INTRODUCTION

Biomethanation is defined as a process of converting complex organic matter under anaerobic conditions mostly to methane and carbon dioxide, with possible emission of trace amounts of hydrogen sulfide, hydrogen, and carbon monoxide. Methane fermentation requires the activity of various populations of microorganisms, responsible for a proper course of consecutive process phases. The following phases of biodegradation are distinguished according to the subsequent organic substance conversions (Figure 1): (a) hydrolysis, where complex organic compounds, such as carbohydrates, proteins, and lipids, undergo hydrolytic transformations with the catalytic participation of enzymes. These processes lead to the production of mostly simple sugars, higher fatty acids, glycerol, and amino acids. Two phyla dominate among hydrolyzing bacteria; Bacteroidetes and Firmicutes, and they include most of the known species. (b) Acidic fermentation, where acidogenic bacteria convert products of the hydrolysis to VFAs, which include acetate, propionate, butyrate and isobutyrate, and valerate and isovalerate. Besides VFAs, alcohols, lactate, formate, CO₂, and H₂ are produced. These two stages are carried out by bacteria of the genera 

Abstract

Methane fermentation is an attractive practice in waste processing, which enables one to both control pollution and recover energy. This kind of anaerobic digestion is exposed to inhibitors, which can retard the process and cause failure. The mechanism causing toxicity of these substances and their impact on the efficiency of the process are already known, but there is still not much information about their influence on methane fermentation microorganisms’ activity and the composition of microbiota. In this review, based on 168 articles, we present a summary of the up-to-date research on the inhibition of anaerobic processes by some specific toxicants: ammonia, sulfides, ions of light metals, heavy metals, antibiotics, ethylene and acetylene, chlorophenols, halogen aliphatic hydrocarbons, aliphatic nitro compounds, and long-chain fatty acids. This review principally focuses on the impact of these inhibitors on the microorganisms involved in the process. More accurate recognition of methane fermentation inhibition mechanisms, with particular emphasis on the microbiological aspect, can help to improve the efficiency of the process.

KEYWORDS
anaerobic digestion, inhibitors, methane production efficiency, methanogens
extent, by *Streptococcus* sp. and *Enterobacterium* sp. and others.\(^7,8\) (c) Acetogenesis, where acetogenic bacteria (including *Syntrophomonas* sp., *Syntrophobacter* sp.) produce acetic acid. Methanogens can use acetate, formate, H\(_2\), CO\(_2\), and methyl compounds directly, but other intermediates formed by acidogenesis have to be additionally biodegraded by other microorganisms, which enables methanogens to use them in order to produce methane. Syntrophic acetogenesis is the process in which these intermediates are further biotransformed to form acetate, H\(_2\), and CO\(_2\). With regard to thermodynamics, this is one of the most difficult stages. What is needed here is the syntrophy of acetogenic and methanogenic bacteria, where one group of microorganisms produces and the other consumes hydrogen. Hydrogenotrophic methanogens live in syntrophy with acetogens and consume H\(_2\) provided from the latter.\(^7,5,10\) A recent study has shown direct interspecies electron transfer performed by some microorganisms using electrically conductive pili. Electrons can be transferred in this way from *Geobacter* to *Methanoseta*, for example.\(^4,10-12\) (d) Methanogenesis, where methanogenic microorganisms under anaerobic conditions convert products of the preceding phases, releasing methane, carbon dioxide, and water. Hydrogenotrophic methanogens are critical for anaerobic digestion because of their ability to scavenge H\(_2\) and keep the partial pressure low. The most frequently observed hydrogenotrophic methanogens in anaerobic digesters belong to the genera *Methanobacterium*, *Methanobrevibacter*, *Methanoculleus*, *Methanospirillum*, and *Methanothermobacter*. Acetoclastic methanogens belong to the genera *Methanoseta* and *Methanosarcina*.\(^9,10,13-15\)

Methane fermentation is an attractive practice in waste processing, which enables us to both control pollution and recover energy. As reported by Scarlat et al.,\(^17\) in 2015, in Europe alone, there were about 17,000 biogas plants of different sizes and types, and the total biogas production reached more than 650 PJ of primary energy. Biogas production has achieved an important growth recently. However, the same researchers state that there are frequent problems due to the low efficiency of methane production (eg, caused by a decrease in the activity of various groups of microorganisms involved in anaerobic digestion, including methanogenes) and instability of the entire process, which prevents the widespread implementation of this technology. The major reason why methane fermentation process is inhibited is the diversity of substances present in various concentrations in different types of waste.\(^2,17,18\) This review article is dedicated to the identification of factors and mechanisms causing the inhibition of methane fermentation, with particular emphasis on the microbiological aspect. First, however, the characteristics of the process of biogas production and methanogenic microorganisms are discussed.

### 1.1 General introduction to the anaerobic digestion process

The global energy consumption and demand for power are constantly growing. Meanwhile, most of the resources, such as coal, natural gas, or crude oil, are not sustainable energy sources. The contemporary critical phase in the human population growth requires increasingly larger energy inputs. These circumstances substantiate the growing interest in renewable energy.\(^19\)

Except for solar and wind energy, biogas is among the most promising bioenergy alternatives to the energy based on fossil fuels. Many types of biodegradable waste can be used as feedstocks for biogas production, thus relieving the pressure on the natural environment and limiting the total area of landfills.\(^20\) Biodegradable waste most often consists of by-products from agricultural production (including

![Figure 1](https://example.com/f1.png)

**Figure 1** Process of methane fermentation according to Chen et al.\(^{16}\)
postharvest residues, excess biomass, roots, and leaves), waste from the agricultural and food processing industries (pressed fruit pomace, extracts, pulp, sediments, filtration, and extraction leftovers), and from abattoirs and meat processing plants. Other substrates used for methane fermentation are sewage sludge and municipal waste as well as dedicated energy crops (maize, amaranthus, sorghum, oilseed rape, sugar beet, fodder beet, and others), algae and seaweed (water base) and by-products from the production of ethanol and biodiesel.

Animal farms generate waste and by-products, which have various impacts on the natural environment. They can also serve as feedstocks for methane fermentation. Slurry is certainly the type of raw material considered for methane production that is available in large quantities in many parts of the world. Data on methane productivity from different feedstocks submitted to fermentation are given in Table 1.

Methane can be an alternative to fossil fuels in thermal and electric power generation, but it can also serve as a fuel for vehicles. By replacing a natural fossil fuel with a renewable one, we can produce a beneficial influence on the environment and achieve greater diversification of sources of energy. Sustainable development of the human population requires both restraining our addiction to fossil fuels and limiting environmental pollution, and methane fermentation is one of several technologies able to achieve both aims.

The site and method of biogas production have a significant influence on its quality and quantity. Biogas from different sources can have different methane content and therefore different values of energy parameters as well as the content of pollutants. It has been demonstrated, for example, that biogas from landfills is characterized by a highly variable methane content, and biogas from fermentation tanks at wastewater treatment plants as well as on farms is more stable. Typical landfill biogas contains between 25% and 67.9% of methane, and its calorific value ranges between 16.0 and 23.5 MJ/m³. The content of methane in biogas from WTPs is between 57% and 67%, and its calorific value varies from 20.5 to 23.4 MJ/m³. Biogas with the highest calorific value, from 18.7 to 30.6 MJ/m³, can be obtained from agricultural biogas plants, where the methane content varies within 56%-70%.

### Table 1: Biogas and methane yield from different types of substrates

| Type of organic waste                      | Organic content (%) | Biogas production (mL/g) | Methane yield (Unit of methane yield measurement) | References                      |
|------------------------------------------|---------------------|--------------------------|-------------------------------------------------|--------------------------------|
| Cellulose                                | —                   | —                        | 73.4 %                                          | Barlaz et al141                |
| Hemicellulose                            | —                   | —                        | 17.1 mL/g                                       | Li et al142                    |
| Fruit and vegetable waste                | —                   | —                        | 326 mL/g                                        | Cassie et al143                |
| Grass silage                             | —                   | 80                      | 238                                             | Li et al142                    |
| Wheat straw                              | —                   | 25-30                   | 192.4                                           | Cassie et al143                |
| Cotton stock                             | —                   | 190                     | 138                                             | Cassie et al143                |
| Chicken manure                           | —                   | 265                     | 714                                             | —                              |
| Pig/cow manure                           | —                   | 120                     | 40-60                                           | —                              |
| Corn silage                              | —                   | 714                     | 450-600                                         | —                              |
| Brewery waste                            | 15-20               | —                        | 40-60                                           | —                              |
| Bakery waste                             | 3-4                 | —                        | 150-240                                         | —                              |
| Fish-oil sludge                          | 7-10                | —                        | 40-55                                           | —                              |
| Source sorter organic household waste    | 80-85               | —                        | 800-1000                                        | —                              |
| Whey                                     | 90                  | —                        | 40-55                                           | —                              |
| Soy oil/Margarine                        | 3-4                 | 15-20                    | 297-370                                         | —                              |
| Sewage sludge                            | 7-10                | —                        | 247-375                                         | —                              |
| Concentrated sewage sludge               | 86-91               | 382-506                  | 85-110                                          | —                              |
| Forage mix                               | 90                  | 85-110                   | 297-370                                         | —                              |
| Maize                                    | 90-93               | 85-110                   | 297-370                                         | —                              |
| Barley                                   | 20-30               | —                        | 150-240                                         | —                              |
| Rye                                      | 90                  | —                        | 403-404                                         | —                              |
| Sugar beet                               | 90                  | —                        | 504                                             | —                              |
Methanopyrales, and Methanocellales of methanogens have been recognized: 

1. The biogas yields received from animal manure and animal slurry vary from 370 m³ per ton dry matter cattle manure to 450 m³ per ton dry matter pig manure.

Unlike natural gas, biogas can contain various types of pollutants. These can be chemical (sulfides, ammonia, chloride, and fluorine compounds, silanes), mechanical (e.g., silicon, dust), and biological (bacteria, fungi) pollutants. The contaminants found in biogas may have an adverse impact on its quality and combustion. Recognizing and understanding the aspects connected with this problem could certainly support attempts to create strategies for developing the technology and reinforce its credibility as an alternative energy source. The continued improvement of existing biomethanation technologies and the development of new technologies can enhance the effectiveness and stability of these processes. Methane fermentation is a process in which technical solutions must respond to the following considerations: (a) only the organic fraction undergoes degradation, (b) the nature of the biological process imposes certain restrictions, such as the process's temperature, pH, composition of feedstocks, presence of toxic substances, (c) anaerobic digestion requires a sealed container (reactor), and (d) the product (biogas) contains other components apart from methane and carbon dioxide.

1.2 Microorganisms involved in methane production

Archaeal methane metabolism has a significant role in the global carbon cycle, with methane produced by archaea corresponding to over a half of all methane produced in the world per year. Methane is produced by methanogenic archaea in the last step of organic matter fermentation under anaerobic conditions. All methane-synthesizing microorganisms have a specific functional gene, mcrA, which encodes the α-subunit of methyl-coenzyme M reductase and is a better tool for analysing their biodiversity changes than 16S rRNA. The analysis of methanogens and their analysis based on 16S rRNA as a marker gene is limited because methanogenic Archaea are not monophyletic. Several orders of methanogens have been recognized: Methanosarcinales, Methanococcales, Methanomicrobiales, Methanobacterales, Methanopyrales, and Methanocellales.

The overall cell structure of the Archaea representatives resembles the structure of a bacterial cell. The cytoplasm lacks mitochondria, lysosomes, endoplasmatic reticulum, or the Golgi apparatus. The cell is typically enveloped by a cell wall and membrane. The cell wall in archaea does not contain peptidoglycan (murein), and its stability and stiffness depend on the presence of other polymers. A paracrystalline protein cover layer, commonly referred to as the S-layer, is present in almost all described archaea. S-layers are formed of only one or two proteins and create various lattice structures. This is a superficial, 5- to 25-nm-thick layer that envelopes the cell, thus helping it to maintain the proper shape and protecting it from unfavorable changes in the environment. It is fairly smooth on its outer surface, with a more corrugated internal surface. In some archaea, S-layer proteins are the sole cell cover component, while in others the cell cover consists of various polymers, including the polysaccharides pseudomurein and methanochondroitin, and can also include additional S-layer proteins. Same as in certain bacteria, the S-layer of archaea is composed of proteins and/or glyco-proteins, distinguished by a large content of acidic and hydrophobic amino acids. However, the structure of archaea is clearly different from that of bacteria. Representatives of the Archaea also have some specific surface structures, including archaea, pilus, hami, and cannuca. Many microorganisms from the domain Archaea have intercellular organelles of motion, which used to be called flagella, like organs of locomotion in bacteria. Nowadays, it is known that these organelles have a structure different from that of bacterial flagella or of cilia, characteristic for many cells of eukaryotic organisms. These organelles are now referred to as archaella. Unlike bacterial flagella, archaella do not have rings that would enable them to anchor in the cell wall and membrane. Archaea are characterized by a considerable natural resistance to antibiotics, the presence of nucleotides in tRNA molecules, absent in cells of other microorganisms, and by an atypical structure of RNA polymerase, dependent on the DNA. Genes linked to cellular divisions and metabolism in archaea resemble the ones occurring in the genome of bacteria, whereas genes participating in the processes of replication, transcription and translation are more similar to their counterparts in eukaryotic cells. Archaea reproduce asexually, by cell division or budding, and they exchange genetic material in a way similar to generalized transduction in bacteria, but also through the processes of conjugation and transformation. This is possible because archaea, like bacteria, possess additional genetic material in the form of plasmids. Most of the Archaea recognized until now multiply by cell division. Although the last two decades have witnessed an enormous progress in our knowledge and understanding of cellular structures, including the complete structure of archaeal cells, some of the functions and mechanisms responsible for stability in extreme ambient conditions still await clarification. Microorganisms which belong to the domain Archaea are isolated from various environments, often from particularly extreme habitats. Archaea are typical microbiota of oceans, seas, lakes, soils, the rumen, and also biogas reactors. This domain is characterized by a large share of thermophilic and hyperthermophilic organisms, isolated from hot springs and from hydrothermal chimneys situated on the bottom of the
oceans. Other archaea are representatives of typical psychrophilic and psychrotrophic organisms, isolated from waters and soils at temperatures close to 0°C. Another group of archaea is composed of halophilic organisms, growing in habitats with extremely high salinity, there are also ones dwelling in habitats with extremely high or low pH (alkaliphiles and acidophiles). Methanogenic archaea are also a constituent part of the microbiome of many animals and humans. Methanogenic organisms colonize mostly the digestive tract, including the large bowel.

Archaea can produce methane in three ways (Figure 2), different in the carbon substrates and sources of reduction potential. The most common methanogenesis among methanogenic archaea is the hydrogenotrophic pathway, where carbon dioxide is reduced with the participation of hydrogen as an electron donor. It consists of seven stages, leading to the production of methane. Another substrate used on this pathway is formate, which is the source of both carbon and electrons. The two other types of methanogenesis are the acetoclastic and methylotrophic pathway, which occur among representatives of the order Methanosarcinales. In the former pathway, acetic acid is decomposed to carbon dioxide and a methyl group. CO is gradually oxidized, which coincides with the release of electrons, necessary to reduce the methyl group to methane. The methylotrophic pathway has been also observed among representatives of the Methanobacteria. In its thus far best explored variant, one-carbon compounds (ie, methylamines or methanol) are used simultaneously as a donor and acceptor of electrons. One C-1 molecule of the compound is oxidized to obtain electrons, which serve to reduce three consecutive molecules until the final product, that is, methane, is obtained.

The process of methane synthesis is participated by many unique co-enzymes (tetrahydromethanopterin, methanofuran, co-enzyme F420, HS-coenzyme B, co-enzyme M) and electron carriers (ie, methanophenazine). Additionally, over 200 genes are responsible for encoding the synthesis of co-enzymes, enzymes, and prosthetic groups participating in the process of reducing carbon dioxide to methane and its coupling with ADP phosphorylation. It is maintained that the primordial group in the evolution consisted of hydrogenotrophic methanogens. This is confirmed by the presence of genes responsible for production of methane in all species, in an almost unchanged form.

It is worth underlining that methanogenesis can occur in a wide range of temperatures, and its efficiency depends primarily on the conditions in which particular representatives of Archaea dwell. As Mikucki et al report, methane is synthesized at different temperatures. Mesophilic as well as thermophilic species are responsible for its production. Methane can be synthesized by hyperthermophilic species, for example, Methanococcus jannaschii and Methanopyrus kandleri. For instance, the mesophilic species Methanoculleus submarinus synthesizes methane as hydrates at a temperature as low as 15-16°C. Some psychrophilic methanogens have also been reported, such as Methanogenium frigidum and Methanosarcina lacustris. It is therefore evident that methanogens play a significant role in the carbon cycle in nature, by synthesizing methane from various simple inorganic and organic compounds.

Table 2 presents microorganisms responsible for conducting consecutive stages of biogas production.

## 2 | INHIBITORS OF METHANE FERMENTATION

Inhibitors of methane fermentation can be divided into specific and nonspecific ones. Specific inhibitors cause the process to stop by affecting only the group of methanogenic microorganisms, active in the last stage of fermentation, whereas nonspecific inhibitors influence the activity of both methanogens and other groups of microorganisms. There are numerous studies reporting on various chemical substances which inhibit methane production by archaea, at different densities of microbial populations and concentrations of inhibitors.

### 2.1 Ammonia

Although ammonia is an essential nutrient for the growth of bacteria, if present in very high concentrations it can inhibit methanogenesis during anaerobic digestion. According to Yenigun and Demirel, ammonia is considered to be a potential inhibitor during biogas production, especially in composite substrates, such as manure or the organic fraction of municipal waste. Ammonia is generated during the biological degradation of nitrogenous matter, mostly proteins, and urea. Ammonia ions (NH₄⁺) and free ammonia (NH₃) are the two main forms of inorganic ammonia nitrogen in aqueous solution. It is suggested that free ammonia is the main cause of the inhibition of methanogenesis because it can freely permeate through cell membranes. The relative concentration of molecular ammonia (NH₃) and ammonia in the form of the ammonium ion (NH₄⁺) depends on the pH and temperature. An increase in pH and temperature values favors the formation of toxic molecular ammonia. Several mechanisms of free ammonia inhibition after diffusion into a cell have been described: change of the intracellular pH, proton imbalance, rise in maintenance energy demand, and inhibition of specific enzymatic reactions.

It is commonly maintained that ammonia concentrations of approximately 200 mg/L are beneficial for anaerobic processes because nitrogen is an essential nutrient for anaerobic microorganisms. However, large concentrations of
The total ammonia nitrogen can limit microbial activities.\textsuperscript{69} The literature also provides information about the sensitivity of methanogens to ammonia nitrogen. As reported by Yenigun and Demirel,\textsuperscript{65} the influence of ammonia on the maximum rise in the growth of hydrogen-consuming methanogenic microorganisms was investigated at different pH levels and temperatures. The maximum noninhibited rate of the growth of methanogens in sewage sludge was 0.126 hour\textsuperscript{-1} at pH equal 7.0 and temperature of 37°C. The maximum growth rate under these conditions was depressed to nearly a half of this value at 350 mmol/L ammonia. Besides, it has been shown that an increase in pH from 7.0 to 7.8 at 37°C seemed to have reinforced the inhibitory action of ammonia. During anaerobic digestion of liquid manure, the activity of methanogens was inhibited at high concentrations of total nitrogen, which was confirmed by changes in the parameters of acetate consumption. In a study on the fermentation of poultry litter, the maximum rate of growth of acetogenic bacteria was
| Stages of methane fermentation | Bacteria | References |
|--------------------------------|----------|------------|
| Hydrolysis | Bacteroides sp. | Li et al\textsuperscript{145} |
| | Bacillus sp. | Lo et al\textsuperscript{146} |
| | Bifidobacterium sp. | Venkata et al\textsuperscript{147} |
| | Cellulomonas sp. | Wiącek and Tys\textsuperscript{148} |
| | Clostridium sp. | Yu et al\textsuperscript{25} |
| | Enterobacterium sp. | Ziemiński and Frąc\textsuperscript{8} |
| | Erwinia sp. |  |
| | Micrococcus sp. |  |
| | Peptococcus sp. |  |
| | Pseudomonas sp. |  |
| | Ruminococcus sp. |  |
| | Streptococcus sp. |  |
| | Thermomonospora sp. |  |
| Acidogenesis | Aerobacter sp. | Ariesyady et al\textsuperscript{149} |
| | Alcaligenes sp. | Bertsch et al\textsuperscript{150} |
| | Bacillus sp. | De Bok et al\textsuperscript{151} |
| | Bacteroides sp. | de Bok et al\textsuperscript{152} |
| | Butyribacterium sp. | Detman et al\textsuperscript{153} |
| | Clostridium sp. | Imachi et al\textsuperscript{154} |
| | Escherichia sp. | Li et al\textsuperscript{145} |
| | Flavobacterium sp. | Schmidt et al\textsuperscript{155} |
| | Micrococcus sp. | Sousa et al\textsuperscript{156} |
| | Propionibacterium sp. | Wiącek and Tys\textsuperscript{148} |
| | Pseudomonas sp. | Ziemiński & Frąc\textsuperscript{8} |
| | Ruminococcus sp. |  |
| Acetogenesis | Acetobacterium sp. |  |
| | Methanobacterium propionicum |  |
| | Methanobacterium suboxydans |  |
| | Pelobacter sp. | Albers and Meyer\textsuperscript{41} |
| | Pelotomaculum sp. | de Bok et al\textsuperscript{152} |
| | Smithilela sp. | Jarrell et al\textsuperscript{157} |
| | Sporomusa sp. | Korzeniewska et al\textsuperscript{158} |
| | Syntrophobacter sp. | Kouzuma et al\textsuperscript{159} |
| | Syntrophomonas sp. | Lira-Silva et al\textsuperscript{160} |
| | Syntrophus sp. | Liu et al\textsuperscript{63} |
| | Methanobacterium thermoautotrophicum | Mikucki et al\textsuperscript{60} |
| | Methanococcales burtonii | Yenigun and Demirel\textsuperscript{65} |
| | Methanococcus jannaschii | Zhang et al\textsuperscript{161} |
| | Methanococcus voltae | Ziemiński and Frąc\textsuperscript{8} |
| | Methanocorpusculum sinense |  |
| | Methanogenium cariaci |  |
| | Methanolacina paynteri |  |
| | Methanopyrus candleri |  |
| | Methanoseta concilii |  |
| | Methanosarcina barkeri |  |
| | Methanosarcina mazei |  |
| | Methanosarcina thermophila |  |
| | Methanospirillum hungatei |  |
| | Methanothermobacter thermautotrophicus |  |
| | Methanothermobacter thermoflexus |  |
| | Methanothermobacter wolfei |  |
| | Methanosarcina flavescens |  |
| | Methanobacterium formicicum |  |

(Continues)
observed at a concentration of total nitrogen between 7700 and 10 400 mg/L and pH between 7.8 and 7.93.\textsuperscript{65,70-72} It has been reported that pH and total nitrogen concentration are the factors that inhibit acetogenic bacteria.\textsuperscript{65} In a study by Hendriksen and Ahring\textsuperscript{73} dealing with the impact of ammonia on methanogenic microorganisms consuming hydrogen, including \textit{M thermouautotrophicum}, \textit{Methanobacterium thermoformicicum}, and \textit{Methanogenium} sp., initial inhibition was detected at a total nitrogen concentration in a range of 3000-4000 mg/L, and when it rose to 6000 mg/L, the growth of microorganisms declined by 50\%. Moreover, slow growth and formation of aggregates of \textit{M thermoformicicum} were noticed by the same authors at a total nitrogen concentration equal 9000 mg/L. Based on the research results, it was concluded that thermophilic methanogens are less sensitive to ammonia than their mesophilic forms.\textsuperscript{65,73}

The literature describes a wide range of inhibitory concentrations of ammonia, with inhibitory concentrations of ammonia nitrogen in a range of 1700 do 14 000 mg/L causing a 50\% reduction in the production of methane.\textsuperscript{74} Sung and Liu informed that methanogenic activity in soluble non-fat dry milk digestion was heightened at TAN concentrations lower than 1500 mg/L, whereas methane fermentation was obviously inhibited at TAN concentrations higher than 4000 mg/L.\textsuperscript{74,75}

The differentiating role in the inhibition due to ammonia concentrations can be attributed to the type of substrate submitted to fermentation, environmental conditions (temperature, pH), and acclimation periods. When waste with high concentrations of ammonia nitrogen is being processed, pH affects the growth of microorganisms and the form of nitrogen that appears under such conditions.\textsuperscript{76} The accumulation of volatile fatty acids (VFA) causes a decrease in pH, and therefore, it reduces concentrations of ammonia but raises the content of ammonia ions. According to Chen et al,\textsuperscript{75} ionized ammonium nitrogen is an important inhibitor during food waste methane fermentation with uncontrolled pH. The inhibition effect occurred with the ammonium concentration of 2000 mg/L. The inhibition effects of high ammonium concentrations on anaerobic digestion led to VFA increase and pH decrease. These factors repressed the acetoclastic pathway and activity of \textit{Methanoseta} sp. The same authors reported that the ammonium concentration of 6000 mg/L inhibited the metabolism of the hydrogenotrophic methanogens, such as \textit{Methanobacterium} sp. and \textit{Methanospirillum} sp.\textsuperscript{75}

Interactions between the form of ammonia nitrogen, VFA, and pH can lead to “the inhibition of the established state,” where the fermentation process runs in a stable manner but generates less methane.\textsuperscript{77} As for temperature, in general, a higher temperature of the process has a beneficial effect on the rate of metabolic changes achieved by microorganisms, but it also causes an increase in the concentration of toxic free ammonia. The study of Hansen et al\textsuperscript{78} has demonstrated that fermentation of waste with a high content of ammonia is less stable and more strongly inhibited at thermophilic rather than at mesophilic temperatures. However, the acclimation of microorganisms as a factor can influence the rate of inhibition of methane fermentation by ammonia. Adaptation can result from internal changes among dominant methanogenic species and consequently within the whole population of methanogens.\textsuperscript{84} Conclusions derived from the research into ammonia effect on anaerobic digestion are summarized in Table 3.

Two physicochemical methods can be applied to remove ammonia from a feedstock: ammonia stripping with air, and chemical titration. Both methods have proven to be feasible at high ammonia concentrations and complex compositions of substrates.\textsuperscript{79} A popular approach to limiting the inhibition of methane fermentation due to ammonia consists in the dilution of a feedstock (mostly slurry) up to the final ammonia concentration of 0.5\% to 3.0\%. However, the resultant increase in the volume of waste to be processed makes this method economically unattractive.\textsuperscript{80} Another approach is to increase the retention time of a substrate in a reactor. It has been found that the methane productivity in a continuous stirred-tank reactor (CSTR) could be improved when a stirrer is switched on half an hour before and after feeding the substrate. This solution is thought to be promising because it is easy and economically viable.\textsuperscript{78}

### 2.2 Sulfides

Sulfate is a common component of many types of industrial wastewater. In anaerobic reactors, sulfate is reduced to sulfides by sulfate-reducing bacteria (SRB). Sulfate is reduced by two major groups of SRB, that is, the ones which reduce such compounds as lactate to acetate and CO$_2$, and the ones which completely decompose acetate to CO$_2$ and HCO$_3^-$. Sulfate-reducing bacteria are highly varied in terms of metabolic pathways. The compounds which can be partly or completely degraded by SRBs comprise branched and long-chain fatty acids, ethanol and other alcohols, organic acids, and aromatic compounds. Because of the various ways in which substrate can be used, SRBs compete for organic substrates or hydrogen with other fermentation microorganisms, that is, methanogens and acetogens, acidogens, and hydrolytic bacteria.\textsuperscript{81-83} The outcome of such competition between SBRs and other anaerobic microbes determines the concentration of sulfides in the reactor. Sulfides in different concentrations can be toxic not only to methanogens but also to sulfate-reducing bacteria themselves.\textsuperscript{84} Thus, degradation of sulfates in sewage sludge is a highly undesirable process because of both the depressed methane productivity and the unpleasant odor due to H$_2$S release.\textsuperscript{84,85}

As reported by Chen et al,\textsuperscript{64} H$_2$S is the most toxic form of sulfide because it is capable of diffusing through cell
membranes and, as a result, it causes denaturation of proteins by forming disulfide bridges between polypeptide chains, thus disturbing metabolism. Moreover, the presence of H₂S in biogas significantly decreases the potential use of biogas and its economic value because the H₂S is an acidic and toxic gas, which causes powerful corrosion on pipes, combustors and instruments. Therefore, the H₂S in biogas must be removed before its use, which minimizes the corrosion.⁸⁶-⁸⁸

In order to control the methanogenesis inhibitory effect of sulfides, certain processes are implemented that remove these compounds from the substrate. A possible measure to prevent the toxicity of sulfides is by diluting a stream of wastewater, although an unwanted consequence is the enlarged total volume of the wastewater that undergoes processing. An alternative solution is to remove sulfides during the entire wastewater processing. Technologies include physicochemical solutions (stripping), chemical reactions (coagulation, oxidation, titration), and biological conversions (partial oxidation of sulfur to its elemental form).⁹⁰ A commonly used procedure to remove sulfides is to add iron salt solutions to the wastewater, which results in the precipitation of sulfide from the solution. As reported Ahmad et al., the maximum sulfide elimination efficiency of the Fe⁺²/Fe⁺³ treatment was around 70%. These researchers found the sulfide precipitation method promising for effective sulfidic wastewater treatment in various industries.⁹¹ According to Krayzelova et al., various processes are available to remove high sulfide content from biogas, too. There are physicochemical (which involve high costs and energy) and biological methods. The latter one, more economically advantageous, rely on the oxidation of sulfides to sulfates, thiosulfates, and elemental sulfur. The same authors state that microaeration is one of the available biological methods that has recently gained much attention owing to its simplicity and high efficiency. Microaeration takes place in the anaerobic digester and involves the dosing of little amounts of air into it. In effect, sulfides oxidize to elemental sulfur as a result of the activity of sulfide oxidizing bacteria (SOB), which includes, for example, *Thiobacillus* sp.⁹¹,⁹²

### Table 3

| Substrate                        | Temperature (°C) | pH  | Critical or specified TAN concentration (mg/L) | Critical or specified FAN concentration (mg/L) | Organisms affected/present | References               |
|----------------------------------|-----------------|-----|-----------------------------------------------|-----------------------------------------------|---------------------------|--------------------------|
| Chicken manure                   | 35-73           | —   | —                                             | >250 (100% inhibition)                         | —                         | Bujoczek et al¹⁶²        |
| Soluble nonfat dry milk          | 55              | —   | 4000                                          | 10 000 (100% inhibition)                       | —                         | Sung and Liu⁷⁴           |
| Livestock waste                  | 55              | 7.2-7.3 | 3000-4000                                    | —                                             | *Methanosarcina* sp.     | Angelidaki and Ahring¹⁶³ |
| Sewage sludge                    | 35              | —   | 6000                                          | —                                             | *Methanobacterium* sp.    | Sawayama et al¹⁶⁴        |
| Food waste                       | 37              | —   | 2000                                          | —                                             | *Methanoseta*             | Chen et al⁷⁵             |
| Swine waste                      | 25              | —   | ≥3500                                         | —                                             | *Methanosarcina* sp.      | Angenent et al¹⁶⁵         |
| Synthetic wastewater             | 35              | 8.0  | 6000 (100% inhibition)                        | >700 (100% inhibition)                         | *Methanosarcina* sp.      | Calli et al¹⁶⁶           |
| Sodium acetate                   | —               | —   | 7000 (acclimated)                             | —                                             | *Methanosarciaceae* spp.  | Fotidis et al¹⁶⁷         |
| Cattle excreta + olive mill waste| 37/55           | —   | 1300                                          | —                                             | *Methanosarcina* sp.      | Goberna et al¹⁶⁸          |

Note: Critical concentration—the concentration at which inhibition begins.
Abbreviations: FAN, free ammonia nitrogen; TAN, total ammonia nitrogen.

### 2.3 | Ions of light metals

Toxicity of salts towards microorganisms has been investigated in microbiology for decades. High concentrations of
salts lead to the dehydration of bacterial cells due to a change in osmotic pressure. Although salt cations in a solution must be always bound to anions, it has been found that the toxicity of salts is largely determined by ions with a positive charge. Ions of light metals, including sodium, potassium, calcium, and magnesium, are present in fermentation tanks. As Chen et al reported, they can be released as a result of the decomposition of organic substances in the substrate or added with pH-regulating substances. While moderate concentrations stimulate the growth of microorganisms, excessive amounts of light metal ions will decelerate the multiplication of microbes, cause inhibition of their activity and have a toxic influence, as a result of which they can eventually destabilize cell membranes, disrupt functions of buffers and inhibit the production of biogas. At relatively low salinities (about 100-150 g/L), processes like the transformation of acetate and higher fatty acids, reduction of sulfate, aceticotrophic, and hydrogenotrophic methanogenesis are difficult.

Data from the literature concerning the effect of aluminum on methane fermentation are very scarce. It has been implicated, however, that the inhibitory effect of aluminum may arise from its competition with iron and magnesium, or from the adhesion to the membranes/walls of bacterial cells, which may have an impact on the growth of bacteria. Cabriol et al showed that the activity of aceticogenic and methanogenic microorganisms becomes inhibited when Al(OH)₃ has been added to a fermentation mixture. After the exposure to Al(OH)₃ in a concentration of 1000 mg/L for 59 days, the specific activity of methanogenic and acetic microorganisms decreased by 50% and 72%, respectively.

As Chen et al reviewed, very little is known about the toxicity of calcium ions in an anaerobic system. It has been demonstrated that the optimal concentration of Ca²⁺ needed for methanation of acetic acid is 200 mg/L. Calcium ions produced a moderate inhibitory effect when present in concentrations of 2500-4000 mg/L, although strong inhibition was demonstrated at a concentration of 8000 mg/L. High levels of potassium ions in fermentation tanks are also undesirable. The passive influx of K⁺ ions neutralizes the membrane potential. It has been shown that low potassium concentrations (below 400 mg/L) cause an increase in the methane fermentation productivity, in both thermophilic and mesophilic processes. At higher concentrations of this ion, there is an inhibitory effect, clearly seen in thermophilic processes.

Wastewater with a high sodium concentration is generated in the food-processing industry. At low concentrations, sodium is essential for methanogens, probably due to its role in the generation of adenosine triphosphate or in NADH oxidation. The optimal conditions for the growth of mesophilic methanogens include a concentration of sodium ions up to 350 mg/L. Higher concentrations of sodium are likely to affect the activity of microorganisms and disturb their metabolism, except for halophilic archaea (haloarchaea) which belong to the order Halobacteriales and thrive in environments with salt concentrations nearing saturation. A comparison of the sensitivity of bacteria able to decompose volatile fatty acids showed that sodium was more toxic to acidogetic than to acetoogenic microorganisms. Gradual adaptation of methanogens to high sodium concentrations can improve their tolerance and shorten the lag phase before the onset of methane production.

2.4 Heavy metals

Heavy metals are an important class of compounds with an inhibitory effect towards methanogens. The impact of heavy metals on the activity of cultures of methanogens is well described in literature. The development of several industries, like manufacture of glass and ceramics, metal plating, mining, as well as production of paper, pesticides, and storage batteries, has raised the heavy metals concentration in wastewater. The presence of heavy metals in larger concentrations is detectable in industrial and municipal wastewater as well as in sewage sludge. The most common heavy metals are zinc (Zn), lead (Pb), copper (Cu), mercury (Hg), cadmium (Cd), chromium (Cr), iron (Fe), nickel (Ni), cobalt (Co), and molybdenum (Mo). The main characteristic of heavy metals is that—unlike many other toxic substances—they are not biodegradable and can accumulate in cells. The toxicity of heavy metals is one of the main causes of disruptions and low productivity during methane fermentation processes. An important effect of a disturbance during anaerobic digestion induced by the presence of heavy metals is the reduction in biogas production and the accumulation of intermediate organic compounds. The toxic effect of heavy metals arises from the way they interfere with the functions and structures of bacterial enzymes by binding with thiol and other groups of protein molecules or by replacing the naturally occurring metals in enzymatic prosthetic groups. It is known that heavy metals inhibit the activity of anaerobic microorganisms, including acidogenic, acetoogenic, and methanogenic ones as well as sulfate reducing bacteria. The heavy metal concentrations that cause a 50% decrease (IC₅₀) values in the hydrogen production by acidogenic bacteria were as follows: 3300 mg/L for Cd, 3000 mg/L for Cr, 30-350 mg/L for Cu, 1300 mg/L for Ni, >500-1500 mg/L for Zn, and > 5000 mg/L for Pb. The activity of methanogens was inhibited in 50% by concentrations of: 36 mg/L, 27 mg/L, 8.9-20.7 mg/L, 35 mg/L, and 7.7 mg/L for Cd, Cr, Cu, Ni, and Zn, respectively. An inhibitory effect of heavy metals on methanogenic microorganisms was also confirmed by the study of Sariglu et al, who evaluated the effect of Cu, Ni, Zn, and Pb
during biomanization of wastewaters from a yeast factory. The decline in methane production for heavy metal concentrations over 0.16 mmol/L of Cu, 0.17 mmol/L of Ni, 0.15 mmol/L of Zn, and 0.05 mmol/L of Pb was observed.

Many heavy metals are contained in the structure of essential enzymes, which drive numerous anaerobic reactions. Whether heavy metals stimulate or inhibit anaerobic microorganisms depends on their total concentrations in substrates or on their chemical forms. Toxicity of heavy metals largely depends on ambient parameters, too, e.g., pH, redox potential, and others.

### 2.5 Organic compounds

As reported by Chen et al., a wide range of organic compounds can inhibit anaerobic processes. Organic substances which are weakly dissolved in water or adsorbed on the surface of sediments can accumulate in large concentrations within fermentation tanks. The accumulation of non-polar organic compounds in bacterial membranes makes the membranes demonstrate a disrupted gradient of ions, which may eventually lead to the cell’s lysis. The same authors informed that factors which influence the toxicity of organic compounds include the concentration of a toxic substance, the concentration of biomass, exposure duration, age of a cell, acclimation, and temperature. Same as with other inhibitory substances, the adaptation of microorganisms to the presence of organic substances is an important factor to consider in an evaluation of their inhibitory effect. Mutually related mechanisms have been proposed through which such adaptation can be achieved. These are (a) enrichment of reactors with microorganisms which can degrade toxic compounds, (b) induction of specific degradation enzymes, and (c) genetic engineering. Acclimation of microorganisms participating in methane fermentation enhances their tolerance to the presence of toxic organic substances and biodegradability of these substances.

There is still little knowledge about the exact mechanism of action of most of these organic inhibitors, and the literature on this issue is scarce and requires more credibility, especially in the microbial aspect.

#### 2.5.1 Antibiotics

Every year, thousands of tonnes of antibiotics and products of their metabolism enter wastewater treatment plants, having been excreted by humans and animals, or disposed of if unused. Antibiotics present in waste can induce the inhibition of waste treatment processes, including methane fermentation. Antibiotics can affect microorganisms in different ways. The action of these compounds can rely on the inhibition of DNA replication, RNA transcription, SOS response, or ATP generation. Antibiotics can also impair cell division, protein translation (by inhibition of aminoacyl tRNA binding to ribosome or the setback of elongation and translocation steps), and cell wall synthesis or nucleotide biosynthesis.

Ionophore antibiotics accumulate in the bacteria’s cell membranes and interfere with the ion gradients required to generate a proton motive force and transport nutrients. A study by Sanz et al. revealed how different methanogen populations are inhibited by different antibiotics. The researchers chose several antimicrobial agents: ampicillin, chloramphenicol, erythromycin, hygromycin B, kanamycin, novobiocin, rifampicin, chlorotetacycline, gentamicin, neomycin, penicillin G, spectinomycin, streptomycin, tylosin, and doxycycline. The study showed some regularity: (a) some antibiotics, such as the macrolide erythromycin, are characterized by any inhibitory effect on the process of biogas production, (b) some antimicrobial agents, with different specificities (especially the aminoglycosides), have partial inhibitory effects on biomethanization and decrease methane production by suppressing the activity of bacteria which degrade propionic acid and butyric acid; and (c) the protein synthesis inhibitors, like chlorotetacycline and chloramphenicol, strongly inhibit methane fermentation. The majority of the chosen antibiotics inhibited the activity of acetogenic bacteria. Chloramphenicol and chlorotetacycline are able to cause complete inhibition of the aceticlastic methanogenic archaea. Rusanowska et al. conducted a study to determine to what extent methane fermentation of sewage sludge could be inhibited due to β-lactams, tetracyclines, fluoroquinolones, sulfonamides, and metronidazole contained in this feedstock. According to amounts of generated biogas, no significant differences were determined between the control and the analyzed samples. In another study, Aydin et al. analyzed a long-term effect of mixtures of antibiotics: (a) erythromycin, tetracycline and sulfamethoxazole (ETS), and (b) sulfamethoxazole and tetracycline (ST) on communities of anaerobic microorganisms, and the influence of these antibiotics on processes in bioreactors. It was demonstrated that the activity of acetogens in the presence of either of the antibiotic combinations was higher than that of methanogens. The biogas productivity and the stability of a bioreactor were higher in a bioreactor fed a feedstock with the ETS rather than with the ST set of antibiotics. Mutual interactions and activities of acetogens and methanogens were of key importance to the processes occurring in both bioreactors. Coban et al. too, showed mutual relationships between structures of microbial assemblages and the presence of an antibiotic (oxytetracycline) in fermentation tanks, which had a direct impact on the production of biogas.

Mitchell et al. found no effect of sulfamethazine or ampicillin on the total yield of biogas once the concentration of these antibiotics in the substrate reached 280 and 350 mg/L, respectively. However, an inhibitory effect of ampicillin on biogas production was observed at its earlier stages. On
the other hand, tylosin at concentrations between 130 and 913 mg/L decreased the biogas yield by 10%-38%, whereas the presence of florfenicol in a bioreactor at a concentration of 6.4, 36, and 210 mg/L lowered the output of biogas by 5%, 40%, and 75%, respectively. Reyes-Contreras and Vidas analyzed the effect of the methanogenic toxicity of chlorotetracycline in different concentrations and demonstrated that this antibiotic at a concentration of 10 mg/L inhibited the activity of methanogenic bacteria by 50%. Moreover, values of volatile fatty acids (VFA) achieved at the termination of the experiment showed that the presence of chlorotetracycline in the bioreactor also affected the efficiency of methanogenesis.

2.5.2 Ethylene and acetylene

It has been demonstrated that ethylene, the simplest unsaturated hydrocarbon from the homologous series of alkenes, at its concentration of 0.07% in the gaseous form inhibits by 50% the production of methane by pure cultures of Methanospirillum hungatei, Methanothrix soehngenii and Methanosarcina barkeri. This inhibition is reversible, and the activity of methanogens is completely recuperated after ethylene has been removed from the bioreactor. Acetylene, which is the simplest unsaturated hydrocarbon among alkenes, also shows an inhibitory influence on methanogenesis. Acetylene inhibited methane production even more efficiently: 50% inhibition was noted with 0.015% (Schink, 1985).

2.5.3 Chlorophenols

Chlorophenols comprise monochlorophenols (CPs), dichlorophenols (DCP), trichlorophenols (TCP), tetrachlorophenols (TeCPs), and pentachlorophenol (PCP). Chlorophenols are popular as pesticides, herbicides, antiseptics, and fungicides. They are also used as wood preservatives, or added to glues, paints, plant fabrics, and leather goods. These compounds are toxic to anaerobic microorganisms. Their high hydrophobicity promotes the adhesion of these compounds onto the bacterial membranes, which produces an effect by interfering with the gradient of protons of the cell membranes and the transduction of energy in cells.

Based on the research, it can be stated that PCP is the most toxic chlorophenol, and there is evidence indicating that the toxicity of chlorophenols is associated with hydrophobicity through a linear dependence between the logarithm of the partition coefficient n-octanol/water (log P) and the EC50 values. There are many reports indicating various degrees of inhibition caused by organic compounds which belong to the above group. A concentration of PCP within 0.5-10 mg/L inhibited the activity of acidogenic and methanogenic populations. It was demonstrated in an experiment conducted by Jin and Bhattacharya that TCP were more toxic than DCP and CP. The toxicity induced by DCP and TCP is associated with the degradation of both propionate and acetate and depended on where in the benzene ring chlorine atoms were substituted. The inhibitory activity of chlorophenols seems to be directly connected with the preservation of the division into lipophilic groups. Disturbances of the membrane gradient of protons caused by this group of compounds, as well as transduction of cellular energy, result in certain irregularities in cellular catabolic and anabolic reactions.

2.5.4 Halogen aliphatic hydrocarbons

Most halogen aliphatic hydrocarbons, which are products of halogen reactions with chain hydrocarbons, are potent inhibitors of methanogenesis. Bromine compounds are stronger inhibitors towards methanogens than their chlorinated analogues. It has also been shown that tri- and tetrachloride forms of these compounds are more toxic than dichloride forms. Compared to their saturated counterparts, unsaturated chlorinated aliphatic hydrocarbons are less toxic.

2.5.5 Aliphatic nitro compounds

Aliphatic nitro compounds are reactive toxic substances, which include nitrobenzene, nitrophenol, aminophenol, and aromatic amines. Their reactive toxicity is due to specific chemical interactions with enzymes and disturbances they cause in metabolic pathways.

A greater number of nitro groups do not have any substantial influence resulting in an elevated toxicity of nitrobenzens. On the other hand, the presence of more than one amino group in aminophenoles adds to the inhibitory effect on methane fermentation induced by these compounds. At the same time, an additional amino group in aniline led to a lesser inhibition of the said process.

Anderson et al noted that methane production was markedly reduced by additions of aliphatic nitro compounds during ruminal fermentation, and maximal inhibition was reached at concentrations of 12 mmol/L of nitroethane.

2.5.6 Long chain fatty acids

Methane fermentation of substrates with a high content of the fatty fraction is often inhibited by long-chain fatty acids. These compounds are highly toxic to methane fermentation microorganisms, retarding their growth and making the cell membranes rupture due to absorption.

Inhibition of a methane fermentation process by long chain fatty acids (LCFA) depends on the type of LCFA, population...
of microorganisms, and temperature.\textsuperscript{133} It has been revealed that thermophilic microorganisms involved in methane fermentation are more sensitive to long chain fatty acids than mesophilic microorganisms, most probably because of having a different composition of cell membranes.\textsuperscript{134} Oleic, palmitic, and stearic acids have been described as LCFAs with the most potent inhibitory effect on thermophilic microorganisms.\textsuperscript{135} If the microbial population’s activity is disturbed the most potent inhibitory effect on thermophilic microorganisms is correlated with the number of double bonds in the LCFAs.\textsuperscript{136} The inhibition of LCFAs is positively correlated with the number of double bonds in the LCFAs.\textsuperscript{136,137}

The mechanism of inhibiting methanogenesis by long-chain fatty acids mainly consists in the adsorption of LCFAs to the cell membrane or wall, and affecting the metabolic transport.\textsuperscript{138,139} This decelerates the production of methane. The mechanism can be prevented by providing a competitive, synthetic adsorbent (eg, bentonite).\textsuperscript{140} Due to detergent properties, LCFAs can solubilize the lipid bilayer or membrane proteins, leading to enzyme activity inhibition, electron transport chain disruption or even cell lysis. The LCFAs structure influences its inhibitory effect. LCFAs with longer carbon chains affect microbial activity more than LCFAs with shorter carbon chains. The inhibition of LCFAs is positively correlated with the number of double bonds in the LCFAs.\textsuperscript{136}

Among the factors that can counteract the inhibitory influence of the presence of organic compounds on the process of methane fermentation is the adaptation of the microorganisms engaged in this process. Studies based on the degradation of oleic acid in bioreactors with immobilized substrate showed that acclimation of microorganisms had a positive influence on their resistance to oleate and improved the ability to degrade the substrate. It was also demonstrated that addition of calcium diminishes the inhibitory effect of long chain fatty acids by forming insoluble salts.\textsuperscript{138}

3 | SUMMARY

Methane fermentation is an efficient method of processing waste, as it enables us to reduce the volume of waste and to generate renewable energy such as biogas. Depending on the origin of the waste, its composition can include inhibitory and toxic substances. All the factors described in this paper that inhibit the course of methane fermentation are often mutually connected. Thus, it is extremely important to establish proper parameters in a bioreactor’s fermentation tank so as to ensure the highest possible efficiency of this process. This review paper is based on 168 articles, of which 15.5% had been published prior to the year 2000 (Figure S1). Many subsequent publications on the inhibition of the methane fermentation process are still based on outmoded data. Furthermore, nearly all cited papers deal with the effect of inhibitors on parameters of biogas generation; meanwhile, our knowledge about inhibition of the microbiota engaged at particular steps of methane fermentation is still rather scanty. Ammonia is the only type of a methane fermentation inhibitor for which the literature provides information on the impact on the activity of microorganisms involved in the process, as well as changes in the structure of their population. In the case of the other inhibitory compounds mentioned in this review, these data are very scarce and require verification (ions of light metals, heavy metals, antibiotics, ethylene and acetylene, chlorophenols), or the literature does not provide any information about them (sulfides, halogen aliphatic hydrocarbons, aliphatic nitro compounds, long-chain fatty acids). Therefore, more research is required in order to identify the influence of inhibitory and toxic substances present in waste on the activity of methane fermentation microbiota, which will allow us to ensure the optimal conditions for the growth and development of these microorganisms. Such research should rely on some modern research tools, for example, NGS sequencing.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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