed the pH decrease to iron precipitation during the reaction with OH⁻ ions.

The pretreatment method influenced changes in the TS of the samples. A reduction in TS was observed for the thermochemical method with NaOH above a dose of 1.8 g/L. A similar trend was observed for samples with the addition of iron, regardless of its dose (Figure 2e, Table S5). The reduction in TS could probably be caused by the release of VFAs that had been oven drying during indicator measurement [50]. In turn, in the case of thermochemical method 2 (addition of iron), it could be identified by dosing metallic iron in powder. The changes in TS were reflected in the content of VS. Only for the thermochemical test with the addition of sodium hydroxide at the level of 3.5 g/L was the value of the indicator higher than for the control sample (untreated co-digestion mixture) (Figure 2f, Table S6). Rahman et al. [51] also observed an increase in ash content with a simultaneous decrease in VS during the co-digestion of poultry droppings and briquette wheat straw at mesophilic and thermophilic conditions.

Extracellular polymeric substances (EPS) are essential in the matrix of sludge and other organic wastes. EPS are complex mixtures of high-molecular-weight compounds secreted by microorganisms, playing a pivotal role in bioaggregation and in the formation of biofilms. Understanding and manipulating EPS within pretreatment processes can significantly impact the hydrolysis step of anaerobic digestion, the solubilization of organic matter and the accessibility of substrates to microbial action. Sewage sludge forms a flocculent structure because bacterial micelles are grouped into the given structure due

to the secretion of extracellular polymers substances (EPS) into the environment. In the existing literature, three fractions are distinguished in the structure of extracellular polymers substances: soluble EPS (S-EPS or SB-EPS), loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS) (Figure S1). In this context, how the composition of the mentioned fractions changed depending on the conditioning method was discussed [52–54]. As part of this work, the concentration of ammonium nitrogen, NPOC, protein, and carbohydrate content was examined in individual fractions (Figures 3–6, Tables S7–S26).

Tukey's test, carried out as part of this research, showed statistically significant differences in the concentration of NPOC ($p < 0.05$) in the extracellular polymers. Moreover, it proved that the pretreatment method significantly impacted the distribution of NPOC in individual EPS fractions (Tables S12–S16, Figure 3). This observation is consistent with the literature reports [55]. For all the tested methods for the first two fractions (EPS-S, EPS-LB), a significant increase in the NPOC concentration was noted compared to the control sample (Tables S13 and S14). Meanwhile, for the fraction of tightly bound EPS (EPS-TB), the trend for NPOC was utterly different. The NPOC concentration was lower than for the control sample for all the samples (Table S15). The highest degree of liquefaction of organic matter measured by NPOC changes was recorded for the thermochemical method, precisely the Na5.4 sample, i.e., with the addition of 5.4 g NaOH/L (Figure 3).

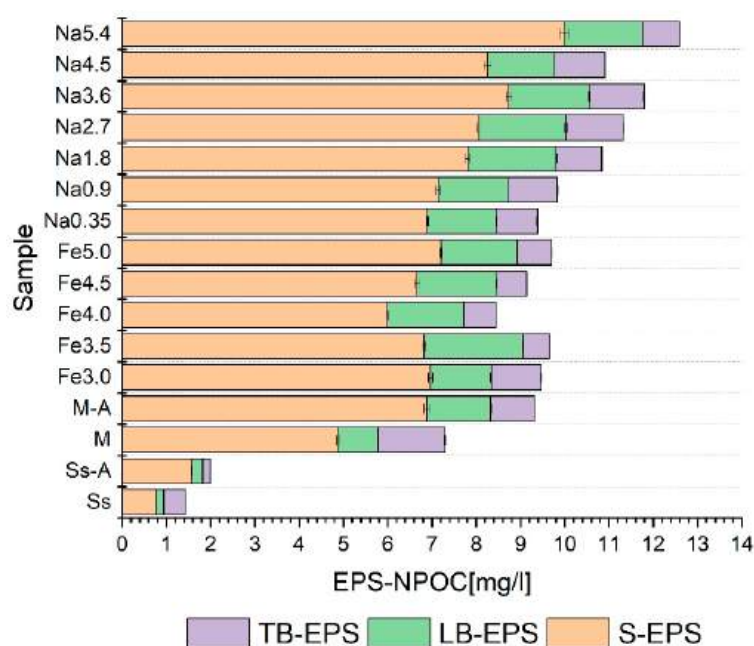


Figure 3. The influence of the pretreatment methods on the composition of the EPS measurement of changes in the NPOC concentration. SB-EPS—soluble EPS; LB-EPS—loosely bound EPS; TB-EPS—tightly bound EPS; Ss—untreated sewage sludge; Ss-A—sewage sludge treated with the thermal method; M—untreated co-digestion mixture of sewage sludge, poultry manure, and grease trap sludge (control sample); M-A—co-digestion mixture treated with the thermal method; Fe3 Fe3.5, Fe4, Fe4.5, and Fe5—co-digestion mixture treated with the thermochemical methods with iron addition, where the digit is the amount of iron used in the study, i.e., respectively, 3, 3.5, 4, 4.5, and 5 g/L; Na0.35, Na0.9, Na1.8, Na2.7, Na3.6, Na4.5, and Na5.4—co-digestion mixture treated with the thermochemical methods with NaOH addition, where the digit is the amount of NaOH used in the study, i.e., respectively, 0.35, 0.9, 1.8, 2.7, 3.6, 4.5, and 5.4 g/L.

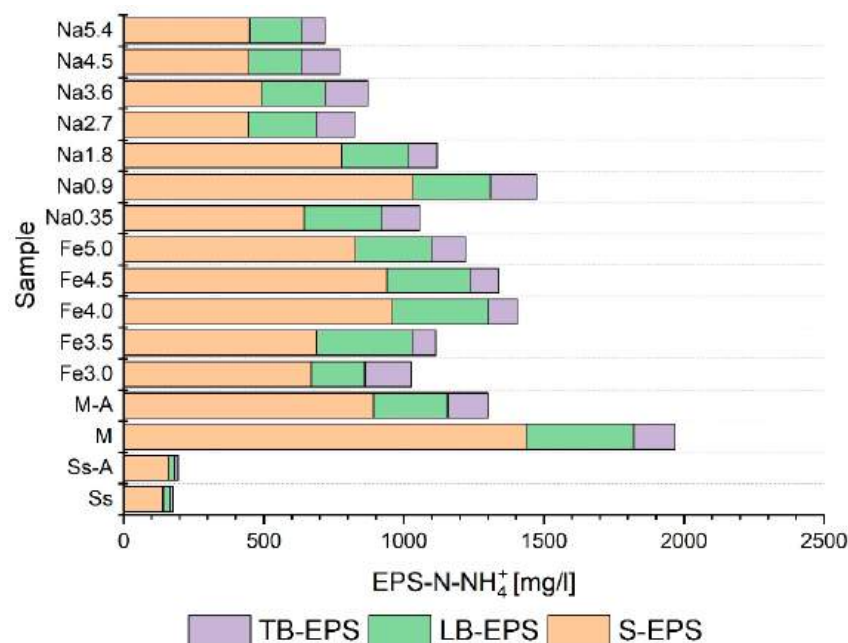


Figure 4. The influence of the pretreatment methods on the composition of the EPS measurement of changes in the N-NH_4^+ concentration. SB-EPS—soluble EPS; LB-EPS—loosely bound EPS; TB-EPS—tightly bound EPS; Ss—untreated sewage sludge; Ss-A—sewage sludge treated with the thermal method; M—untreated co-digestion mixture of sewage sludge, poultry manure, and grease trap sludge (control sample); M-A—co-digestion mixture treated with the thermal method; Fe3, Fe3.5, Fe4, Fe4.5, and Fe5—co-digestion mixture treated with the thermochemical methods with iron addition, where the digit is the amount of iron used in the study, i.e., respectively, 3, 3.5, 4, 4.5, and 5 g/L; Na0.35, Na0.9, Na1.8, Na2.7, Na3.6, Na4.5, and Na5.4—co-digestion mixture treated with the thermochemical methods with NaOH addition, where the digit is the amount of NaOH used in the study, i.e., respectively, 0.35, 0.9, 1.8, 2.7, 3.6, 4.5, and 5.4 g/L.

A one-way analysis of variance showed that the pretreatment method affected the ammonium nitrogen content in individual fractions of extracellular polymers (Tables S8–S11, Figure 4). EPS's highest ammonium nitrogen concentration was recorded for the control sample (Figure 4, Table S11). In turn, the most significant reduction in the total ammonium nitrogen content in EPS compared to the control was recorded for the thermochemical method, when the NaOH dose ranged from 2.7 to 5.4 g/L. Only for tightly EPS (TB-EPS), in two samples, there was an increase in the nitrogen concentration recorded compared to the control sample. These were the Na0.9 and Fe3 samples (Table S10).

Tukey's test showed that the pretreatment method had a statistically significant impact on the changes in the carbohydrate concentration in individual EPS fractions (Tables S16–S21, Figure 5). For all the samples treated with the conditioning agent, it was noted that the total carbohydrate content in the fractions was higher than in the control sample (Table S21, Figure 4). However, in relation to the iron, the dose of the chemical reagent did not affect the concentration of the mentioned indicator. It was also noted that, in regard to the carbohydrates determined in the EPS-S and EPS-LB fractions, the indicator values for all the conditioned samples were higher than for the control sample. The opposite trend was observed for the carbohydrates determined in the EPS-TB fraction.

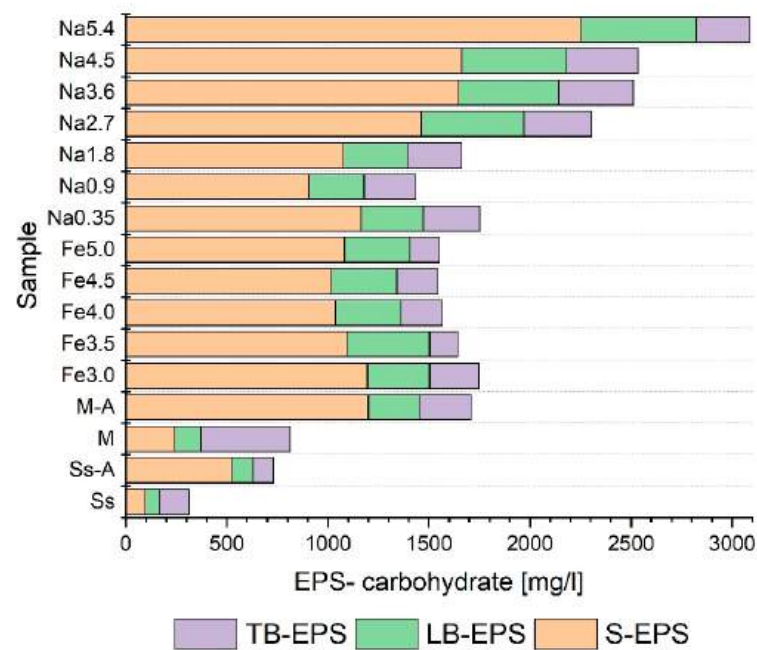


Figure 5. The influence of the pretreatment methods on the composition of the EPS measurement of changes in the carbohydrate concentration. SB-EPS—soluble EPS; LB-EPS—loosely bound EPS; TB-EPS—tightly bound EPS; Ss—untreated sewage sludge; Ss-A—sewage sludge treated with the thermal method; M—untreated co-digestion mixture of sewage sludge, poultry manure, and grease trap sludge (control sample); M-A—co-digestion mixture treated with the thermal method; Fe3, Fe3.5, Fe4, Fe4.5, and Fe5—co-digestion mixture treated with the thermochemical methods with iron addition, where the digit is the amount of iron used in the study, i.e., respectively, 3, 3.5, 4, 4.5, and 5 g/L; Na0.35, Na0.9, Na1.8, Na2.7, Na3.6, Na4.5, and Na5.4—co-digestion mixture treated with the thermochemical methods with NaOH addition, where the digit is the amount of NaOH used in the study, i.e., respectively, 0.35, 0.9, 1.8, 2.7, 3.6, 4.5, and 5.4 g/L.

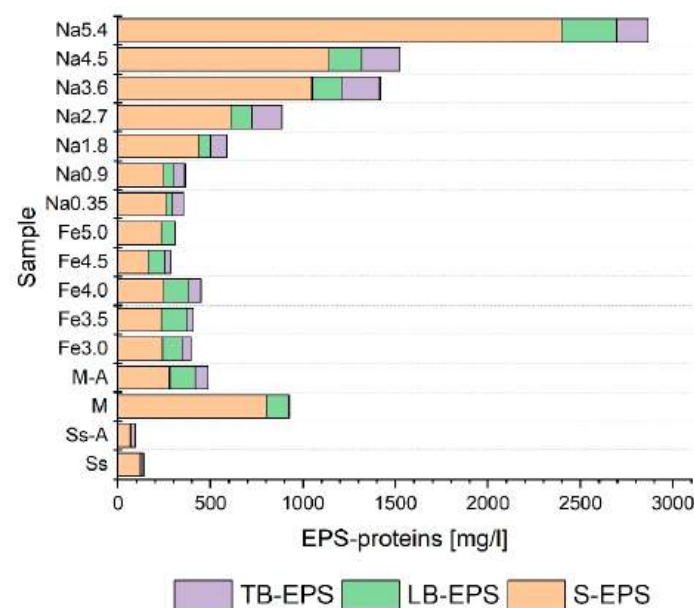


Figure 6. The influence of the pretreatment methods on the composition of the EPS measurement of changes in the protein concentration. SB-EPS—soluble EPS; LB-EPS—loosely bound EPS; TB-EPS—tightly bound EPS; Ss—untreated sewage sludge; Ss-A—sewage sludge treated with the thermal method; M—untreated co-digestion mixture of sewage sludge, poultry manure, and grease trap sludge

(control sample); M-A—co-digestion mixture treated with the thermal method; Fe3, Fe3.5, Fe4, Fe4.5, and Fe5—co-digestion mixture treated with the thermochemical methods with iron addition, where the digit is the amount of iron used in the study, i.e., respectively, 3, 3.5, 4, 4.5, and 5 g/L; Na0.35, Na0.9, Na1.8, Na2.7, Na3.6, Na4.5, and Na5.4—co-digestion mixture treated with the thermochemical methods with NaOH addition, where the digit is the amount of NaOH used in the study, i.e., respectively, 0.35, 0.9, 1.8, 2.7, 3.6, 4.5, and 5.4 g/L.

As in the case of the other components, Tukey's test showed for the proteins that the pretreatment method affected the distribution of the indicator in individual fractions of extracellular polymers (Tables S22–S26). The highest protein concentrations were determined in the first fraction (Figure 6). Small fluctuations in the total protein content were observed for dosed iron samples. In turn, for thermochemical method 2, an increase in the indicator concentration was observed with an increase in the NaOH dose. Compared to the control sample (Figure 6 and Table S26), only in mixtures to which at least 3.6 g/L of NaOH had been added, an increase in the total protein content was determined in the three EPS fractions.

3.4. Methane Production and VS Removal

Based on the results of series 3, several conditions for the pretreatment process were selected for further research. Their impact on biogas production and VS removal is shown in Figures 7 and 8. For the untreated co-digestion mixture, the methane yield was 340 mL/g VS. After the thermal pretreatment, an increase in the methane yield of 29% was recorded. Meanwhile, for the mixture treated with the thermochemical method with the application of sodium hydroxide to the feedstock, an increase to 501 mL/g VS was recorded; thus, it was 47% higher than that of the control sample (Figure 7). The method used did not statistically significantly affect VS removal ($F = 0.076$; $p = 0.99$) (Figure 8). The higher methane yields in the pretreatment samples could be associated with changes in the EPS structure. This phenomenon was particularly well observed in the liquefaction of organic matter measured by NPOC. This indicator increased significantly compared to the unconditioned co-digestion mixture. Furthermore, a significant decrease in its concentration in tightly bound EPS was observed in favor of an increase in the remaining fractions weakly bound to the cells. The higher methane yield for the samples conditioned with sodium hydroxide may have been related to an increased protein concentration in all the EPS fractions (Table 9). It has been estimated that 1 g of carbohydrates and proteins could theoretically obtain 830 mL (CH_4 of 50%) and 921 mL (CH_4 of 68.8%) of biogas. Saponification plays a crucial role in anaerobic digestion by increasing organic waste's solubility in the presence of a high lipid content, namely, the fraction with the highest methane potential (1425 mL/g, CH_4 of 69.5%) [56]. Moreover, saponification pretreatment with alkali reactants significantly increases the rates of hydrolysis, acidogenesis, and methanogenesis, ultimately improving the overall efficiency of the process [57,58].

For all the pretreatment co-digestion mixtures, co-digestion performance index (CPI) values higher than 1 were recorded (1.04 to 1.19). This clearly indicated the synergistic effect of AcD and the pretreatment methods used.

Due to the fact that no one had so far tested the possibility of improving methane production from poultry manure using a hybrid solution combining anaerobic co-digestion with a pretreatment, there were no direct data to which the obtained results can be compared. Therefore, the effectiveness of the process was compared with the pretreatment of poultry manure itself, its co-digestion with other waste streams, or data on the possibility of using hybrid solutions (Table 10). Generally, a much higher efficiency of methane production for poultry manure was achieved if it was digested with other waste than if it had been conditioned. Often, combining several unit operations in a hybrid system significantly improved process efficiency, although this was strongly dependent on the composition of the co-digestion mixture.

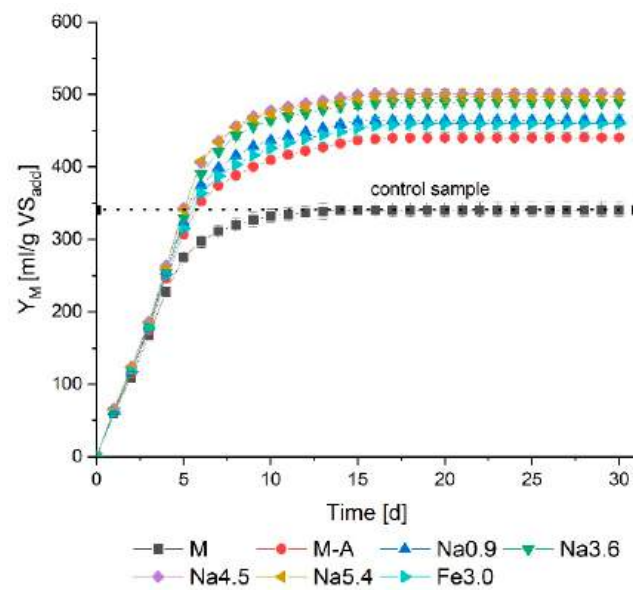


Figure 7. The influence of the selected pretreatment methods on the methane yield. M—untreated co-digestion mixture of sewage sludge, poultry manure, and grease trap sludge (control sample); M-A—co-digestion mixture treated with the thermal method; Na0.9, Na3.6, Na4.5, and Na5.4—co-digestion mixture treated with the thermochemical methods with NaOH addition, where the digit is the amount of NaOH used in the study, i.e., respectively, 0.9, 3.6, 4.5, and 5.4 g/L; and Fe3—co-digestion mixture treated with the thermochemical method with an iron addition of 3 g/L. Results obtained with an I/S ratio equal to 2.

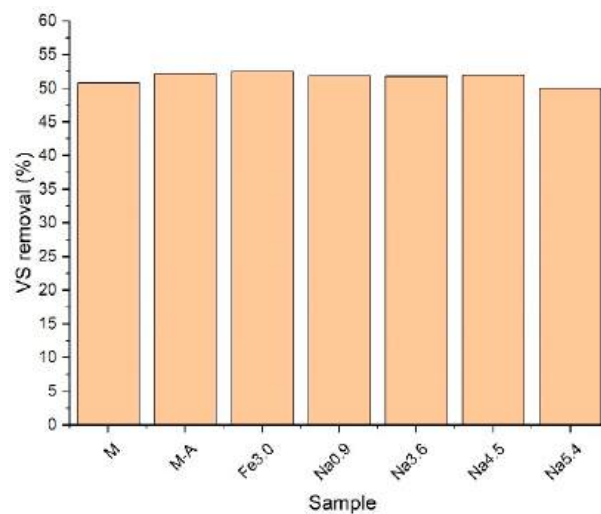


Figure 8. Impact of the pretreatment method on VS removal. M—untreated co-digestion mixture of sewage sludge, poultry manure, and grease trap sludge (control sample); M-A—co-digestion mixture treated with the thermal method; Na0.9, Na3.6, Na4.5, and Na5.4—co-digestion mixture treated with the thermochemical methods with NaOH addition, where the digit is the amount of NaOH used in the study, i.e., respectively, 0.9, 3.6, 4.5, and 5.4 g/L; and Fe3—co-digestion mixture treated with the thermochemical method with an iron addition of 3 g/L. Results obtained with an I/S ratio equal to 2.

Table 9. The influence of the pretreatment methods on the feedstock compared to the untreated co-digestion mixture.

Indicator		Sample					
		M-A	Na-0.9	Na-3.6	Na-4.5	Na-5.4	Fe-3.0
VFAs N-NH ₄ ⁺ NPOC		↓	↑	↓	↓	↓	↓
		↓	↑	↓	↓	↓	↓
		↑	↑	↑	↑	↑	↑
N-NH ₄ ⁺	SB-EPS	↓	↓	↓	↓	↓	↓
	LB-EPS	↓	↓	↓	↓	↓	↓
	TB-EPS	↓	↑	-	↓	↓	↑
	Sum	↓	↓	↓	↓	↓	↓
NPOC	SB-EPS	↑	↑	↑	↑	↑	↑
	LB-EPS	↑	↑	↑	↑	↑	↑
	TB-EPS	↓	↓	↓	↓	↓	↓
	Sum	↑	↑	↑	↑	↑	↑
carbohydrate	SB-EPS	↓	↓	↓	↓	↓	↓
	LB-EPS	↑	↑	↑	↑	↑	↑
	TB-EPS	↓	↓	↓	↓	↓	↓
	Sum	↑	↑	↑	↑	↑	↑
Protein	SB-EPS	↓	↓	↑	↑	↑	↓
	LB-EPS	↑	↓	↑	↑	↑	↓
	TB-EPS	↑	↑	↑	↑	↑	↑
	Sum	↓	↓	↑	↑	↑	↓

SB-EPS—soluble EPS; LB-EPS—loosely bound EPS; TB-EPS—tightly bound EPS; ↓—lower than the control sample; and ↑—higher than the control sample.

Table 10. The impact of various strategies on methane production from poultry manure (pretreatment and/or in a mixture with various types of organic waste).

Feedstock	Pretreatment	AD Conditions	Remarks	Reference
Chicken manure	Extraction (by shaking) of chicken manure with water in the amount of 5 g fresh weight of manure per 100 mL of deionized water.	Batch assay, 2 L reactor, 38 ± 2 °C	Without pretreatment Y _B : 362.5 mL/g VS With pretreatment Y _B : 420.6–527.8 mL/g VS Enhancement: +45%	[59]
Chicken litter	5% of NaOH 90 min, 120 °C +3% of H ₂ SO ₄ 90 min, 120 °C		With pretreatment Y _M : 137 mL/g VS Enhancement: -	[60]
Chicken litter and chicken feathers	Ca(OH) ₂ at 90 °C, 1.27 bar pressure		With pretreatment Y _M : 101 mL/g VS Enhancement: -	[61]
Poultry manure	<i>Clostridium cellulolyticum</i> , <i>Clostridium saccharolyticum</i> , and <i>Clostridium thermocellum</i> as bioaccumulation strains	Batch assay, 50 mL bottles, 37 °C	With pretreatment Y _M : 102 mL/g VS Enhancement: +15%	
Food waste + poultry manure	120 °C, 10 bar, 30 min 2450 MHz, power 1460 W, wavelength of 12.24 cm, and cavity size of 470 Ultrasonication: 20 kHz, 130 W, 30 min	Batch assay, 2.0 L bottles, 30 d	Untreated: 8921 mL Enhancement: +4–11%	[62]

Table 10. Cont.

Feedstock	Pretreatment	AD Conditions	Remarks	Reference
Poultry droppings + kitchen waste	The addition of silica gel as a catalyst	26–31 °C, 8% TS	With pretreatment Y _M : 10,545 mL/kg Enhancement: +31.12% compared to trial without silica gel	[63]
Chicken litter (CL) + wheat straw (WS) + food waste (FW)	NaOH (1–5% <i>w/v</i>), 30–90 min, 60–120 °C	Batch assay, 1 L, 37 °C	CL Y _M : 321.1 mL/g VS _{added} With pretreatment Y _M : 440–590 mL/g VS _{added} Enhancement: up to 83.7%	[60]
	Sequential alkaline acid pretreatment 1%, 2%, and 3% (<i>w/v</i>) H ₂ SO ₄		With pretreatment Y _M : 470–625 mL/g VS _{added} Enhancement: up to 94.6%	
Poultry manure	150 °C/5 min and 4.8 ba	AMPTS device (Bio-Process control, Lund, Sweden), 39 ± 1 °C	With pretreatment Y _M : 288 mL/g VS _{added} Enhancement: up to 14.4%	[64]
Chicken manure	hyper-thermophilic (70 °C) pretreatment	continuously stirred tank reactors, 12 L, 100 rpm, 55 °C	With pretreatment Y _M : 518 mL/g VS _{added} Enhancement: up to 54.6%	[65]
Chicken manure (CM)	pretreated at 100 °C, 125 °C, 150 °C, 175 °C, 200 °C, and 225 °C	Batch assay, 35 ± 1 °C	CM: Y _M : 340 mL/g VS _{added} Enhancement: −7.86	[66]
Co-digestion (AcD) a mixture of 60% sewage sludge, 20% chicken manure, and 20% sugarcane waste (SCW)	Acid pretreatment with different doses of acetic acid (2%, 3%, and 4% <i>v/v</i>); duration: 30–90 min	Batch assay, 450 mL bottles, 30 ± 4 °C, 150 rpm	Raw agroindustrial waste: Y _M : 426.9 mL/g VS _{rem} Enhancement: up to 1392.9 mL/g VS _{rem}	[67]
Chicken manure (CM) and sewage sludge (SS)	Iron oxide nanoparticles at dosages of 40, 80, 120, and 160 mg/L	Batch assay, 1 L, 35 °C	SS Y _M : 278.8 mL/g VS CM: SS (70:30 on VS base): 3389.7 mL/g VS AcD with iron: Y _M : 421.9–560.6 mL/g VS	[68]
Poultry manure (PM) and lignocellulosic materials	-	Batch assay, 120 mL bottles, 35 °C, 180 rpm	The best result was achieved for a food-to-inoculum ratio equal to 0.5 Y _M for PM: 13–302 mL/g VS Y _M for co-digestion PM with rice straw, corn cob, peanut shell, sawdust, coffee husks, or sugarcane bagasse lower than for AD of PM alone	[8]
Kitchen waste (KW) and poultry manure (wet weight basis) at ratios of 1:0, 1:1, 2:1, and 3:1	-	Batch assay, 1 L bottles, 28 and 37 °C	KW Y _B : 312 mL/g VS (28 °C); 532 mL/g VS (37 °C) AcD: 360–396 mL/g VS (28 °C); 452–920 mL/g VS (37 °C)	[9]
Poultry droppings (PDs) with two different ratios of physicochemical sludge (20% and 40%, based on the VS)		Continuously stirred anaerobic digester, 12 L, 37 °C, HRT 30 d	PD, Y _M : 270 mL/g VS Enhancement: up to 54%	[69]

Table 11 summarizes the results of the kinetic studies. An increase in the methane production rate was recorded for all the tested methods. Another important factor determining the co-digestion process' effectiveness was the adaptation phase's duration (λ). As shown by the data in Table 11, the pretreatment of the mixture usually resulted in an extension of the adaptation time of the microorganisms. Moreover, it was found that, with an increase in the dose of sodium hydroxide, the adaptation time of the microorganisms was prolonged. A similar trend was noted by Almomani and Bhosale during the anaerobic co-digestion of agricultural waste with cow dung (CD) and the use of alkaline pretreatment with sodium bicarbonate [70]. In turn, Wu et al. [71] observed that saponification with calcium enhanced the methane yield for fat, oil, and grease as well as reduced the lag phase. Despite the longer lag time phase in anaerobic co-digestion reactors, the results clearly showed that methanogens consumed organic acids quite quickly in these reactors, which consequently led to an increase in the rate of methane production and a higher methane yield, thus confirming previous observations from the literature according to which the carefully selected addition of co-substrates to digesters can help improve the rate of methane production during co-digestion [72–75].

Table 11. Summary of the estimated kinetic parameters.

Sample	P [mL CH ₄ /g VS]	R _m [mL CH ₄ /(g VS d)]	λ [d]	R ²
Modified Gompertz equation (MG)				
M	340.3624	63.49502	0.235276	0.99933
M-A	438.4465	66.90778	0.265849	0.99895
Na0.9	463.6653	72.92838	0.389854	0.99915
Na3.6	488.7484	77.75526	0.548816	0.99912
Na4.5	501.0150	81.16676	0.552048	0.99900
Na5.4	496.8261	81.76055	0.589216	0.99862
Fe3.0	457.6325	68.74786	0.290088	0.99895
Logistic function model (LF)				
M	338.9935	58.93189	0.177459	0.99879
M-A	435.8587	62.60483	0.213661	0.99705
Na0.9	461.0132	68.71367	0.373178	0.99800
Na3.6	485.9906	73.89803	0.575518	0.99902
Na4.5	498.2668	77.18721	0.581775	0.99908
Na5.4	494.0965	78.13411	0.636446	0.99896
Fe3.0	454.8350	64.42861	0.242888	0.99693
Transference function (TF)				
M	342.8943	114.4020	0.609348	0.99474
M-A	443.7058	117.5256	0.673899	0.99564
Na0.9	469.2031	126.1682	0.721710	0.99437
Na3.6	494.8274	131.5803	0.786065	0.99226
Na4.5	507.0182	137.4199	0.781930	0.99190
Na5.4	502.7924	137.5215	0.792250	0.99115
Fe3.0	463.4191	120.1680	0.691750	0.99576

All the models showed a very good fit to the experimental results and estimated parameters, as evidenced by the value of the coefficient of determination ($R^2 > 0.99$).

4. Conclusions

This study's primary objective was to investigate the impact of selected pretreatment techniques on the efficiency of anaerobic digestion (AD) of a three-component co-digestion mixture. In addition to conducting biochemical methane potential (BMP) tests, the analysis focused on examining the characteristics of the supernatant post pretreatment and identifying the most suitable inoculum-to-substrate ratio. This study's findings led to several key conclusions:

- Waste with a high fat content has the highest potential for methane production;

- The addition of a third co-substrate to the mixture of sewage sludge and poultry manure resulted in a substantial increase in methane production;
- The inoculum-to-substrate ratio (I/S) has a statistically significant impact on the methane yield;
- The highest value of methane yield was obtained for an I/S equal to 2;
- Pretreatment of the three-component co-digestion mixture frequently led to a reduction in volatile fatty acids (VFAs) within the digestion broth;
- All the pretreatment methods used resulted in an increase in the degree of liquefaction of organic matter measured by changes in the NPOC;
- With an increase in the dose of sodium hydroxide, an increase in the NPOC parameter was observed;
- Iron did not result in statistically significant changes in the NPOC levels;
- The pretreatment generally did not impact the total solid (TS) and volatile solid (VS) content of the three-component co-digestion mixture;
- The pretreatment methods had a significant statistical impact on the distribution of the analyzed indicators in individual EPS fractions;
- The samples treated using thermochemical methods exhibited lower total ammonium nitrogen contents in extracellular polymers than the control sample;
- The use of pretreatment on the co-digestion mixture usually resulted in an increase in the degree of liquefaction of organic matter (measured by changes in NPOC, protein, and carbohydrates) in all the fractions of extracellular polymers;
- Pretreatment intensifies methane production (for all the tested methods, the methane yield was higher than for the control sample);
- The most substantial methane yield was observed following thermochemical pretreatment with 4.5 g NaOH/L (501 mL/g VS);
- An increase in the sodium hydroxide concentration prolonged the adaptation time of microorganisms (lag phase extension);
- The pretreatment methods used slightly improved VS removal. This indicator compared to the unconditioned co-digestion mixture increased from 50.8 to 52.5%; however, the one-way analysis of variance showed that this change was not statistically significant. It is worth emphasizing that, compared to the AD of sewage sludge itself, for which VS removal is usually around 30–40%, the values obtained in the tests were satisfactory.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/en17112679/s1>: Figure S1 Structure of extracellular polymers, based on [76], where SB-EPS-soluble EPS; LB-EPS-loosely bound EPS; TB-EPS-tightly bound EPS Table S1 Tukey test for VFAs result; Table S2 Tukey test for N-NH_4^+ result; Table S3 Tukey test for NPOC result; Table S4 Tukey test for pH result; Table S5 Tukey test for TS result; Table S6 Tukey test for VS result; Table S7 Results of one-way analysis of variance for ammonium nitrogen content in three fractions of extracellular polymers (EPS- N-NH_4^+); Table S8 Tukey test for ammonium nitrogen content in SB-EPS; Table S9 Tukey test for ammonium nitrogen content in LB-EPS; Table S10 Tukey test for ammonium nitrogen content in TB-EPS; Table S11 Results of one-way analysis of variance for ammonium nitrogen content in three fractions of extracellular polymers (EPS- N-NH_4^+); Table S12 Results of one-way analysis of variance for NPOC content in three fractions of extracellular polymers (EPS-NPOC); Table S13 Tukey test for NPOC in SB-EPS; Table S14 Tukey test for NPOC in LB-EPS; Table S15 Tukey test for NPOC in TB-EPS; Table S16 Results of one-way analysis of variance for NPOC content in three fractions of extracellular polymers (EPS-N-NPOC); Table S17 Results of one-way analysis of variance for carbohydrates content in three fractions of extracellular polymers (EPS-NPOC); Table S18 Tukey test for carbohydrates in SB-EPS; Table S19 Tukey test for carbohydrates in LB-EPS; Table S20 Tukey test for carbohydrates in TB-EPS; Table S21 Results of one-way analysis of variance for carbohydrates content in three fractions of extracellular polymers; Table S22 Results of one-way analysis of variance for proteins content in three fractions of extracellular polymers; Table S23 Tukey test for proteins in SB-EPS; Table S24 Tukey test for proteins in LB-EPS; Table S25

Tukey test for proteins in TB-EPS; Table S26 Results of one-way analysis of variance for proteins content in three fractions of extracellular polymers.

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**Anna Jasińska, Anna Grosser, Erik Meers,
Dagmara Piłyp (2025)**

*Evaluation of saponification and chemical
pretreatments for poultry manure co-digestion:
methane production, EPS fraction analysis
and kinetic study*

Manuscript prepared for publication

Evaluation of saponification and chemical pretreatments for poultry manure co-digestion: methane production, EPS fraction analysis and kinetic study

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Abstract

This study investigates the influence of alkaline (NaOH) and thermo-alkaline (saponification at 70 °C + NaOH) pretreatments on the anaerobic co-digestion of poultry manure (PM), sewage sludge (SS), and grease trap sludge (GTS) at a 30:40:30 volatile solids (VS) ratio. The research combines methane yield analysis, extracellular polymeric substances (EPS) fraction profiling, and kinetic modelling. Pretreatments significantly altered the EPS matrix and enhanced substrate solubilization. Saponification induced the most substantial transformation of tightly bound EPS (TB-EPS) into soluble EPS (S-EPS), increasing soluble carbohydrates and proteins by 95–120 % while reducing TB-EPS by 25–45 %. Organic matter solubilization (NPOC, VFAs) was substantially higher than under chemical pretreatment. Methane yield increased linearly with NaOH dose during saponification ($Y_m = 14.9 \times \text{dose} + 476.98$; $R^2 = 0.95$), reaching 556 ml CH₄/g VS at 4.5 g NaOH/l. In contrast, the purely alkaline variant showed an optimum at approximately 3 g/l, beyond which yield declined, likely due to volatile fatty acids (VFAs) and ammonium nitrogen accumulation. Two-way ANOVA confirmed significant effects of method, NaOH dose, and their interaction ($p < 0.001$). Correlation and PCA analyses demonstrated strong positive associations between S-EPS, NPOC, and VFAs ($r \approx 0.8$), and inverse relationships between S-EPS and TB-EPS, confirming the solubilization mechanism. Saponification improved methane yield (+29 %), shortened the lag phase, and increased the co-digestion performance index (CPI = 1.29), indicating synergistic enhancement beyond additive effects. The results highlight saponification as an efficient and controllable pretreatment strategy to improve anaerobic biodegradability and methane productivity of mixed organic wastes.

Key words: anaerobic co-digestion, biowaste, saponification, thermo-alkaline pretreatment, poultry manure; sewage sludge; grease trap sludge; extracellular polymeric substances (EPS), methane yield, biogas production, circular bioeconomy.

Abbreviations

AD, anaerobic digestion; ARGs, antibiotic resistance genes; BMP, biochemical methane potential; CPI, co-digestion performance index; DM, dry mass; EPS, extracellular polymeric substances; GTS, grease trap sludge; HRT, hydraulic retention time; IQR, interquartile range; ISR, inoculum-to-substrate ratio; LB-EPS, loosely bound extracellular polymeric substances; MG, modified Gompertz model; NaOH, sodium hydroxide; N-NH₄⁺, ammonium nitrogen; NPOC, non-purgeable

organic carbon; PCA, principal component analysis; PM, poultry manure; R^2 , coefficient of determination; R_m , maximum methane production rate; SS, sewage sludge; S-EPS, soluble extracellular polymeric substances; TB-EPS, tightly bound extracellular polymeric substances; TF, transfer function model; THP, thermal hydrolysis process; TS, total solids; TKN, total Kjeldahl nitrogen; VFAs, volatile fatty acids; VS, volatile solids; WWTP, wastewater treatment plant.

1. Introduction

Anaerobic digestion (AD) is a cornerstone technology in the circular bioeconomy, combining organic waste stabilization with the recovery of renewable energy in the form of methane. [1]. Traditionally, sewage sludge has been the principal substrate for AD. However, in recent years, animal manure — particularly poultry manure have gained increasing attention as suitable feedstocks due to the rapid growth of the poultry industry [2,3]. Nevertheless, the high total solids content, ammonium nitrogen levels and presence of complex, poorly biodegradable compounds make mono-digestion of poultry manure challenging. The process is prone to instability, inhibition, and low methane yields [3–5]. To overcome these limitations, various strategies have been explored to enhance the biodegradability of poultry manure and improve biogas productivity. These include both substrate pretreatments and co-digestion with complementary organic wastes, which can mitigate inhibition and optimize nutrient balance through synergistic effects [3,6–8]. Literature reports indicate that poultry manure can be effectively co-digested with sewage sludge, grease trap sludge, kitchen residues, lignocellulosic biomass, food industry by-products, or even sheep manure [9,10].

The efficiency of both co-digestion and pretreatment is strongly dependent on the composition and behavior of extracellular polymeric substances (EPS), which form the principal matrix of bioflocs and play a decisive role in the digestion process [11,12]. Understanding the structure and the possibilities for modifying it in EPS is therefore essential to improving anaerobic digestion performance. EPS are complex mixtures of polysaccharides, proteins, nucleic acids, lipids and humic-like substances secreted by microorganisms or released during cell lysis [12,13]. In activated sludge and biofilms they form a three-dimensional structural matrix that stabilizes flocs and mediates intercellular interactions [11]. On the one hand, they are essential for the proper functioning of the microbiocenosis in wastewater treatment plants, ensuring adhesion and sorption capacity toward metals and micropollutants [12,14]. On the other hand, they form a diffusion barrier that limits the access of enzymes to organic material [15,16]. In particular, tightly bound EPS (TB-EPS) exhibit high structural density, thereby reducing hydrolysis rates and overall biodegradability [17]. Conversely, EPS also play a protective role by binding heavy metals [18], pesticides, or antibiotics, thereby mitigating their toxicity toward methanogenic consortia [19]. Recent studies further show that EPS protect microbial aggregates from nanoparticles and microplastics—emerging contaminants increasingly detected in wastewater streams [20–22]. Moreover, EPS influence the dispersion and transport of micro/nanoplastics (MP/NP) and are closely linked with antibiotic resistance genes (ARGs) associated with sludge matrices [16,20,23]. Controlled degradation of EPS may thus help reduce ARG propagation, improving environmental safety [24].

This dual functionality of EPS highlights the importance of analyzing its content and distribution to understand the limitations of anaerobic digestion and to design effective enhancement strategies. Among various pretreatment approaches, those that destabilize the EPS matrix, increase organic matter solubilization, and improve substrate bioavailability are particularly

promising. Thermal and thermo-alkaline pretreatment [25–27], acid or alkaline hydrolysis[28], oxidative reagents [29,30], and ultrasonic disintegration [31] have all been tested with varying success.

Within this context, alkaline-based processes, especially saponification, have attracted growing interest. Saponification not only converts lipid fractions into more biodegradable forms but also modifies the EPS network [25,27,32]. NaOH addition disrupts hydrogen bonds and cross-linking within the EPS matrix, leading to the conversion of TB-EPS into LB- and S-EPS and the release of soluble organic compounds [16,33]. Such transformations enhance substrate bioavailability, accelerate hydrolysis, and increase methane yield. Both EPS removal [34] and EPS mobilization [35] have been shown to influence digestion efficiency. Moreover, EPS themselves can serve as a valuable feedstock for producing volatile fatty acids, alginate-like polymers, or biofloculants [36–38].

The novelty of the present study lies in its integrated evaluation of saponification and chemical pretreatment on the co-digestion of poultry manure with sewage sludge and grease trap waste. The analysis simultaneously addresses (i) methane yield, (ii) the distribution of EPS fractions (S-, LB-, TB-EPS), and (iii) kinetic parameters of digestion. This combined biochemical–engineering perspective links EPS destabilization and substrate solubilization with process kinetics and stability, offering new insights into optimizing AD systems for higher efficiency. Given the growing scale of poultry production and the need to valorize organic residues while expanding renewable energy generation, developing efficient, economically viable pretreatment strategies is of significant practical relevance [39]. Incorporating EPS fraction analysis provides an advanced diagnostic dimension that is often overlooked in prior studies, which typically focused solely on biogas yield [5,25,40]. This research underscores the fundamental role of EPS as both a limiting and co-determining factor in methanogenesis. Such a perspective deepens understanding of the interactions between the biological matrix structure and microbial activity, providing a foundation for the further development of AD technologies to optimize both process efficiency and stability. Here, EPS are recognized not merely as passive by-products, but as active determinants of methane generation pathways and system stability.

Accordingly, this study aims to comprehensively assess the impact of saponification and chemical pretreatment on the anaerobic co-digestion of poultry manure, sewage sludge, and grease trap waste. The analysis encompasses three key aspects: (i) methane production efficiency, (ii) the transformation and redistribution of EPS fractions (S-EPS, LB-EPS, TB-EPS), and (iii) kinetic characteristics of the digestion process. These findings provide a mechanistic understanding of how EPS structural modification enhances methanogenesis and support the practical implementation of pretreatment-based optimization strategies for poultry manure co-digestion.

2. Materials and Methods

2.1. Raw materials

The substrates used in this study consisted of poultry manure (PM), sewage sludge (SS), and grease trap sludge (GTS), mixed at a 30:40:30 volatile solids (VS) ratio. The sewage sludge, comprising both primary and waste-activated sludge, was collected from a municipal wastewater treatment plant located in the Silesian Voivodeship (Poland). Poultry manure originated from a laying hen farm in the same region and was homogenized before use to ensure compositional uniformity. Grease trap sludge was sourced from local meat-processing facilities. The substrate

composition was determined based on previous optimization studies conducted by Jasińska et al. (2024).

The inoculum used in biochemical methane potential (BMP) tests was digested sludge from a mesophilic anaerobic reactor operating with a hydraulic retention time (HRT) of 30 days, treating a mixture of primary and waste-activated sludge. The inoculum was collected from the heat exchanger section of the same wastewater treatment plant (WWTP). Table 1 presents the basic physicochemical characteristics of the substrates and inoculum used in the study.

Table 1. Physicochemical characteristics of raw materials and inoculum

Substrate	TS (%)		VS (% TS)		VS (%)	VS/TS	pH (–)	
	Average	SD	Average	SD	Average	–	Average	SD
GTS	14.62	0.02	89.05	0.56	13.02	0.89	5.44	0.01
PM	27.44	0.24	73.58	0.81	20.19	0.74	8.5	0.01
Ss	3.22	0.01	74.38	0.19	2.4	0.74	5.76	0.01
I	1.78	0.01	62.25	0.4	1.11	0.63	8.41	0.01
Mixture GTS:SS:PM	7.43	0.05	79.73	0.46	5.92	0.8	6.50	0.01

2.2. Experimental procedure

The co-digestion mixture was subjected to two alkaline pretreatment variants: (i) chemical/alkaline pretreatment, performed using NaOH at concentrations ranging from 0.9 to 5.4 g/l at ambient temperature; and (ii) thermo-alkaline pretreatment (saponification), conducted at 70 °C with the same NaOH doses. Each pretreatment lasted one hour and included a control (without NaOH addition).

All experiments were carried out in triplicate.

Following pretreatment, the mixtures were centrifuged, and the resulting supernatant was filtered (3 W soft filter). The filtrate was analyzed for non-purgeable organic carbon (NPOC), ammonium nitrogen ($\text{NH}_4^+\text{-N}$), volatile fatty acids (VFAs), and pH. Total solids (TS) and volatile solids (VS) were measured gravimetrically, while extracellular polymeric substances (EPS) were fractionated into loosely bound (LB-EPS) and tightly bound (TB-EPS) fractions. Each fraction was analyzed for carbohydrates, proteins, NPOC, and $\text{NH}_4^+\text{-N}$ content.

A schematic of the pretreatment procedure is presented in Figure 1.

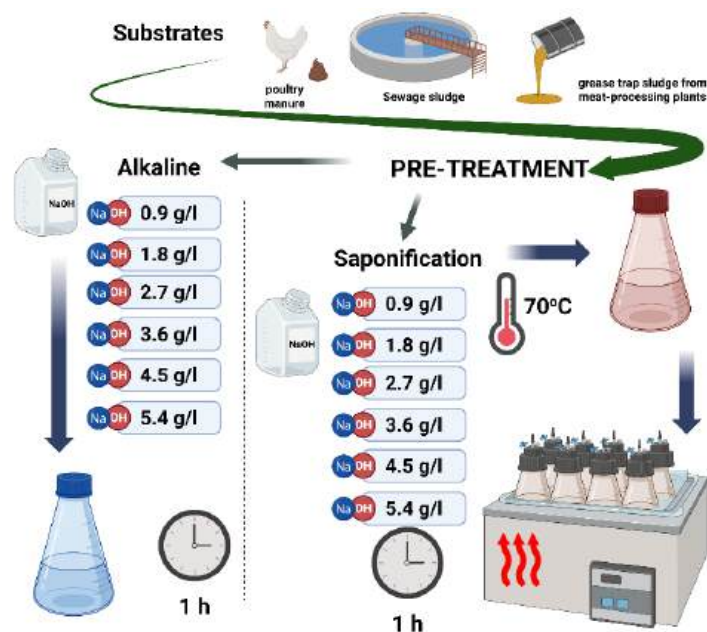


Figure 1. Scheme of pre-treatment

2.3. Biochemical methane potential (BMP) tests

Based on pretreatment outcomes, selected conditioned mixtures were subjected to BMP testing using an AMPTS II system (Automatic Methane Potential Test System, Bioprocess Control, Sweden). The system comprises: Unit A, a thermostated water bath with 15 sealed, mechanically stirred bioreactors; Unit B, a CO₂/H₂S absorption system (Duran bottles) filled with NaOH solution containing thymolphthalein as a pH indicator; and Unit C, a volumetric piston-type gas meter that records methane volume after the removal of acidic gases.

All BMP tests were performed in triplicate under mesophilic conditions at 37 °C for 21 consecutive days. The inoculum-to-substrate ratio (ISR) was maintained at 2 (VS/VS), with a working volume of 400 mL per reactor. Inoculum blanks were included to correct background methane production, and gas volumes were normalized to standard conditions (0 °C, 1 atm). Methane production data were used to estimate kinetic parameters through nonlinear model fitting as described in [9].

To evaluate synergistic effects among co-substrates, the co-digestion performance index (CPI) was calculated according to the methodology of Ebner et al. [41].

2.4. Analytical methods

Volatile fatty acids (VFAs) and ammonium nitrogen (N-NH₄⁺) were determined by indirect steam distillation using a Büchi K-355 distiller, following Standard Methods [42]. Non-purgeable organic carbon (NPOC) was measured using a Multi N/C H1300 analyzer (Analytik Jena, Germany) after dry combustion. Total solids (TS) and volatile solids (VS) were determined gravimetrically, and pH was measured potentiometrically, both according to APHA [42]. Extracellular polymeric substances (EPS) were extracted and fractionated using the methodology proposed by [43]. Carbohydrates were determined spectrophotometrically using the phenol-sulfuric acid method (Nielsen [44], while proteins were quantified using the Bradford method with the Thermo Scientific™ Coomassie Protein Assay Kit (No. 23200). Ammonium nitrogen in EPS fractions was analysed using the phenol-hypochlorite method [45].

2.5. Statistical analysis

The effects of pretreatment method (Chemical vs. Saponification) and NaOH dose were assessed using two-way ANOVA after confirming the assumptions of normality (Shapiro–Wilk test) and homogeneity of variance (Levene’s test). For variables violating these assumptions, non-parametric tests (Kruskal–Wallis with post hoc Dunn’s tests) were applied. Where significant differences occurred, pairwise comparisons were performed using Tukey’s HSD test. Effect sizes (η^2 , ω^2 , and Cohen’s d) were calculated to quantify the magnitude of effects beyond statistical significance. Pearson and Spearman correlation analyses were used to evaluate relationships among process parameters, which were visualized in heatmaps and correlation networks. To identify multidimensional patterns, Principal Component Analysis (PCA) was conducted. Statistical significance was accepted at $p < 0.05$. All analyses and visualizations (boxplots, biplots, heatmaps) were performed in Python 3.11 using the Pandas, NumPy, SciPy, StatsModels, Pingouin, Seaborn, and Matplotlib libraries.

3. Results and discussion

3.1. Selection of pretreatment method based on selected indicators

To determine the influence of pretreatment type on the behaviour of the co-digestion mixture, variations in the concentrations of general process indicators (NPOC, VFAs, pH, N-NH_4^+) and individual EPS fractions were analyzed as a function of pretreatment method and NaOH dose. Under chemical pretreatment, moderate but distinct changes were observed in all analyzed parameters. NPOC increased from the control value of 5.1 mg/l (untreated sample) to 8.7 mg/l at 5.4 g NaOH/l, corresponding to a 70.5% increase. VFAs exhibited fluctuations relative to the control, with a 7% decrease at 1.8 g/l and a 9% increase at 2.7 g/l. The total EPS content also changed dynamically: total protein-bound EPS ($\text{EPS}_{\text{sum-prot}}$) increased from 673 mg/l to 1276 mg/l (+90%), whereas carbohydrate-bound EPS ($\text{EPS}_{\text{sum-carb}}$) decreased by approximately 20% (from 1334 mg/l to 1066 mg/l), indicating a compositional shift toward proteinaceous fractions. The most pronounced alterations were recorded for the LB-EPS fraction. Ammonium nitrogen in LB-EPS increased almost fourfold (from 128 mg/l to 497 mg/l), while LB-EPS-carbohydrates doubled (from 161 mg/l to 327 mg/l). Such a pronounced mobilization of the loosely bound fractions suggests that chemical pretreatment induced partial remodelling of the polymer matrix while preserving the predominance of the TB-EPS fraction.

Far more pronounced changes were observed in saponification, where the synergistic action of NaOH and elevated temperature (70 °C) led to greater solubilization of organic matter. Thermal pretreatment alone increased NPOC from 5.1 mg/l to 9.7 mg/l (+93%). The addition of NaOH further enhanced solubilization by an additional 13%. A distinct trend was noted for VFAs: after thermal treatment alone, VFA concentration decreased by about 10% (from 7350 mg/l to 6600 mg/l), whereas with saponification, VFAs reached a maximum of 7894 mg/l (+19% relative to the thermal control and +7.5% relative to the chemical variant). EPS fractions exhibited even more substantial transformations than under the alkaline method. Soluble EPS (S-EPS) proteins more than doubled (from 457 mg/l to 883 mg/l), while carbohydrate fractions increased by 95–120%. Simultaneously, the content of tightly bound EPS (TB-EPS) decreased by 25–45%, clearly indicating conversion of strongly bound polymers into soluble forms. Stronger correlations between EPS-carbohydrates and EPS-proteins in this variant suggested that elevated

temperature promoted the release of EPS as protein-carbohydrate complexes. Figures 2-6 show the changes in the analyzed indicators.

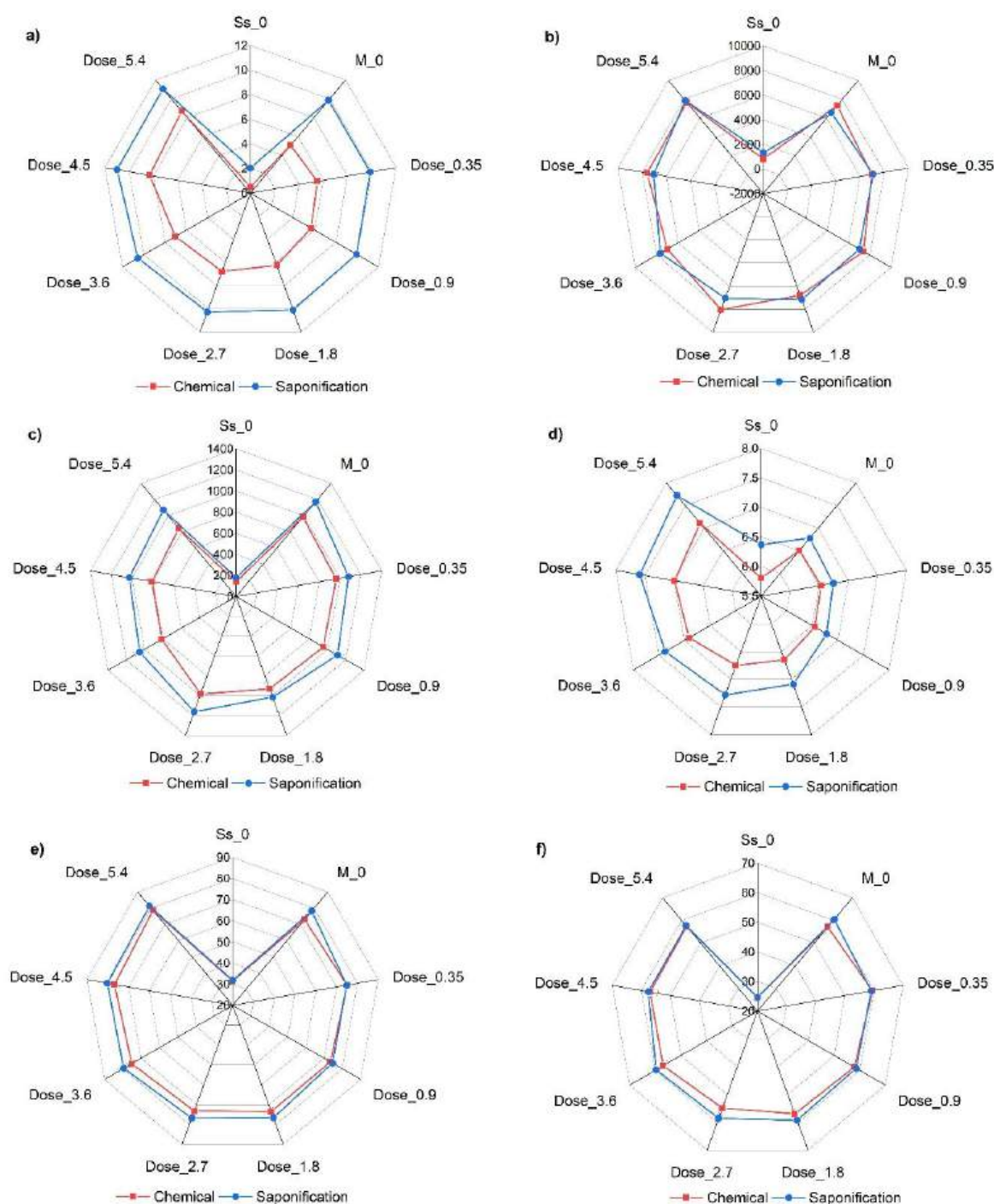


Figure 2. The impact of chemical method and saponification on selected parameters: a) non-purgeable organic carbon (NPOC); b) volatile fatty acids (VFAs); c) ammonium nitrogen (N-NH₄⁺); d) pH level; e) total solids (TS); and f) volatile solids (VS); Ss_0 denotes untreated sewage sludge; M_0 indicates the untreated co-digestion mixture. Dose with the numeral reflecting the amount of NaOH employed in the investigation, specifically 0.35, 0.9, 1.8, 2.7, 3.6, 4.5, and 5.4 g/l, respectively.

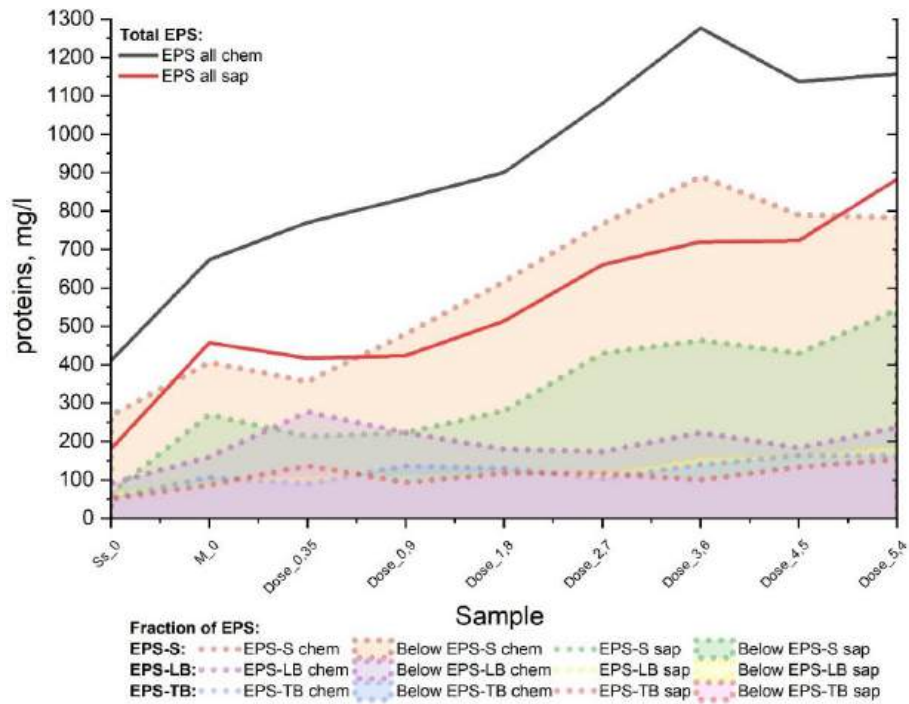


Figure 3. Distribution of protein content within EPS fractions (S-EPS, LB-EPS, TB-EPS); S-EPS—soluble EPS; LB-EPS—loosely bound EPS; TB-EPS—tightly bound EPS; Ss_0 denotes untreated sewage sludge; M_0 indicates the untreated co-digestion mixture. Dose with the numeral reflecting the amount of NaOH employed in the investigation, specifically 0.35, 0.9, 1.8, 2.7, 3.6, 4.5, and 5.4 g/l, respectively

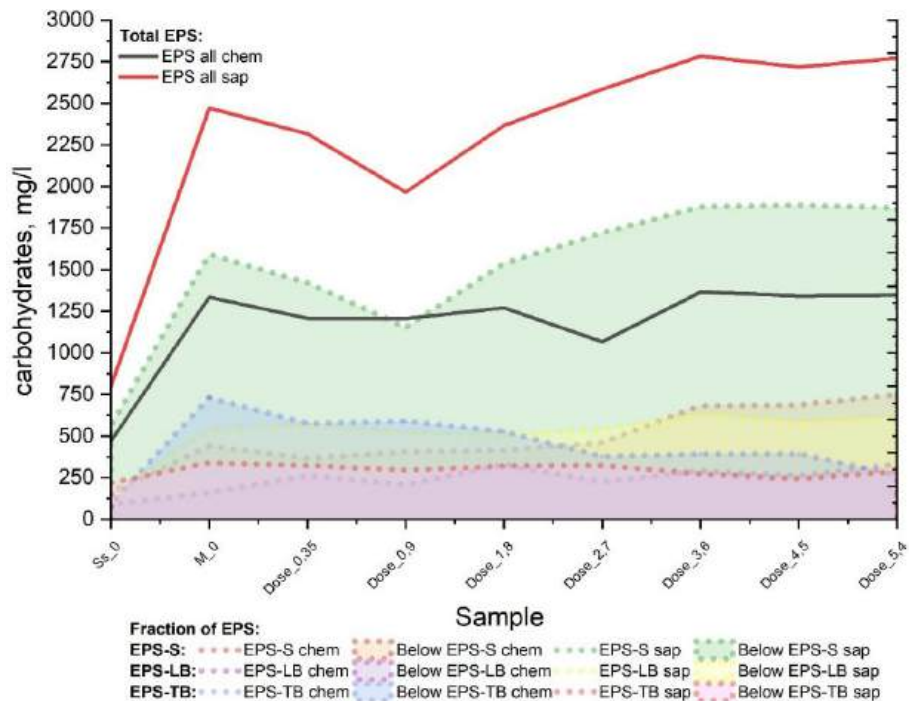


Figure 4. Distribution of carbohydrate content within EPS fractions (S-EPS, LB-EPS, TB-EPS); S-EPS—soluble EPS; LB-EPS—loosely bound EPS; TB-EPS—tightly bound EPS; Ss_0 denotes untreated sewage sludge; M_0 indicates the untreated co-digestion mixture. Dose with the numeral reflecting the amount of NaOH employed in the investigation, specifically 0.35, 0.9, 1.8, 2.7, 3.6, 4.5, and 5.4 g/l, respectively

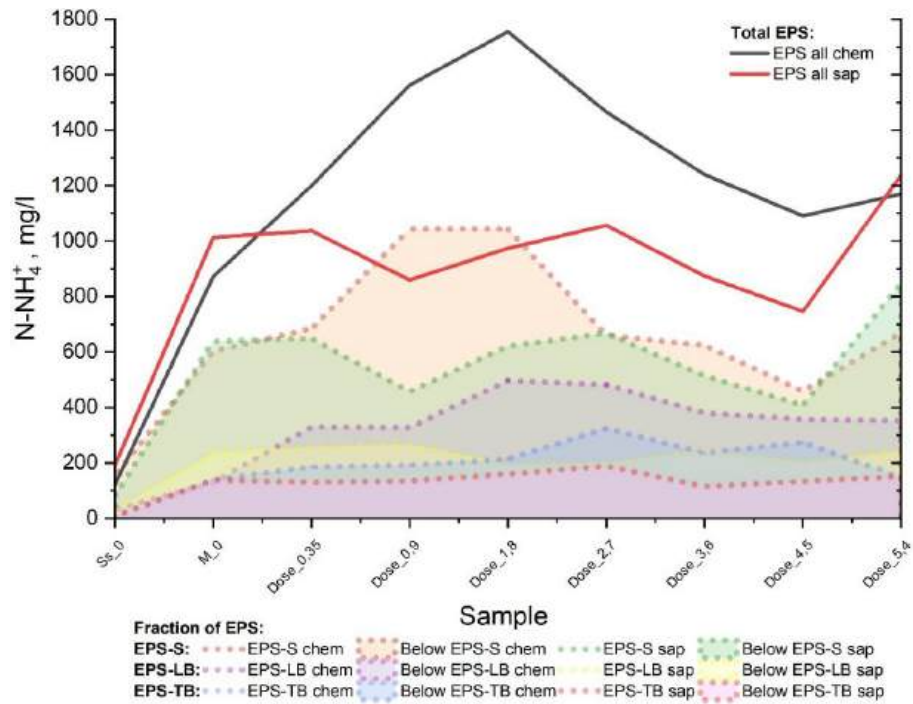


Figure 5. Distribution of ammonium nitrogen within EPS fractions (S-EPS, LB-EPS, TB-EPS); S-EPS—soluble EPS; LB-EPS—loosely bound EPS; TB-EPS—tightly bound EPS; Ss_0 denotes untreated sewage sludge; M_0 indicates the untreated co-digestion mixture. Dose with the numeral reflecting the amount of NaOH employed in the investigation, specifically 0.35, 0.9, 1.8, 2.7, 3.6, 4.5, and 5.4 g/l, respectively

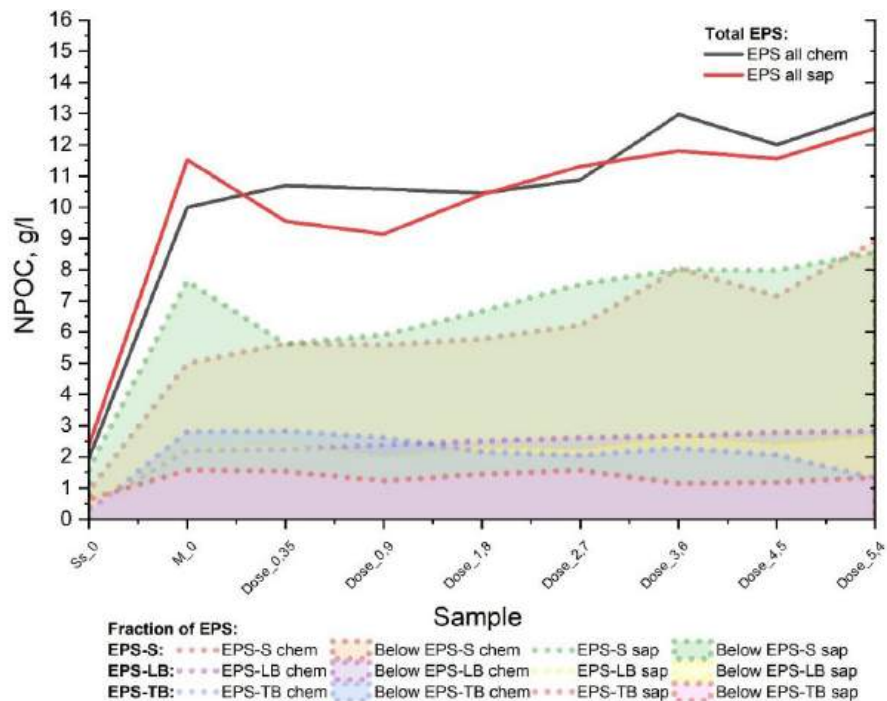


Figure 6. Distribution of NPOC within EPS fractions (S-EPS, LB-EPS, TB-EPS); S-EPS—soluble EPS; LB-EPS—loosely bound EPS; TB-EPS—tightly bound EPS; Ss_0 denotes untreated sewage sludge; M_0 indicates the untreated co-digestion mixture. Dose with the numeral reflecting the amount of NaOH employed in the investigation, specifically 0.35, 0.9, 1.8, 2.7, 3.6, 4.5, and 5.4 g/l, respectively

These results confirm that the combined thermal and alkaline effect of saponification leads to a more profound restructuring of the EPS matrix and greater substrate solubilization than NaOH treatment alone. The observed changes in the organic matter indicators (NPOC, VFAs) were consistent with this mechanism, reflecting increased availability of soluble organic carbon and nitrogen sources that fermentative microorganisms can directly utilize.

The fractionation of EPS (Figures 3–6) confirmed that saponification systematically increased the proportion of soluble EPS (S-EPS) at the expense of tightly bound EPS (TB-EPS). The percentage differences between methods were evident, with S-EPS content higher by +2.9% (at 0.9 g/l NaOH) to +14.9% (at 0.35 g/l). Concurrently, TB-EPS decreased by 6.6% to 19.7% relative to the chemical treatment, while LB-EPS remained relatively stable (−4.8% to +6.5%). In the chemical method, the TB-EPS fraction remained considerably higher (up to 34% in the absence of NaOH), whereas S-EPS accounted for only 45–53% of the total, depending on the dose (Figure 7).

The overall pattern of EPS redistribution supports the hypothesis that thermo-alkaline treatment facilitates the breakdown of hydrogen bonding and cross-linking in TB-EPS, converting them into more mobile and bioavailable fractions. This enhanced solubilization increases substrate accessibility to hydrolytic enzymes, thereby promoting biodegradation. In contrast, the milder chemical pretreatment predominantly mobilized LB-EPS without substantial structural breakdown, which likely explains the lower solubilization efficiency. These findings are consistent with reports that heat and alkali act synergistically to enhance sludge disintegration and solubilization [34]. The present results further demonstrate that saponification effectively destabilises the EPS matrix in complex substrates, such as poultry manure mixtures, thereby improving organic matter conversion efficiency before anaerobic digestion.

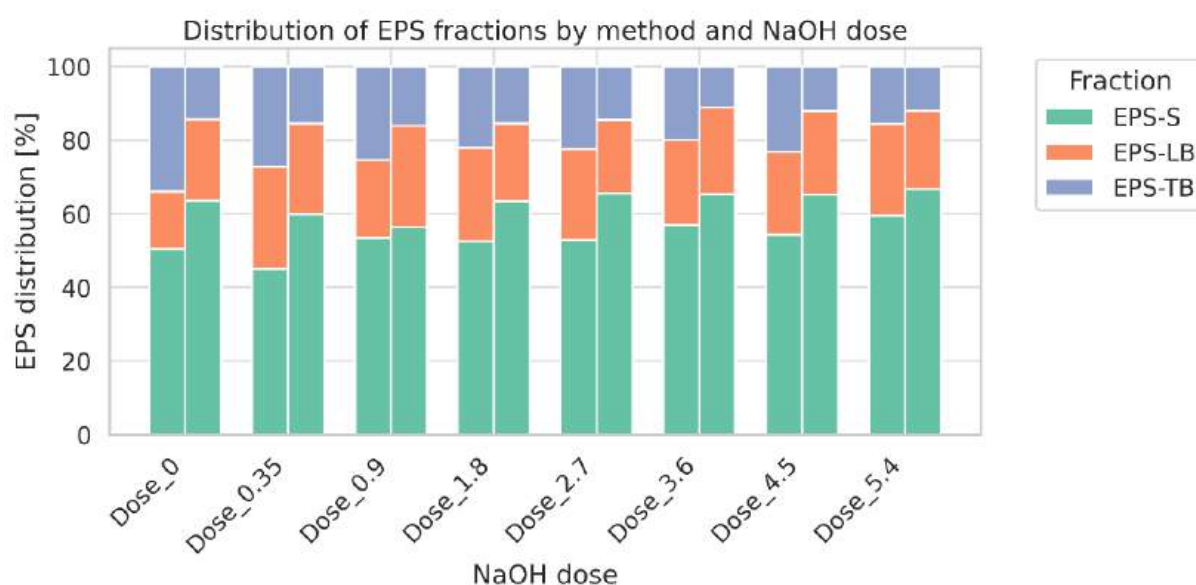


Figure 7. Relative contribution of EPS fractions to total EPS pool in response to NaOH dose under chemical and saponification pretreatment. Bars represent the relative contribution of each fraction to the total EPS pool (mean values). Colors indicate EPS fractions, while paired bars at each dose correspond to the two pre-treatment methods (left = Chemical, right = Saponification).

3.2. Correlation and statistical analysis of EPS transformations

Correlation analysis revealed several significant relationships between the examined parameters and EPS fractions, with distinct patterns emerging between the chemical and saponification treatments. In both cases, strong positive correlations ($r > 0.9$, $p < 0.001$) were observed among variables describing organic matter content (TS, VS) and total EPS (both carbohydrate and protein fractions, as well as NPOC). These findings confirm that the organic matter concentration in the co-digestion mixture directly determines EPS abundance, regardless of pretreatment method. A moderate positive correlation between NPOC and VFAs ($r \approx 0.5$ – 0.6) was also identified, suggesting a shared origin through hydrolysis and solubilization processes.

The most notable differences between the pretreatment methods emerged when examining individual EPS fractions. Under chemical pretreatment, strong correlations were found between LB-EPS and TB-EPS ($r = 0.74$ – 0.79), indicating a stable polymeric structure and limited transformation of tightly bound polymers into soluble forms. Significant positive correlations between pH and proteinaceous EPS fractions, including EPS_{sum}-prot ($r = 0.84$ – 0.86) and TB-EPS-prot ($r \approx 0.74$), suggest that higher alkalinity favored the release of protein-based polymers from the EPS matrix. In contrast, saponification produced markedly different correlation patterns. The strength of relationships between soluble EPS (S-EPS) and indicators of organic matter decomposition (NPOC, VFAs) increased substantially ($r \approx 0.7$ – 0.8), indicating that the combined action of alkali and temperature promoted extensive solubilization and release of soluble polymers. Furthermore, significant negative correlations were observed between TB-EPS and S-EPS ($r \approx -0.66$), which were absent in the chemical variant, confirming the conversion of tightly bound polymers into soluble fractions. Saponification also strengthened the coupling between carbohydrate and protein components within EPS ($r > 0.85$), implying that elevated temperature facilitated the release of EPS as mixed protein–carbohydrate complexes.

These correlations were corroborated by both Pearson's and Spearman's coefficients (Figures 8 and 9). For strongly linear relationships (e.g., TS, VS, total EPS), Pearson's analysis provided a good fit; however, for nonlinear or weaker associations (e.g., NPOC–VFA, TB-EPS–S-EPS), Spearman's rank correlations offered a more representative depiction of data structure. The consistency between both methods strengthens the reliability of these observations.

Traditional correlation analysis identifies relationships between parameters, but in complex systems, these relationships are multidimensional and interdependent. A simple table listing correlation coefficients does not fully capture differences between pretreatment methods or reflect the role of individual EPS fractions within the network of process relationships. For these reasons, to further elucidate interdependencies among parameters, a correlation network based on Spearman's coefficients ($|r| > 0.6$, $p < 0.05$) was developed (Figure 10). Each node represents a process parameter or EPS fraction, while edges denote statistically significant correlations.

The network corresponding to the saponification variant was notably denser than that of the chemical method, revealing a higher number and intensity of correlations—particularly among parameters associated with the soluble EPS fraction. This suggests that thermo-alkaline pretreatment increased system connectivity by enhancing the mobilisation of organic matter. In contrast, the chemical treatment displayed a more conservative network dominated by interactions among bound EPS fractions (LB-EPS and TB-EPS), indicating a more stable polymeric matrix with limited transformation of bound polymers.

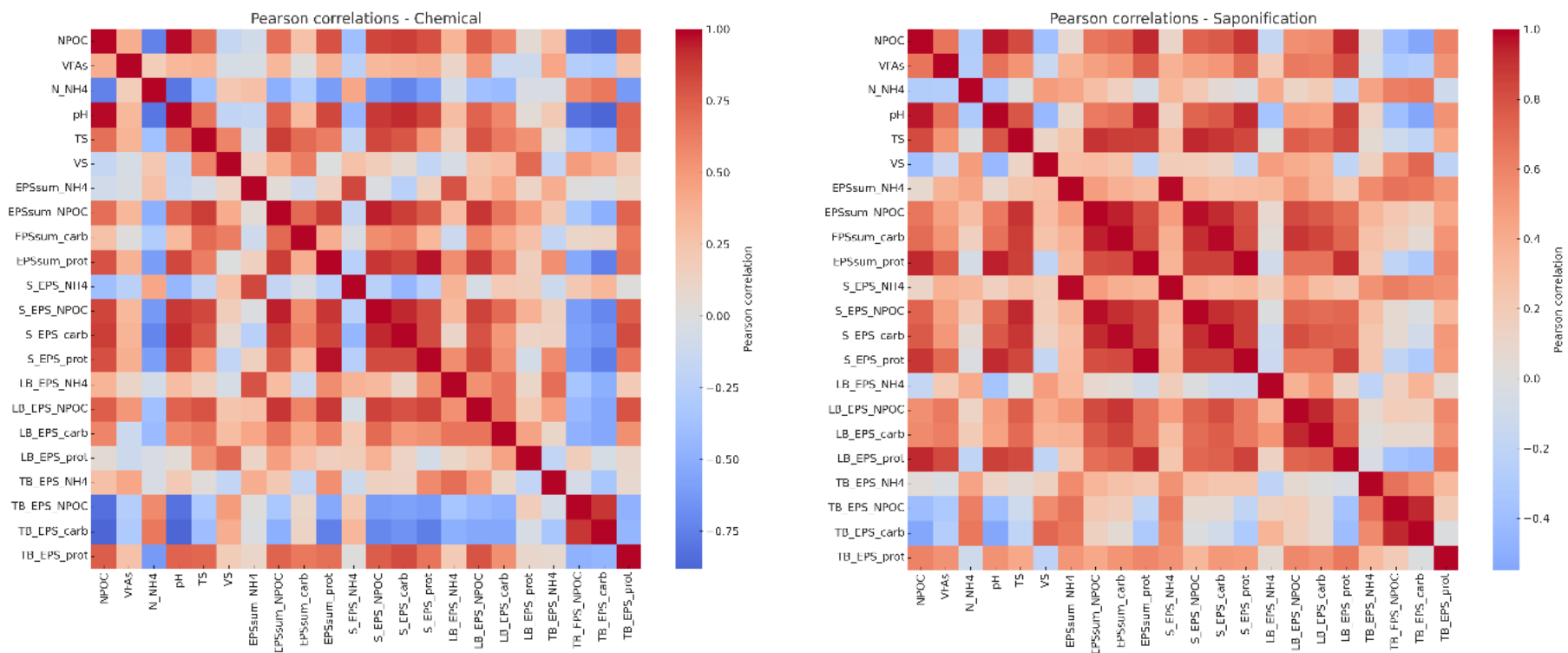


Figure 8. Pearson correlation heatmaps among process indicators under chemical and saponification pretreatment. Red indicates positive correlations, blue indicates negative correlations. Their intensity indicates the strength of the correlation

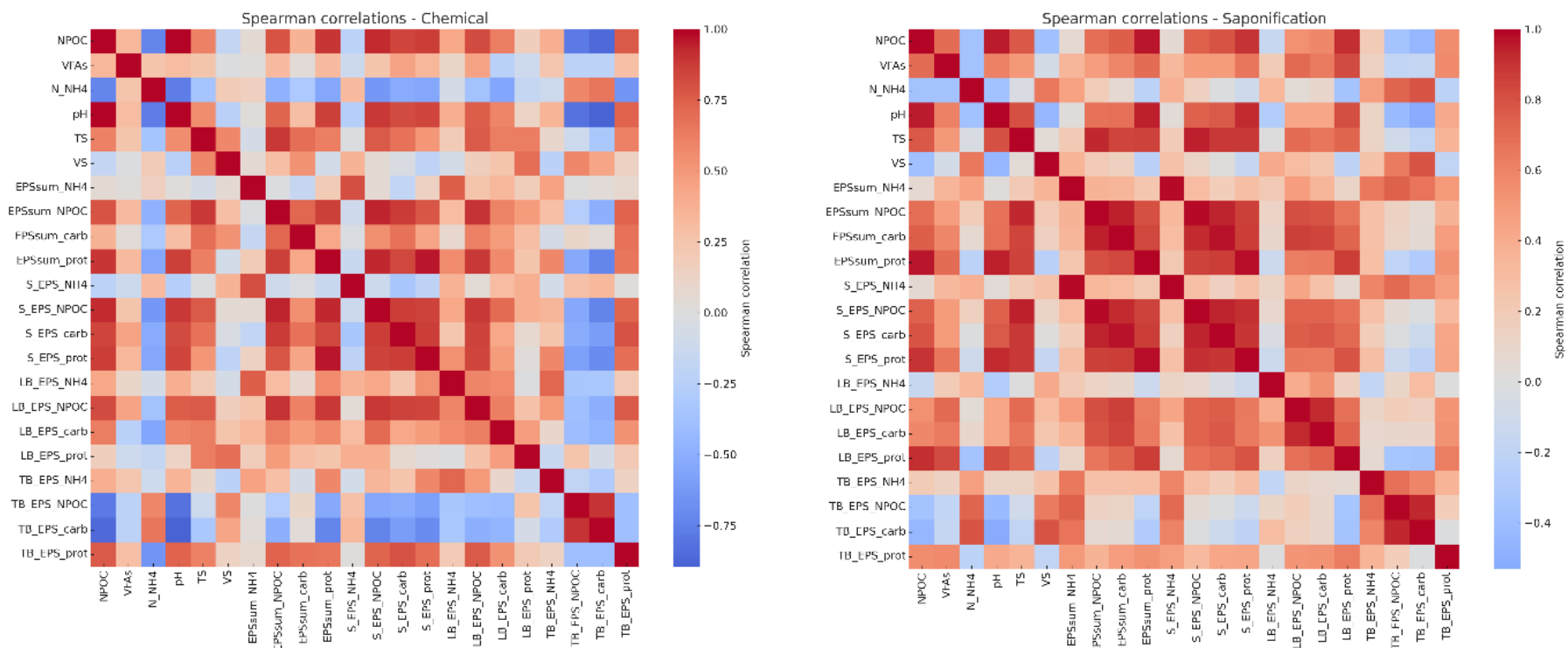


Figure 9. Spearman correlation heatmaps among process indicators under chemical and saponification pretreatments. Red indicates positive correlations, blue indicates negative correlations. Their intensity indicates the strength of the correlation

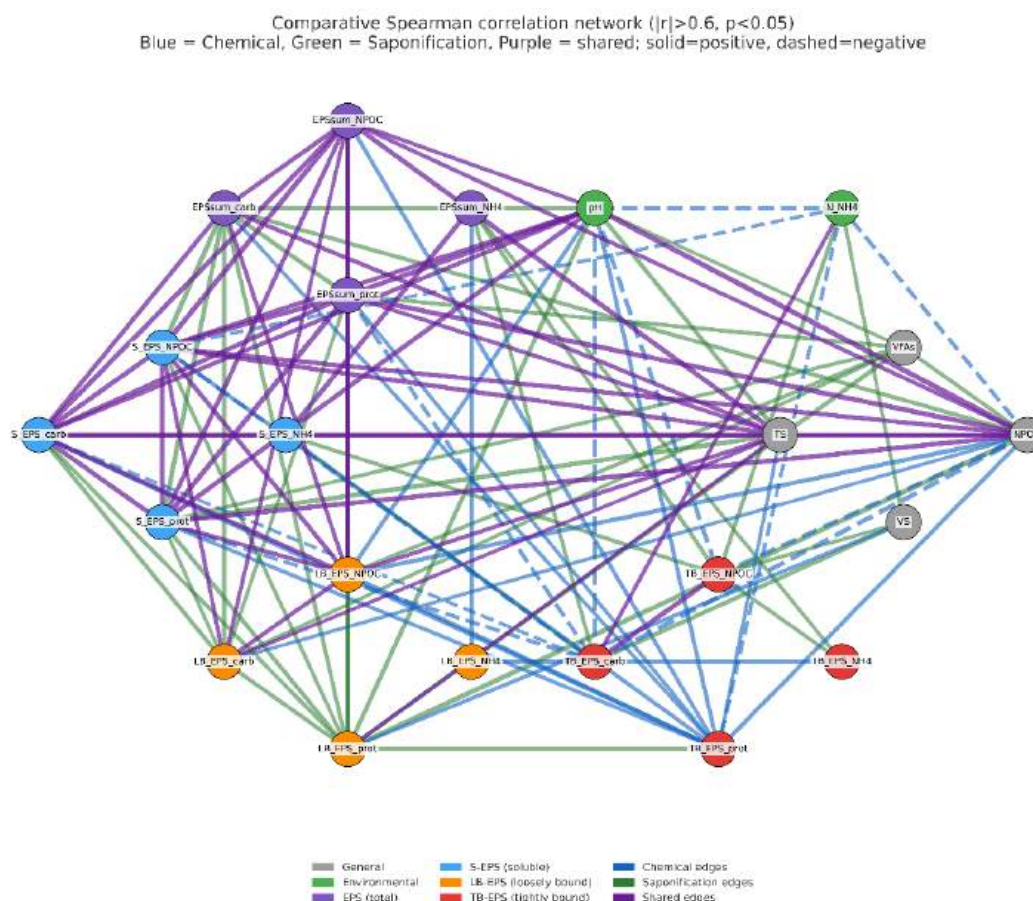


Figure 10. Comparative Spearman correlation network ($|r| > 0.6$, $p < 0.05$) for the chemically conditioned and saponified mixtures. Nodes represent process parameters and EPS fractions (node colors denote variable groups). Edge color encodes the pretreatment method (blue = Chemical, green = Saponification, purple = edges significant in both), edge style encodes the sign (solid = positive, dashed = negative), and edge width is proportional to $|r|$

In both networks, parameters related to organic matter content (TS, VS) occupied central positions and showed strong links with EPS fractions and environmental indicators (pH, N-NH_4^+). However, analysis of the network structure revealed significant differences: in the case of saponification, a synergistic effect was observed, manifested by a greater number and strength of correlations within the soluble fractions (S-EPS) and stronger links between organic parameters and the chemical characteristics of the system. In contrast, the network for chemical pretreatment was more conservative, focusing mainly on EPS-related fractions (LB-EPS, TB-EPS), suggesting a more stable polymer matrix and less dynamic polymer transformation.

To validate these findings, the data were further analyzed using variance-based statistical methods. Both two-way ANOVA and the Kruskal-Wallis nonparametric test were applied, depending on the data distribution and variance homogeneity (Tables 2 and 3). All parameters were analysed using ANOVA (Table 2), whereas variables that violated its assumptions (e.g., pH, N-NH_4^+ , VFAs, EPS fractions) were additionally analysed using a nonparametric test (Table 3). The dual approach minimized potential interpretation bias arising from non-normality while improving statistical robustness.

In addition, the parallel use of ANOVA and nonparametric tests allowed further evaluation of the conclusions. When both tests yielded consistent results (e.g., for pH or some EPS indicators), the results can be considered reliable and robust to statistical limitations. This convergence is a strong argument that the observed effects are real and not an artefact of the analysis method used, while discrepancies—as in the case of VFAs—indicate the sensitivity of some parameters to the fulfilment of assumptions and require more cautious interpretation. A comparison of the results of both statistical approaches is presented in Figure 11.

The analysis of the results confirmed that the pretreatment method was one of the most important differentiating factors, particularly evident in NPOC, TS, and selected indicators describing EPS. The F-statistic values for these parameters were very high ($p < 0.001$), and parallel non-parametric tests confirmed these conclusions in most cases. This emphasizes that the choice of method (chemical pretreatment vs. saponification) is crucial. The NaOH dose also proved to be a significant factor, especially for NPOC, TS, and EPS, indicating a clear dose-dependent relationship between the reagent dose and the scale of the observed changes in the mixture.

Table 2. Results of two-way ANOVA (F and p values) for process and EPS parameters.

Variable	Effect	F	p-value
<i>NPOC</i>	Method	34543.054	4.058e-50
	Dose	1037.084	7.472e-36
	Method x Dose	221.787	2.910e-25
<i>VFAs</i>	Method	9.458	0.004
	Dose	13.947	3.916e-08
	Method x Dose	14.525	2.451e-08
<i>N-NH₄⁺</i>	Method	135.416	4.906e-13
	Dose	8.357	8.673e-06
	Method x Dose	1.633	0.162
<i>pH</i>	Method	225860.210	3.681e-63
	Dose	59734.611	5.511e-64
	Method x Dose	4257.286	1.221e-45
<i>TS</i>	Method	41.291	3.182e-07
	Dose	10.351	1.026e-06
	Method x Dose	1.811	0.119
<i>VS</i>	Method	13.334	9.219e-04
	Dose	1.716	0.140
	Method x Dose	1.453	0.219
<i>EPSSum_N-NH₄⁺</i>	Method	111.466	5.910e-12
	Dose	13.215	7.240e-08
	Method x Dose	14.772	2.014e-08
<i>EPSSum_NPOC</i>	Method	1.507	0.229
	Dose	6.760	5.961e-05
	Method x Dose	1.426	0.229
<i>EPSSum_carb</i>	Method	565.361	6.604e-22
	Dose	5.359	3.937e-04
	Method x Dose	2.936	0.017
<i>EPSSum_prot</i>	Method	307.467	5.699e-18
	Dose	37.459	1.167e-13
	Method x Dose	2.818	0.021
<i>Fraction_EPS_S_N-NH₄⁺</i>	Method	47.696	8.103e-08
	Dose	24.466	3.804e-11
	Method x Dose	25.433	2.283e-11

<i>Fraction_EPS_S_NPOC</i>	Method	14.740	5.489e-04
	Dose	19.042	9.389e-10
	Method x Dose	3.530	0.006
<i>Fraction_EPS_S_carb</i>	Method	1200.363	6.052e-27
	Dose	19.335	7.760e-10
	Method x Dose	3.053	0.014
<i>Fraction_EPS_S_prot</i>	Method	398.707	1.251e-19
	Dose	64.337	4.777e-17
	Method x Dose	6.895	5.025e-05
<i>Fraction_EPS_LB_N-NH₄⁺</i>	Method	244.765	1.512e-16
	Dose	19.783	5.826e-10
	Method x Dose	32.471	8.478e-13
<i>Fraction_EPS_LB_NPOC</i>	Method	6.696	0.014
	Dose	4.691	0.001
	Method x Dose	1.571	0.180
<i>Fraction_EPS_LB_carb</i>	Method	705.035	2.278e-23
	Dose	7.223	3.334e-05
	Method x Dose	3.175	0.011
<i>Fraction_EPS_LB_prot</i>	Method	269.788	3.767e-17
	Dose	18.974	9.817e-10
	Method x Dose	11.426	3.606e-07
<i>Fraction_Fraction_EPS_TS_LS_N-NH₄⁺</i>	Method	209.858	1.319e-15
	Dose	29.322	3.407e-12
	Method x Dose	16.792	4.394e-09
<i>Fraction_Fraction_EPS_TS_NPOC</i>	Method	322.789	2.806e-18
	Dose	16.350	6.059e-09
	Method x Dose	11.704	2.779e-07
<i>Fraction_Fraction_EPS_TS_carb</i>	Method	285.838	1.639e-17
	Dose	31.742	1.158e-12
	Method x Dose	19.326	7.807e-10
<i>Fraction_Fraction_EPS_TS_prot</i>	Method	13.726	7.961e-04
	Dose	19.601	6.542e-10
	Method x Dose	10.484	8.982e-07

For some parameters, such as VFAs and selected EPS fractions, the analyses yielded less clear results. ANOVA indicated the significance of the method effect, while the Kruskal–Wallis test mainly confirmed the impact of the dose and the method $\times$ dose interaction. This observation highlights the complexity of the processes occurring in the mixture. It indicates that the interpretation of the results should be based not only on significance tests, but also on the analysis of effect sizes. The calculated indices (η^2 , ω^2 , Cohen's d), illustrated in the figures (Figure 12–14), confirm that the pretreatment method and NaOH dose are crucial for NPOC, TS, and total EPS indices. In turn, Cohen's d clearly indicates that the effect of method differentiation was particularly strong for NPOC, VFAs, and EPS.

The EPS analysis showed that individual fractions reacted differently to the pretreatment method and NaOH dose. EPSsum–NPOC was strongly dependent on the NaOH dose, and a significant interaction between the method and dose (method $\times$ dose) was observed, indicating a dose-dependent release of organic matter from EPS that differed depending on the pretreatment method. In turn, changes in EPSsum–NH₄⁺ relies primarily on the method, and the dose effect was statistically significant only in the interaction. Similar trends were observed in individual EPS fractions (EPS-carb, EPS-prot), with significant differences between doses, especially at higher NaOH concentrations. This means that the pretreatment process affects not only the overall EPS

content, but also the proportions of individual components, modifying the structure of the EPS matrix. In the case of the TB-EPS (tightly bound) fraction, significant effects of both the method and the dose were observed, as well as an interaction between the two factors. In contrast, for the LB (loosely bound EPS) fraction, a clear effect of the NaOH dose was observed, while the impact of the pretreatment method was less clear. The Kruskal–Wallis results (Table 3) confirmed the dose-dependent nature of the changes, especially at higher concentrations, suggesting that this fraction is most susceptible to chemical loosening of the structure. In contrast, the S fraction (soluble EPS) showed the most significant variation across pretreatment methods. ANOVA (Table 2) showed a significant effect of the method ($p < 0.001$) and Cohen's d (Figure 12) confirmed a strong differentiating effect. This indicates that the soluble EPS fraction is particularly sensitive to the pretreatment method, and its content in the mixture may serve as a sensitive marker of the pretreatment process's effectiveness.

The results for NPOC and TS clearly confirmed the significance of both the method and the dose, consistent with previous observations that these indicators are sensitive to chemical conditions. In turn, TS and VS showed significant differences, mainly between extreme values (lowest vs. highest doses), suggesting that the response of these indicators is less linear and that clear changes occur only at higher NaOH concentrations.

EPSSum NH_4^+	ANOVA only	Both significant	Both significant
EPSSum NPOC	Both significant	Kruskal only	Both not significant
EPSSum carb	ANOVA only	Both significant	Both significant
Fraction EPS LB NPOC	Both significant	Kruskal only	ANOVA only
Fraction EPS LB carb	ANOVA only	Both significant	Both significant
Fraction EPS LB prot	ANOVA only	Both significant	Both significant
Fraction EPS S NH_4^+	Both significant	Both significant	Both significant
Fraction EPS S NPOC	Both significant	Both significant	ANOVA only
Fraction EPS S carb	ANOVA only	Both significant	Both significant
Fraction Fraction EPS TS NPOC	ANOVA only	Both significant	Both significant
Fraction Fraction EPS TS prot	Both significant	Both significant	ANOVA only
N- NH_4^+	ANOVA only	Kruskal only	Both significant
VFAs	Both significant	Both significant	ANOVA only
pH	Both significant	Both significant	Both significant
	Dose	Interaction Effect	Method

Figure 11. Comparison of results obtained by parametric (ANOVA) and nonparametric (Kruskal–Wallis) variance analyses

Table 3. Results of Kruskal–Wallis nonparametric tests (*H* and *p* values) for parameters violating ANOVA assumptions

Variable	H_Method	p_Method	H_Dose	p_Dose	H_Interaction	p_Interaction
<i>VFAs</i>	1.715	1.904e-01	16.148	2.380e-02	40.336	4.034e-04
<i>N-NH₄⁺</i>	30.546	3.261e-08	10.097	1.832e-01	41.701	2.498e-04
<i>pH</i>	15.253	9.403e-05	30.679	7.125e-05	46.969	3.724e-05
<i>EPSSum_N-NH₄⁺</i>	16.002	6.329e-05	12.097	9.742e-02	41.789	2.422e-04
<i>EPSSum_NPOC</i>	0.750	3.865e-01	24.864	8.021e-04	30.575	1.001e-02
<i>EPSSum_carb</i>	35.265	2.877e-09	5.197	6.359e-01	42.051	2.207e-04
<i>Fraction_EPS_S_NH4</i>	4.338	3.727e-02	20.961	3.828e-03	39.938	4.634e-04
<i>Fraction_EPS_S_NPOC</i>	3.444	6.349e-02	30.235	8.599e-05	39.854	4.771e-04
<i>Fraction_EPS_S_carb</i>	35.265	2.877e-09	9.066	2.479e-01	44.827	8.155e-05
<i>Fraction_EPS_LB_NPOC</i>	3.145	7.618e-02	19.276	7.367e-03	28.384	1.928e-02
<i>Fraction_EPS_LB_carb</i>	35.265	2.877e-09	5.577	5.900e-01	43.364	1.382e-04
<i>Fraction_EPS_LB_prot</i>	25.429	4.589e-07	12.287	9.152e-02	43.342	1.393e-04
<i>Fraction_Fraction_EPS_TS_NPOC</i>	25.730	3.928e-07	10.281	1.732e-01	43.541	1.297e-04
<i>Fraction_Fraction_EPS_TS_prot</i>	2.236	1.348e-01	23.658	1.308e-03	41.405	2.773e-04

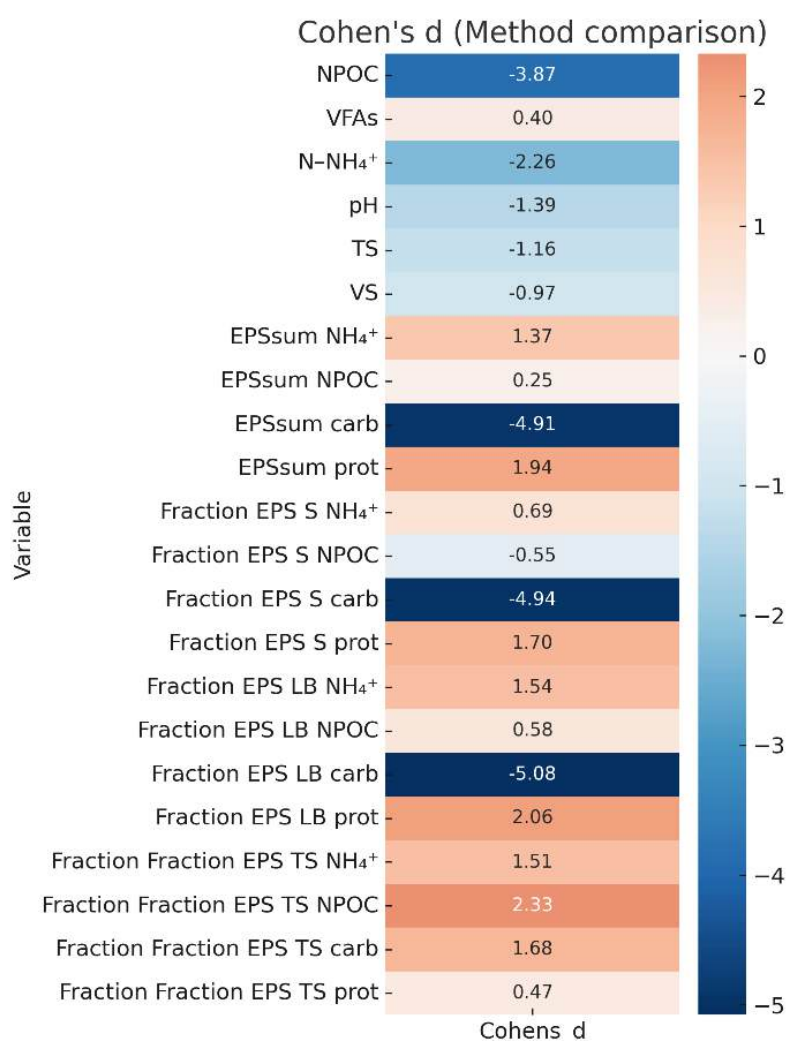


Figure 12. Effect size (Cohen's d) for differences between saponification and chemical pretreatment across process and EPS parameters

Principal Component Analysis (PCA) was subsequently applied to integrate all variables into a multidimensional framework (Figure 15). The first component (PC1, 42% of total variance) was primarily associated with parameters reflecting organic matter mobilization (TS, NPOC, EPS-carbohydrates, EPS-proteins), representing the substrate potential shaped by EPS matrix restructuring. The second component (PC2, 28%) was driven mainly by VFAs, N-NH₄⁺, VS, and pH, corresponding to environmental and stability-related variables.

The spatial distribution of samples along PC1–PC2 clearly distinguished the two pretreatment methods. In the chemical variant, sample displacement occurred mainly along PC1, reflecting gradual organic matter release with limited impact on environmental parameters. Saponification, however, produced pronounced shifts along both PC1 and PC2, indicating that NaOH and elevated temperature jointly increased both solubilization efficiency and accumulation of intermediates (VFAs, NH₄⁺). The largest displacements were observed at higher NaOH doses, confirming strong substrate mobilization but also a potential risk of process instability at extreme conditions.

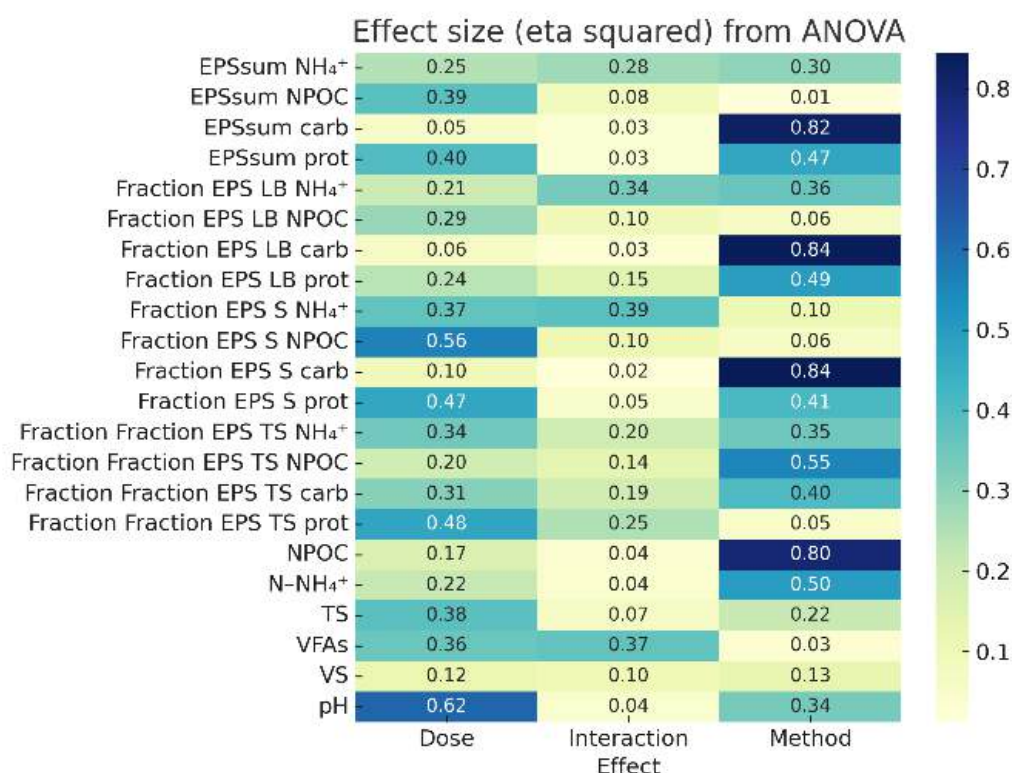


Figure 13. Effect size (η^2) from two-way ANOVA showing the relative contribution of NaOH dose, pretreatment method, and their interaction on process and EPS parameters

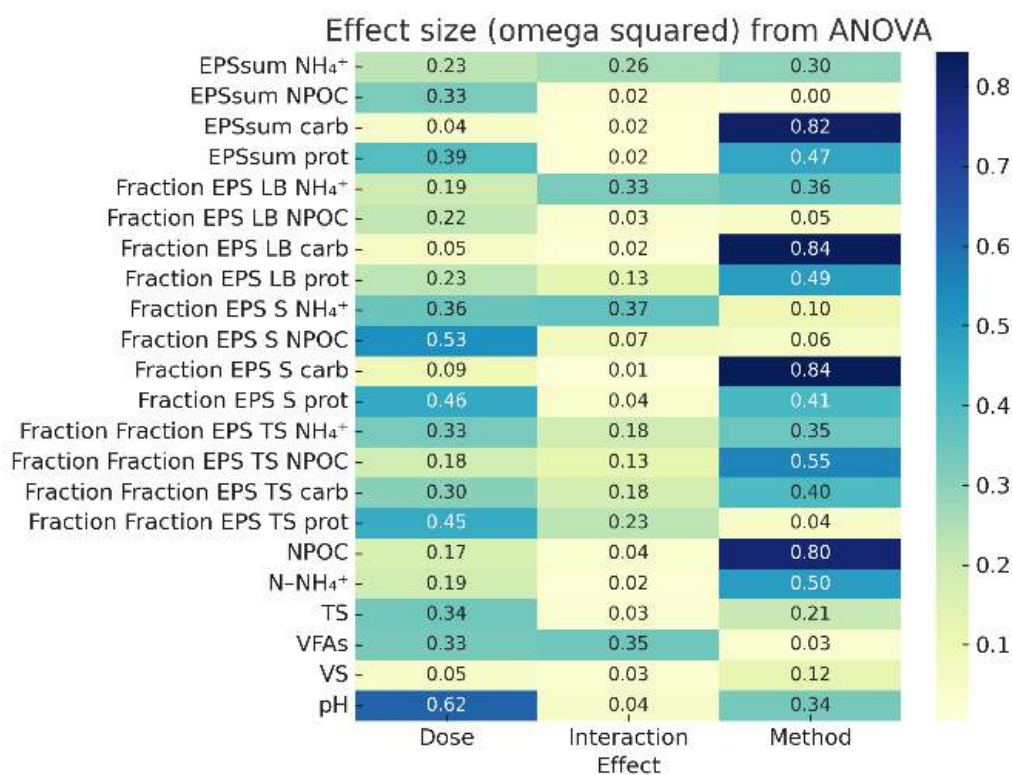


Figure 14. ω^2 effect sizes for dose, method, and interaction effects obtained from two-way ANOVA

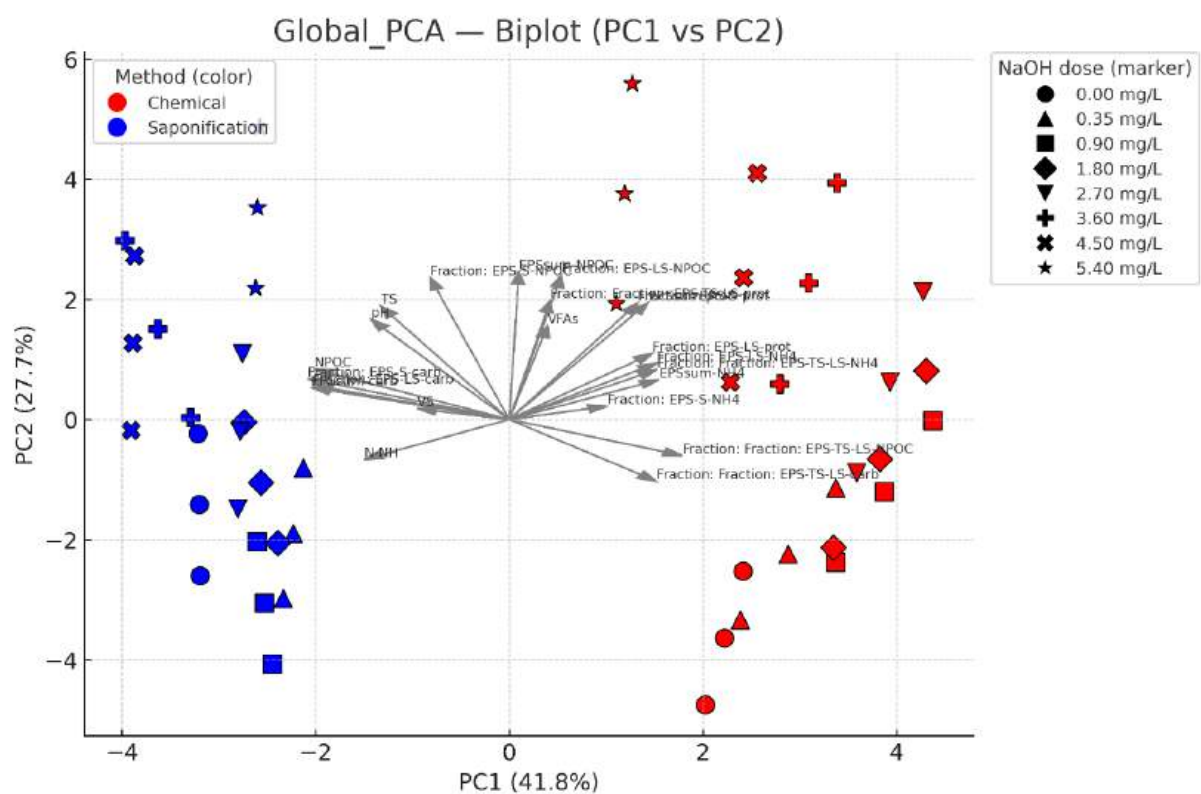
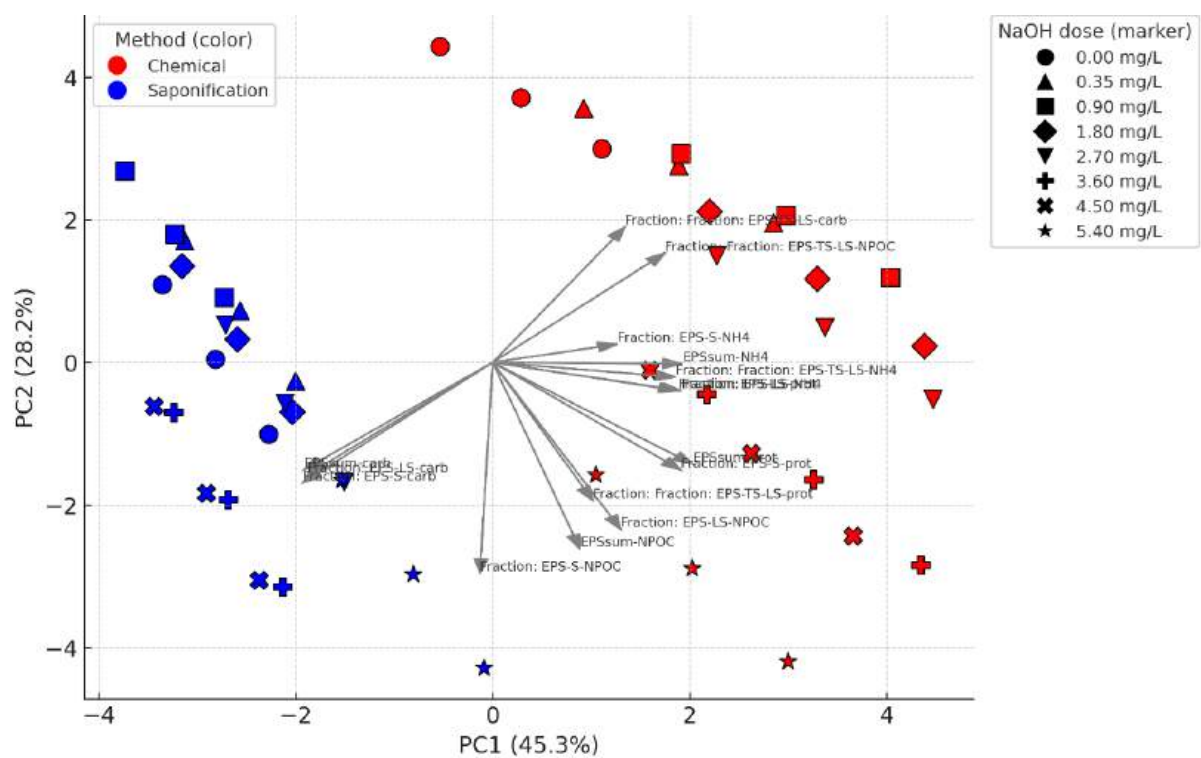


Figure 15. PCA biplot (22 variables) illustrating the differentiation of pretreatment methods and NaOH doses



A detailed PCA analysis performed exclusively for the EPS fraction (Fig. 16) enabled a more precise distinction between the mechanisms underlying changes in the polymer matrix. The first component (PC1, approx. 45% variance) was associated with EPSsum-prot, EPSsum-carb, and the LB-EPS fraction, suggesting a link to structural changes in EPS and to the relationship between protein and carbohydrate fractions. The second component (PC2, approx. 28%) was mainly described by soluble fractions, especially S-EPS-NPOC and S-EPS-prot, suggesting it reflects a direction of change related to the release of mobile, easily accessible EPS fractions. The chemical method primarily induced shifts along the PC1 axis, indicating a structural reorganisation of EPS. At the same time, saponification also resulted in changes along the PC2 axis, associated with the intensive release of soluble fractions.

Additional PCA limited to general process parameters (Figure 17) revealed that TS, NPOC, and pH drove PC1 (49% variance), while PC2 (25%) was associated with VFAs, VS, and N-NH₄⁺. Chemical pretreatment mainly affected PC1 (organic matter mobilization), whereas saponification induced more substantial variation along PC2, consistent with greater accumulation of VFAs and ammonium nitrogen. At low NaOH doses (0–0.9 g/l), the process remained stable with minimal effects; at 2.7 g/l, an optimal balance between solubilization and process control was achieved. Above 3.6 g/l, maximum solubilization was obtained, but with an elevated risk of intermediate accumulation and potential process destabilization, especially under saponification conditions.

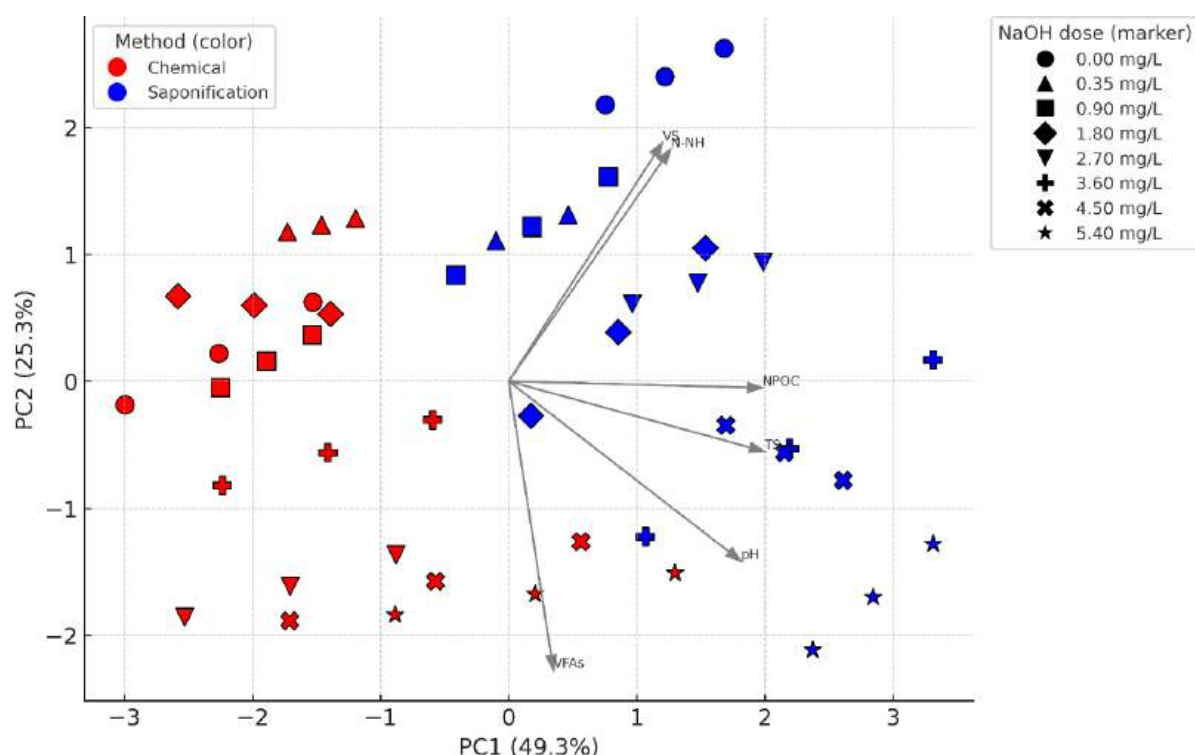


Figure 17. Biplot for other parameter (6 variables)

The obtained relationships are consistent with previous reports indicating that EPS act as reservoirs of organic matter, play a key role in biomass stabilization in biological processes, and serve as a physicochemical barrier protecting cells from abrupt environmental changes [12,14,46]. The most significant differences between the pretreatment methods applied concerned the network structure and the dominant EPS fractions. In the case of chemical pretreatment, the correlations were centered around LB-EPS and TB-EPS, which indicates a more

stable polymer matrix and a limited transformation of bound polymers into soluble fractions. In contrast, saponification led to a pronounced network densification and the emergence of strong associations between S-EPS and the parameters of organic matter degradation (NPOC, VFAs). This phenomenon is consistent with the observations of Huang et al. [11], who emphasized that rapid biomass solubilization and VFA accumulation promote the intensive release of soluble EPS, as well as with more recent studies on thermal and enzymatic treatments, which highlighted the crucial role of EPS modification in improving substrate biodegradability[47].

In the analysis of results, a significant relationship was also observed between the proteinaceous EPS fractions and both pH and N-NH_4^+ —particularly strong in the case of saponification. On one hand, this indicates greater substrate availability; on the other, it points to the potential risk of intermediate (VFAs/ N-NH_4^+) accumulation during the initial phase of AD. These relationships are consistent with previous findings showing that alkaline conditions and the presence of nitrogen compounds promote the release of proteinaceous EPS [15,18]. In saponification, this effect was further intensified by the synergistic action of base and temperature, resulting in higher EPS mobilization and stronger correlations between protein fractions and organic indicators. Jasiński also reported similar mechanisms i wsp. [9], In their research, thermochemical pretreatment with NaOH and elevated temperature (121 °C) increased NPOC by transforming tightly bound polymers (TB-EPS) into soluble fractions (S-EPS, LB-EPS). This phenomenon was also reflected in the network analysis performed as part of this study, which showed negative correlations between TB-EPS and S-EPS, as well as strong positive associations of S-EPS with NPOC and VFAs, during saponification. The observed trend (TB-EPS → S-/LB-EPS and increased S-EPS) is consistent with literature data for thermal-alkaline pretreatment and/or saponification, in which hydrogen bond disruption, partial “de-crosslinking,” and solubilization of protein-carbohydrate polymers promote the formation of soluble fractions [25,48]. From the mechanism's point of view, this corresponds to the well-described role of EPS as a diffusion barrier and floc scaffold—the less TB-EPS and the more S-EPS, the greater the bioavailability of the substrate for hydrolysis [12,15,49]. It is also worth noting that the observed increase in protein fractions within S-EPS during saponification aligns with reports indicating that proteinaceous EPS are more susceptible to alkaline hydrolysis at elevated temperatures [25,33] and, importantly, they can form soluble protein-carbohydrate complexes (higher than in the chemical method correlations between S-EPS-prot and S-EPS-carb), which are then easily fermented [11,47].

These relationships align with the most recent findings that EPS fraction reorganization constitutes a key mechanism of polymer matrix restructuring, leading to an increased share of soluble fractions at the expense of strongly bound polymers. An example is the study on calcium-assisted saponification, which demonstrated a shift in equilibrium toward soluble EPS [29]. Moreover, the review by Huang et al. [11] emphasized that EPS significantly influence the quality of biological processes and their coupling with environmental chemical parameters, which explains the strong associations between EPS fractions and NPOC/VFAs/pH observed in this study. Research on EPS–biochar interactions further indicates that EPS form more complex networks with organic and inorganic matter than previously assumed, reinforcing the interpretative context [50]. The results confirm that the synergistic action of alkali and temperature leads to restructuring of the polymer matrix toward more mobile and bioavailable fractions [51]. In this context, the effects of saponification were more pronounced than those observed for the purely alkaline variant: a greater TB-EPS → S-EPS conversion, stronger NPOC–VFAs coupling, and a more distinct environmental component in PCA (higher VFAs, N-NH_4^+ , and pH loadings in PC2). These

trends are consistent with the literature on Thermal-Alkaline Pretreatment (TAP)/Thermal Hydrolysis Process (THP) [25,27,48] and on saponification of lipid-rich substrates [32]. In both approaches (THP/TAP), intensive solubilization and the release of soluble EPS fractions increase substrate bioavailability, but require careful control of dosage and reaction time to prevent excessive VFA accumulation and increases in N-NH_4^+ and pH. Importantly, the differences between the methods were also confirmed by ANOVA/Kruskal-Wallis results (significant effects of method, dose, and—for selected indicators—the method $\times$ dose interaction), as well as by effect size measures (η^2/ω^2 , Cohen's d), in accordance with guidelines for BMP testing and AD modeling [52–54].

All the changes observed for the co-digestion mixture indicate synergistic effects in terms of substrate solubility and biodegradability, but also highlight the need for system stabilization (through appropriate dosage/operating conditions or the use of additives such as biochar) [6,9,40]. The obtained results—marked increases in S-EPS, TB-EPS conversion, and associated environmental effects (VFAs, N-NH_4^+ , pH)—are consistent with this picture and provide a solid rationale for selecting either more conservative dosages (prioritizing stability) or higher dosages (prioritizing maximal solubilization), depending on the intended operational objective [31,55].

Based on PCA results and statistical testing, sets of conditions representing both the efficiency threshold and the upper operational range for each pretreatment method were selected for batch fermentation. For the chemical method, a threshold dose of 2.7 mg/l NaOH was adopted, providing distinct organic matter release (shifts along PC1) with moderate PC2 changes, and thus associated with limited risk of process disturbance during methanogenesis. Additionally, 5.4 mg/ NaOH was selected as the maximum variant, characterized by the highest mobilization of organic substances and the most pronounced EPS matrix alterations, but also by an increased risk of acidification and intermediate accumulation. In saponification (70 °C + NaOH), four doses were selected: 0 mg/l as a control (to assess the effect of temperature alone), 0.9 mg/l as the threshold dose where the first significant effects appeared (particularly in PC2), 2.7 mg/l as the “equilibrium” dose (moderate and balanced shifts along PC1 and PC2, typical for this method), and 4.5 mg/l as the high-performance variant, ensuring intensive release of organic fractions while maintaining acceptable environmental conditions.

3.3. Impact of pretreatment on methane production

Compared to the AD of sewage sludge alone, for which the methane yield was 307 ml $\text{CH}_4/\text{g VS}$, the introduction of co-substrates into the anaerobic chambers resulted in a significant increase in methane potential to 396.2 ml $\text{CH}_4/\text{g VS}$, which corresponds to an increase of 89.2 ml $\text{CH}_4/\text{g VS}$, or approximately +29% relative to the baseline value. A further increase in yield was achieved as a result of saponification (70 °C + NaOH), during which the methane yield increased systematically with the increase in the dose of the alkaline agent, reaching 471.5 ml $\text{CH}_4/\text{g VS}$ (0 g/l), 492.5 ml $\text{CH}_4/\text{g VS}$ (0.9 g/l), 524.6 ml $\text{CH}_4/\text{g VS}$ (2.7 g/l), and 553.4 ml $\text{CH}_4/\text{g VS}$ (4.5 g/l), respectively. In the chemical pretreatment variant (without the thermal stage), the maximum yield (552.8 ml $\text{CH}_4/\text{g VS}$) was obtained at 2.7 g/l NaOH, whereas further increase to 5.4 g/l reduced methane production to 457.2 ml $\text{CH}_4/\text{g VS}$ (Figure 18).

For the saponification process, a linear increase in methane potential (BMP) was observed as a function of NaOH dose (g/L). The regression model obtained was: $Y_M = 14.90 \cdot \text{Dose} + 476.98$, where the slope coefficient of 14.90 ml $\text{CH}_4/\text{g VS}$ indicates an additive increase in methane yield of approx. 14.9 ml $\text{CH}_4/\text{g VS}$ for each additional 1 g/l of reagent dose. The high value of the coefficient

of determination ($R^2 = 0.951$) confirms the very good fit of the linear model to the experimental data. For the alkaline variant, a non-linear curve was obtained, indicating the existence of an optimal NaOH dose. In this pretreatment variant, the dependence of methane production on the dose was best described by a quadratic model: $Y_M = 17.37 \cdot \text{Dose}^2 + 105.08 \cdot \text{Dose} + 396.29$. The maximum of the function was observed for a dose of ≈ 3.02 g/l, at which the methane production coefficient was 555.21 ml CH_4 /g VS. The coefficient of determination, $R^2 = 0.985$, indicates a very good fit of the model to the experimental data. A similar approach based on linear and polynomial regression to describe the relationship between organic matter load and biogas yield was used by Duong and Lim [56], who developed empirical models predicting biogas production in co-fermentation systems of pig manure and used cooking oil. In their study, the use of second-order polynomial regression improved model fit from $R^2 = 0.7167$ to $R^2 = 0.9656$, confirming the approach's effectiveness in predicting biogas yield.

Two-way ANOVA confirmed significant effects of pretreatment method ($F = 83.91$, $p < 0.001$), NaOH dose ($F = 243.90$, $p < 0.001$), and their interaction ($F = 112.76$, $p < 0.001$). Effect size analysis indicated a dominant influence of NaOH dose ($\eta^2 = 0.627$, $\omega^2 = 0.623$), followed by the method $\times$ dose interaction ($\eta^2 = 0.290$, $\omega^2 = 0.287$), and a moderate but significant contribution from the method itself ($\eta^2 = 0.072$, $\omega^2 = 0.071$). Kruskal–Wallis testing further confirmed significant differences between treatment combinations ($H = 19.15$, $p = 0.0039$). In contrast, post hoc Tukey/Dunn tests identified the most essential contrast between saponification and chemical pretreatment at higher doses (e.g., $S_{4.5}$ vs. $Ch_{5.4}$: $\Delta = 96.1$ mL CH_4 g $^{-1}$ VS; $p < 0.001$). These results unequivocally indicate that saponification is the more effective pretreatment for enhancing methane yield, with the optimal range observed between 2.7–4.5 g L $^{-1}$ NaOH. Beyond this, excessive solubilization may reduce stability by accumulating intermediates (VFAs, NH_4^+ , and pH increases).

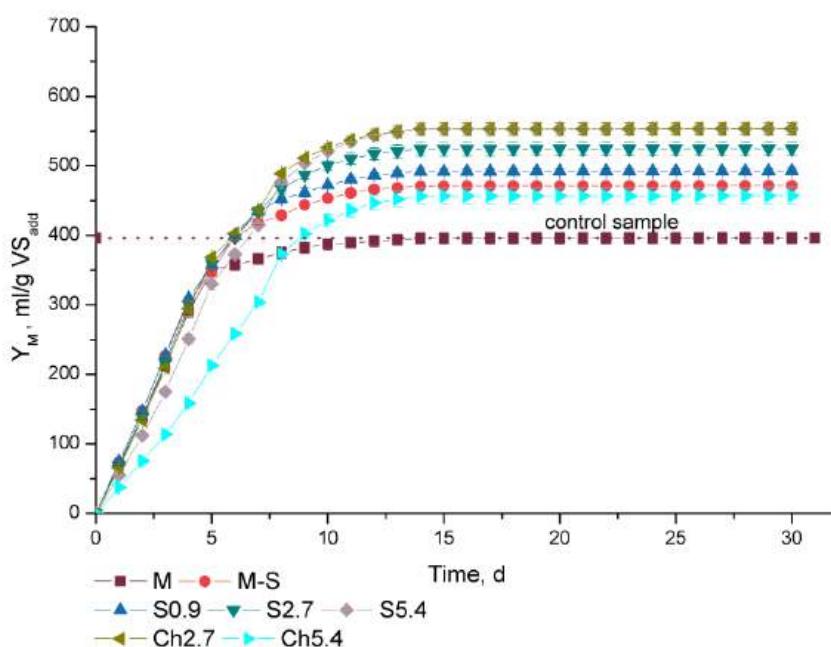


Figure 18. Effect of pretreatment method on methane yield; **M** – unconditioned co-digestion mixture (control sample); **M-S** – mixture subjected to 70 °C for 1 h; **S0.9; S2.7; S4.5** – mixtures subjected to saponification; numbers indicate NaOH dose (0.9, 2.7, 4.5 g/l), **Ch2.7; Ch5.4** – mixtures conditioned by chemical method; numbers indicate NaOH dose (2.7, 5.4 g/l)

The co-digestion performance index (CPI) further supported the synergistic effect of pretreatment. Without pretreatment, $CPI < 1$ indicated the absence of synergy. Thermal treatment alone (70 °C, one h) slightly increased CPI to 1.10, while saponification caused a pronounced dose-dependent rise—from 1.15 at 0.9 g/l to 1.29 at 4.5 g/l NaOH, corresponding to 15–29% higher methane yields compared with the theoretical additive value. In the variant with alkaline treatment only, the maximum was recorded at a dose of 2.7 g NaOH/l ($CPI = 1.29$), followed by a decrease with a further increase in the dose to 5.4 g NaOH/l ($CPI = 1.06$). This pattern of change – a local maximum and a sharp reduction in the index after exceeding the limit dose – is typical for chemical pretreatment processes described in the literature [25,27,48] and is associated with excessive solubilization, leading to the accumulation of volatile fatty acids (VFAs), an increase in free ammonia concentration, and the formation of poorly biodegradable compounds, such as Maillard reaction products (melanoidins), especially with prolonged alkaline exposure and/or the presence of a thermal component.

The observed pattern aligns with literature on thermal–alkaline pretreatment (TAP) and thermal hydrolysis processes (THP), which generally produce 10–30% methane yield increases under optimized conditions but can cause inhibition beyond the optimal alkaline range [21,25,31,35,48]. In saponification, a wider range of tolerated doses is typically observed, as the synergistic action of heat and alkali promotes both TB-EPS conversion to S/LB-EPS and lipid ester hydrolysis, thereby reducing LCFA accumulation. Consequently, the process exhibits a linear or at least monotonic increase in methane potential within the tested range [25,32].

Significantly higher yield increases of 40–90%, reported by Jasińska et al. (2024) and Zhan et al. (2023), apply to mixtures with different compositions or to processes carried out under more aggressive treatment conditions. In the study by [40], thermochemical variants showed a clear advantage over mixtures that had not undergone pretreatment, and in the poultry litter–straw system, alkalization to $pH \approx 12$ combined with aeration resulted in an increase in methane production of approximately +46.7%. These results indicate that thermo-alkaline feed preparation is a key factor in ensuring a stable, lasting synergistic effect, whereas alkaline pretreatment alone requires precise selection of the reagent dose to avoid loss of the beneficial effect.

Kinetic modelling using the modified Gompertz (MG), logistic (LF), and transfer function (TF) models demonstrated excellent agreement with experimental data ($R^2 \approx 0.99$ for all models). As shown in Table 4, pretreatment significantly increased the methane yield of the mixture while maintaining a high maximum methane production rate (R_m) within the range of operationally justified doses. The R_m remained relatively stable across moderate NaOH concentrations (~ 77 – 81 ml CH_4 /g VS d for M–S, S_{0.9}, and S_{2.7}), indicating that the increase in methane potential following saponification was not accompanied by a decline in methanogenic activity. A noticeable decrease in both R_m and methane yield was observed only at excessive alkali dosage (Ch_{5.4}: 60.0 ml CH_4 /g VS d).

Significant changes were recorded in the lag phase (λ), which was strongly affected by pretreatment intensity. Thermal treatment alone reduced the lag phase from 0.31 d (untreated) to 0.12 d, while increasing NaOH concentration led to a gradual extension ($S_{0.9} = 0.16$ d; $S_{2.7} = 0.23$ d; $S_{4.5} = 0.70$ d; Ch_{5.4} = 1.24 d). This type of dose–lag phase relationship, characteristic of alkaline systems, has been previously described by Almomani and Bhosale [56], who observed an extension of the lag phase with increasing alkalinity during the co-fermentation of agricultural

waste with cattle digestate (NaHCO_3 alkalization). Such dose-dependent elongation reflects temporary delays in the adaptation of methanogenic consortia due to elevated VFA and ammonia levels. In contrast, under saponification conditions, the synergistic effect of heat and alkali modifies this relationship. At optimal doses (2.7–4.5 g/l NaOH), both a shortened lag phase and an enhanced methane yield were achieved, indicating improved substrate accessibility without signs of metabolic inhibition. Similar effects were reported by Wu et al. ([29], who demonstrated that calcium-assisted saponification of FOG-containing mixtures can simultaneously increase methane yield and shorten the lag phase by “unblocking” lipid fractions and improving substrate bioavailability.

Table 4. Summary of estimated kinetic parameters derived from the modified Gompertz (MG), logarithmic (LF), and transfer function (TF) models

Sample	P [ml CH_4 /g s.m.o.]	R_m [ml CH_4 /(g s.m.o. d)]	λ [d]	R^2
<i>Modified Gompertz model (MG)</i>				
M	395.0145	84.34147	0.307947	0.99796
M-S	470.8948	79.439445	0.124692	0.99959
S0.9	492.3547	80.79020	0.160348	0.99979
S2.7	525.4415	78.52165	0.230837	0.99969
S4.5	556.4433	77.24405	0.700188	0.99904
Ch2.7	555.0871	80.25975	0.359198	0.99929
Ch5.4	461.8400	60.02645	1.242405	0.99717
<i>Logarithmic model (LF)</i>				
M	393.5631	79.25289	0.291303	0.99768
M-S	468.9017	72.49790	0	0.99759
S0.9	490.2294	73.78093	0.035432	0.99800
S2.7	522.9289	71.73753	0.103162	0.99792
S4.5	553.1907	72.40742	0.696218	0.99882
Ch2.7	552.2713	73.76190	0.260260	0.99791
Ch5.4	458.4612	58.04185	1.390175	0.99930
<i>Transfer function model (TF)</i>				
M	397.5470	151.3363	0.623797	0.99359
M-S	474.7228	145.5487	0.601285	0.99721
S0.9	496.5997	147.0405	0.625620	0.99686
S2.7	530.9472	140.8669	0.690699	0.99616
S4.5	565.0810	129.2638	0.919843	0.99143
Ch2.7	561.6960	141.2386	0.761020	0.99464
Ch5.4	472.6038	91.54493	1.126988	0.98414

Legend: **P** - methane production potential; **R_m** - maximum methane production rate; **λ** - phase lag length; **R^2** - coefficient of determination; **M** – unconditioned co-digestion mixture (control sample); **M-S** - mixture subjected to 70 °C for 1 h; **S0.9; S2.7; S4.5** - - mixtures subjected to saponification; numbers indicate NaOH dose (0.9, 2.7, 4.5 g/l), **Ch2.7; Ch5.4** – mixtures conditioned by chemical method; numbers indicate NaOH dose (2.7, 5.4 g/l).

4. Conclusions

Saponification (70 °C + NaOH) proved to be a more effective and stable pretreatment method than the classic alkalization of a mixture of chicken manure, sewage sludge, and fatty waste. This process led to a profound restructuring of the EPS matrix, increased the solubility of organic matter, and permanently increased methane yield without compromising the system's stability. The combination of kinetic, statistical, and structural analyses provided a comprehensive explanation of the mechanisms of increased substrate bioavailability after thermal-alkaline treatment.

Specific conclusions

1. Mechanism of action:

Saponification restructures the EPS polymer matrix by converting the TB-EPS fraction to S-EPS, thereby increasing the substrate availability to hydrolytic enzymes and improving biodegradation efficiency.

2. Process efficiency:

The release of soluble fractions increased the bioavailability of organic matter, increased microflora activity, and shortened the AD delay phase.

3. Method × dose interaction:

The effectiveness of pretreatment depended on both the NaOH dose and the presence of a thermal component; the same dose generated different effects depending on the method, indicating the synergistic nature of saponification.

4. Statistical confirmation:

The results of ANOVA and nonparametric tests confirmed the significant influence of the method, dose, and their interaction ($p < 0.001$), thereby increasing the reliability of the observed relationships and allowing the effects to be considered real rather than statistical artefacts.

5. Significance of EPS as a biomarker:

EPS fractions showed strong correlations with organic matter degradation parameters (NPOC, VFAs), confirming their potential use as rapid indicators of conditioning effectiveness and substrate “readiness” for fermentation.

6. Synergistic effect:

Saponification caused a lasting synergistic effect ($CPI > 1.2$), resulting from controlled solubilization of organic fractions, rather than excessive decomposition or inhibition of the methanogenic microflora.

7. Practical application:

The optimal dose range (approx. 2.7–4.5 g NaOH L⁻¹) allows a balance to be achieved between the mobilization of organic compounds and the stability of the system, which is crucial when designing processes on a technical scale.

8. Implementation prospects:

The inclusion of EPS markers and NPOC/VFA indicators in the current process diagnostics may enable dynamic control of pretreatment intensity, thereby improving the energy efficiency and stability of biogas plants.

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