



Review

Tetracycline in anaerobic digestion: Microbial inhibition, removal pathways, and conductive material mitigation

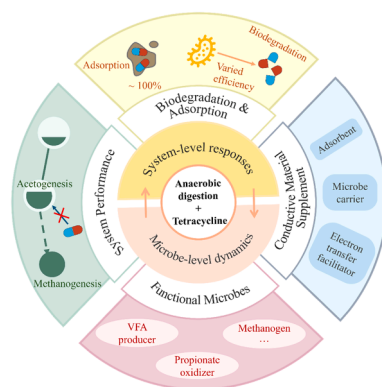
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HIGHLIGHTS

- Tetracycline inhibits methanogenesis mainly by suppressing acetogenesis.
- Removal of tetracycline via adsorption and biodegradation is critically reviewed.
- Conductive materials act as multifunctional enhancers of tetracycline removal.
- Key gaps flagged in microbial trade-offs, metabolite, and conductive material application.

GRAPHICAL ABSTRACT



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ABSTRACT

Tetracycline enters the environment due to its incomplete absorption in humans and animals, posing a significant ecological threat. Tetracycline can hinder the biosystems when treating tetracycline-containing wastewater/waste through anaerobic digestion. This review summarizes the role of tetracycline in inhibiting system performance and related functional microorganisms in holistic process of anaerobic digestion. Tetracycline may primarily inhibit methanogenesis by suppressing acetogenesis, with methane production reductions ranging from 10 % to complete inhibition depending on factors such as tetracycline concentration, inoculum source, substrate composition, and temperature. As a refractory pollutant, tetracycline can be removed in anaerobic digestion systems through adsorption and biodegradation, with removal efficiencies reported between 14.8 % and over 90 %. This review systematically summarizes the mechanisms of tetracycline removal pathways and evaluates the potential contributions. Co-existence of readily biodegradable substrates, extended sludge retention time, and the regulation of environmental parameters such as pH and temperature are potential strategies to enhance tetracycline removal. Moreover, the addition of conductive materials has been identified as a promising strategy to serve as tetracycline adsorbents, facilitate direct interspecies electron transfer and mediate redox reactions, and act as microbial carriers to enhance microbial activity. Finally, this review highlights that the dynamic responses and microbial survival strategies under tetracycline stress deserve further investigation. A deeper

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understanding of these mechanisms will offer clear theoretical guidance for upgrading technologies for tetracycline-containing wastewater/waste treatment.

1. Introduction

Tetracycline is one of the most extensively used antibiotics worldwide in human medicine, livestock farming, and aquaculture [228,39]. For instance, tetracyclines constituted the highest proportion of veterinary antibiotics consumed in Ireland in 2022 at 35.8 % and accounted for 23.5 % within the European Union [48,66]. Tetracycline derived the name from its four hydrocarbon rings and possesses the capability to inhibit the protein synthesis of bacteria, thereby exhibiting comprehensive activity against both Gram-positive and Gram-negative bacteria [34,75]. However, over 50 % of used tetracycline is unmetabolized and excreted in its active form by humans or animals [194]. Thus, tetracycline persists in various environmental compartments, posing potential risks to human health and ecology [92].

Livestock manure, municipal wastewater, and industrial effluents are key sources of tetracycline in the environment [3]. Tetracycline remains as a recalcitrant pollutant in different matrices. Various technologies have been investigated for tetracycline removal, including advanced oxidation processes (AOPs), and aerobic and anaerobic treatment systems. Compared to the high energy consumption and costs of AOPs and aerobic activated sludge systems, anaerobic digestion offers a more cost-effective and sustainable option. Anaerobic digestion not only supports tetracycline removal but also recovers resources through biogas production, reducing fossil fuel dependence and providing significant environmental benefits [17,157].

Recent research has explored the potential of anaerobic digestion for treating tetracycline-containing wastewater. In anaerobic digestion systems, adsorption and biodegradation are the primary removal pathways for tetracycline. Tetracycline readily adsorbs onto sludge due to its high affinity for the solid phase [225,39]. As a result, system retention time is a key factor influencing removal mechanisms. In the short term, adsorption dominates tetracycline removal [53,65]. For example, Cheng et al. [31] observed nearly 100 % tetracycline removal in both anaerobic reactors with non-sterilized and sterilized sludge. However, this high removal efficiency primarily resulted from adsorption, which transfers tetracycline from the liquid phase to the solid phase but without eliminating its environmental risk [39]. When adsorption reaches equilibrium, biodegradation could become the primary pathway. However, reported contributions of biodegradation vary, ranging from 14.8 % to over 90 % [144,21,65]. The underlying mechanisms of tetracycline biodegradation in anaerobic digestion systems and their respective contributions remain unclear.

As an antimicrobial agent, tetracycline can impact anaerobic digestion systems driven by microbial activity. The presence of toxic substances may disrupt metabolic balance by hindering complex substrate degradation, promoting intermediate accumulation, and inhibiting methanogenesis [125]. Tetracycline directly affects the activity of functional microorganisms and exerts selective pressure on microbial community structure [59]. Previous studies have shown that tetracycline exposure at varying concentrations could lead to volatile fatty acids (VFAs) accumulation, particularly propionate, resulting in acidification and methane production inhibition [103,133,65]. Despite these findings, the specific impacts of tetracycline on system performance and microbial community dynamics remain insufficiently characterized. Therefore, current literature still lacks a comprehensive review on tetracycline removal mechanisms, as well as system and microbe responses to tetracycline disturbance during anaerobic digestion.

Anaerobic digestion systems treating tetracycline-containing wastewater/waste require strategies that can simultaneously enhance substrate degradation and tetracycline removal. Conductive materials have been widely applied as additives for anaerobic digestion improvement

by facilitating direct interspecies electron transfer (DIET), serving as redox mediators, and enriching functional microorganisms [42]. Under tetracycline stress, conductive materials have been observed to promote the VFA degradation and methane production [175,222]. Meanwhile, conductive materials may also act as adsorbents to remove tetracycline through adsorption, while simultaneously enhancing its biodegradation [175,32]. However, their specific functions in anaerobic digestion systems under tetracycline stress require systematic investigation.

This review systematically explores the dual role of tetracycline in anaerobic digestion systems, where it acts as an inhibitor that limits system performance and as a recalcitrant pollutant that undergoes adsorption and biodegradation. The main objectives of this review are: (1) to systematically summarize the distinct impacts of tetracycline on each stage of anaerobic digestion; (2) to identify the removal pathways of tetracycline in anaerobic digestion systems, and clarify the underlying mechanisms and influencing factors; and (3) to assess the application of conductive materials as an enhancement strategy to improve system resistance to tetracycline inhibition while facilitating its effective removal. This review provides a useful foundation for optimizing anaerobic digestion in the treatment of tetracycline-containing wastewater/waste.

2. Tetracycline in the environment

Since Watts et al. [177] found 1 $\mu\text{g/L}$ of antibiotics from surface water in England in 1982, tetracycline in various environmental matrices has been increasingly detected in recent years [140,69]. Tetracycline has been found in surface water, groundwater, wastewater treatment plant (WWTP) influents and effluents, sludge, sediments, and livestock manure (Fig. 1). High concentrations of tetracycline are commonly traced in the influent, effluent, and sludge of pharmaceutical WWTPs [3]. Hou et al. [70] reported a tetracycline concentration reaching 2.6 mg/L in the effluent and a maximum amount of 5481.1 mg/kg dry weight in dewatered sludge. Meanwhile, livestock manure serves as a major reservoir of tetracycline. For example, Chen et al. [29] detected tetracycline concentrations as high as 98.2 mg/kg in manure samples collected from swine farms. Detailed tetracycline concentrations detected in environmental matrices are shown in Table S1.

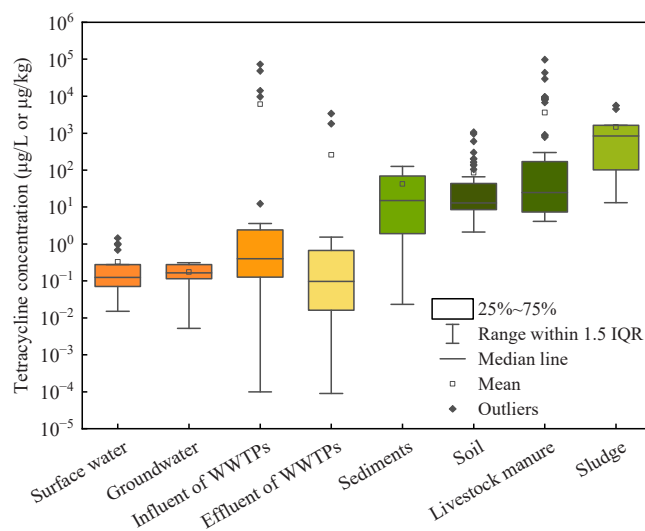


Fig. 1. Detected concentrations of tetracycline in various environmental matrices (liquid: $\mu\text{g/L}$; solid: $\mu\text{g/kg}$).

The pathways through which tetracycline enters the environment are primarily categorized based on their sources: human and veterinary medicine, and industrial sources. Human and veterinary medical sources encompass three main origins: hospitals, farms, and living areas (municipal wastewater) [3]. In agriculture, animal manure serves as a natural fertilizer, facilitating the spread of tetracyclines in soil [130, 216]. Moreover, industrial emissions, particularly from pharmaceutical wastewater, constitute another main component of tetracycline in the environment [3]. Consequently, wastewater discharge, animal manure, and chemical manufacturing plants are the three primary pathways for tetracycline to enter the environment [52] (Fig. 2).

Due to its solubility in water [16,165], tetracycline can diffuse and spread through aqueous media. Additionally, tetracycline can be easily precipitated with cations [141,39]. Unused tetracycline enters municipal wastewater systems through human feces, with sewage treatment plants receiving a large portion of tetracycline excretions from residential populations [216]. Removal efficiencies of antibiotics in wastewater treatment plants can range from 48 % to 77 % [215], and the unremoved fraction continues to enter the environment through wastewater discharge. Tetracycline in the environment could bio-magnify through the food chain, accumulating in higher trophic levels [13]. This promotes the spread of antibiotic resistance genes (ARGs) and negatively impacts ecosystem health [42].

3. Effects of tetracycline on anaerobic digestion processes

Anaerobic digestion is a microbially-driven process composed of sequential steps: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. The balance of these four steps is challenged by the degradation of complex substrates, accumulation of intermediates, and inhibition of methanogenesis. In anaerobic digestion systems, tetracycline could impact microbial communities by disrupting their physiological functions and metabolic activities. Consequently, tetracycline could lead to reduced anaerobic microbial activity, shifted microbial community structure, and declined system performance [103,20]. Therefore, understanding tetracycline's inhibitory effects on both system and cellular levels is essential.

3.1. Mechanism of inhibitory effects on microorganisms

Fig. 3 revealed the inhibitory mechanism of tetracycline in prokaryotic cells. Efflux pumps are a key mechanism from the perspective of

tetracycline resistance, aiming at reducing intracellular tetracycline concentrations and preventing its binding to RNA [143]. Consequently, the cellular uptake and intracellular accumulation of tetracycline require attention. Tetracycline is assumed to cross the cytoplasmic membrane of Gram-positive bacteria in a neutral, lipophilic form, with this process driven by the ΔpH modulated proton motive force [34]. Meanwhile, tetracycline is a strong chelator [141]. Regarding Gram-negative bacteria, tetracycline chelates with metal ions extracellularly, such as Mg^{2+} , forming [M-Tetracycline] complexes [120,124]. These complexes pass through porins into the periplasmic space [120], and abundant hydrogen ions lead to protonation of the negative groups of tetracycline [161,33]. As a result, the [M-Tetracycline] complexes dissociate. The uncharged tetracycline then diffuses across the phospholipid bilayer into the cytoplasm [120]. The higher pH and metal ion concentrations in the cytoplasm compared to the periplasm could facilitate the formation of the [M-Tetracycline] complex as the predominant form of tetracycline [132,26]. These cationic complexes have limited ability to diffuse across the membrane, leading to intracellular accumulation of tetracycline [143]. Thus, pH and metal ion concentrations in the environment are crucial to the inhibitory effects of tetracycline on microorganisms.

The mechanism of the bacteriostatic activity mainly includes hindering protein synthesis and altering cell membrane permeability [158, 30]. Firstly, tetracycline could inhibit bacterial protein synthesis, thereby exerting the antimicrobial effects [143,97]. Ribosomes, composed of proteins and RNAs, are the sites of protein synthesis [180]. In prokaryotes, the 50S large subunit contains 23S and 5S rRNAs, while the 30S small subunit contains 16S rRNA [142,63]. rRNA forms the ribosomal structure, with the 30S subunit decoding mRNA and the 50S subunit catalysing peptide bond formation [85]. It has been reported that there were high-affinity binding sites of tetracycline on the 30S subunit [143,2]. Tetracycline binds to 16S rRNA, inhibiting protein synthesis by hindering the interaction of aminoacyl-tRNA and the mRNA-ribosome complex [34], thus suppressing cellular activity. Additionally, serving structural and functional roles, double-stranded RNA (dsRNA) could be formed through the folding of single-stranded RNA or pairing of sense and antisense RNAs [118,142,35]. dsRNA has been identified as a potential target of interactions between tetracycline antibiotics and RNAs [36]. Furthermore, the binding of tetracyclines to dsRNA may interfere with RNase III processing of rRNAs [36]. Although pre-23S rRNA remains functional, pre-16S rRNA lacks the capacity to participate in protein synthesis [181,35]. Consequently, this disruption

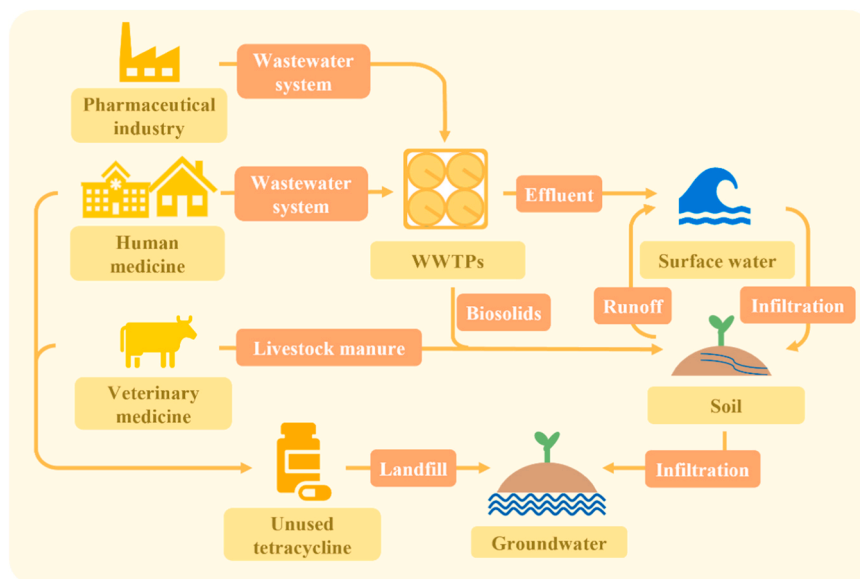


Fig. 2. Main environmental pathways of tetracycline from human, agricultural, and industrial sources.

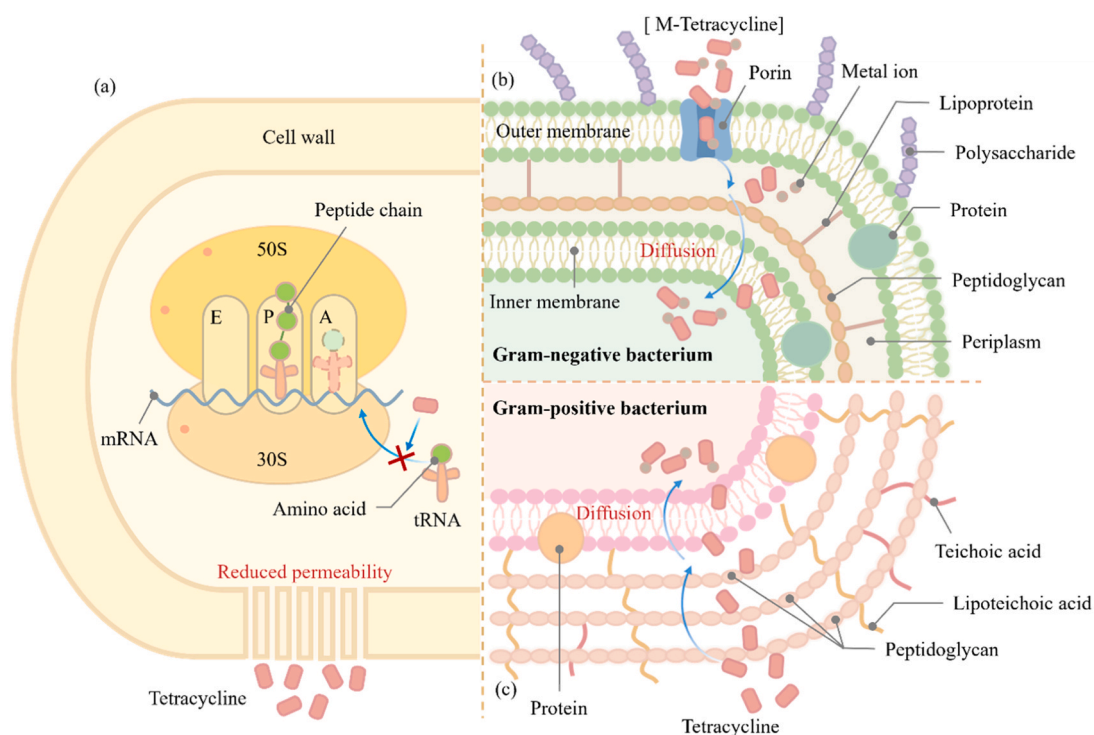


Fig. 3. Tetracycline inhibition and cellular uptake: (a) inhibition mechanism; (b) cellular entry into Gram-negative bacteria; (c) cellular entry into Gram-positive bacteria.

could further inhibit the protein synthesis process.

Tetracycline has been reported to change the cell membrane permeability. This effect was speculated to be an indirect result of protein synthesis inhibition [218]. However, tetracycline has also been found to cause alterations of the cytoplasmic membrane, leading to the leakage of intracellular nucleotides, amino acids, and other substances [128]. Subsequently, Novo et al. [121] cultured *Staphylococcus aureus* and *Micrococcus luteus* with 4 µg/mL tetracycline, observing membrane depolarization after four hours. They noted that this membrane depolarization was time- and concentration-dependent and demonstrated the disruption of membrane permeability using the dye TO-PRO-3 [121]. Furthermore, Wenzel et al. [178] found that 2 µg/mL tetracycline did not cause membrane depolarization within 30 min but observed changes in membrane protein localization, indicating alterations in membrane structure. These changes were subsequently revealed to be independent of ribosomal inhibition [178]. Hence, tetracycline may have a secondary, ribosome-independent antimicrobial activity directly targeting bacterial membranes.

Moreover, there are other indirect inhibitory mechanisms of tetracycline on microorganisms. In the presence of divalent metal ions (e.g., Co^{2+}), tetracycline could cause the generation of reactive oxygen species (ROS) through auto-oxidation [114,89]. In anaerobic digestion systems, oxidative stress in response to antibiotic pressure has been reported, along with an increase in the abundance of genes involved in antioxidant processes, such as alkyl hydrogen peroxide reductase (AhpC/F) [160]. ROS was found to function in cell signalling and homeostasis, and oxidative stress could result in cellular damage under tetracycline stress [148,46]. Besides, increased ROS have been suggested to induce the enrichment and transfer of ARGs [160,47].

3.2. Hydrolysis and acidogenesis (degradation of complex organic carbon)

Table 3 summarizes the reported impacts of tetracycline on anaerobic digestion. Tetracycline has frequently been reported to cause accumulation of intermediate products (e.g., VFAs) [103,193,20,222,

23]. Methanogenesis has been reported to be more sensitive to tetracycline inhibition than hydrolysis and acidogenesis [51]. Nonetheless, negative impacts of tetracycline on the hydrolysis and fermentation of complex substrates have been frequently reported. Moreover, tetracycline addition may inhibit microbial growth [193], potentially reducing substrate metabolic efficiency with low biomass concentrations. Therefore, the research focusing on the first two steps of anaerobic digestion is essential and can help further elucidate the effects of tetracycline on the degradation of complex substrates.

The effects of tetracycline on hydrolysis and acidogenesis depend on its concentration and experimental conditions. Liu et al. [103] found that tetracycline at 4 mg/L and 8 mg/L inhibited both microbial protein hydrolysis and glucose acidogenesis. Meanwhile, this suppression was demonstrated to be independent of tetracycline concentration. Moreover, He et al. [65] reported that 0.03 mg/L tetracycline had no influence on the hydrolysis-acidification process of waste sludge. However, 1.27 mg/L tetracycline reduced the activity of acidogens by 46.4 %, while not affecting protein hydrolysis. Similarly, Wang et al. [169] observed that 25 mg/L tetracycline had a negligible impact on polysaccharide and protein hydrolysis but reduced acidogenesis efficiency by 13.7 %. These findings contradict Liu et al. [103], suggesting that the impact of tetracycline on hydrolysis-acidification may have a threshold concentration, above which the effect becomes dose-independent. Besides, acidogens may be more sensitive to tetracycline than hydrolytic microorganisms. It is worth noting that the influence of tetracycline on hydrolysis and acidification may not be entirely negative. For instance, Shang et al. [144] observed that 50 mg/L of tetracycline enhanced the hydrolysis of bovine serum albumin and glucose acidification, whereas 400 mg/L restricted these processes. These different effects might be due to variations in tetracycline dosage and functional microbial communities, highlighting the necessity of examining the influence of tetracycline on anaerobic digestion systems at the microbial community level.

Functional microorganisms affected by tetracycline are summarized in Table 1. *Firmicutes* are recognized as a hydrolytic-acidogenic phylum capable of effectively withstanding tetracycline stress. Generally, when tetracycline is present at microgram or milligram per litre levels, the

Table 1
Summary of microbial abundance shifts in anaerobic digestion under tetracycline stress.

Tetracycline concentration (mg/L)	Additional operational condition ^a	Microorganism	Taxonomy	Function	Abundance	Condition of the dynamic ^b	Reference	
0.001, 0.15, 20	With 20 mg/L tetracycline, operational period: before day 21 and after day 21	<i>Bacteroidetes</i>	phylum	hydrolysis, acidogenesis	+	0.15 and 20 mg/L tetracycline	[193]	
		<i>Chloroflexi</i>	phylum	hydrolysis, acidogenesis	-	20 mg/L tetracycline		
		<i>Cloacimonetes</i>	phylum	hydrolysis, acidogenesis	-	20 mg/L tetracycline		
		<i>Clostridium aurantibutyricum</i>	species	acidogenesis	+	0.15 and 20 mg/L tetracycline		
		<i>Desulfomicrobium baculatum</i>	species	acetogenesis	+	0.15 mg/L tetracycline		
						-		20 mg/L tetracycline
		<i>Firmicutes</i>	phylum	hydrolysis, acidogenesis	-	0.15 mg/L tetracycline		
		<i>Geobacter argillaceus</i>	species	acetogenesis	-	0.15 and 20 mg/L tetracycline		
		<i>Ignavibacteriae</i>	phylum	hydrolysis, acidogenesis	-	20 mg/L tetracycline		
		<i>Ignavibacterium album</i>	species	acidogenesis	-	20 mg/L tetracycline		
		<i>Macellibacteroides fermentans</i>	species	acidogenesis	+	0.001 and 0.15 mg/L tetracycline		
						-		20 mg/L tetracycline
		<i>Marinithermofilum abyssi</i>	species	acidogenesis	-	0.15 and 20 mg/L tetracycline		
		<i>Methanobacterium</i>	genus	methanogenesis	not influenced	all conditions		
		<i>Methanobrevibacter</i>	genus	methanogenesis	not influenced	all conditions		
		<i>Methanoculleus</i>	genus	methanogenesis	-	20 mg/L tetracycline, before day 21		
						+		20 mg/L tetracycline, after day 21
		<i>Methanomassiliicoccus</i>	genus	methanogenesis	+	20 mg/L tetracycline		
		<i>Methanosarcina</i>	genus	methanogenesis	-	20 mg/L tetracycline, before day 21		
						+		20 mg/L tetracycline, after day 21
		<i>Methanotherix</i>	genus	methanogenesis	not influenced	all conditions		
		<i>Microbacter margulisiae</i>	species	acidogenesis	+	0.15 and 20 mg/L tetracycline		
		<i>Petrimonas sulfuriphila</i>	species	acidogenesis	-	0.15 and 20 mg/L tetracycline		
		<i>Porphyromonas pogonae</i>	species	acidogenesis	+	20 mg/L tetracycline		
		<i>Proteiniphilum acetatigenes</i>	species	acidogenesis	-	20 mg/L tetracycline, before day 21		
						+		20 mg/L tetracycline, after day 21
		<i>Proteobacteria</i>	phylum	hydrolysis, acidogenesis	-	20 mg/L tetracycline		
		<i>Spirochaetes</i>	phylum	hydrolysis, acidogenesis	+	20 mg/L tetracycline		
		<i>Syntrophobacter wolinii</i>	species	acetogenesis	-	20 mg/L tetracycline		
		<i>Syntrophomonas</i>	genus	acetogenesis	+	20 mg/L tetracycline		
<i>Syntrophorhabdus</i>	genus	acetogenesis	-	20 mg/L tetracycline				
<i>Treponema zuelzeriae</i>	species	acidogenesis	+	20 mg/L tetracycline				

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Table 1 (continued)

Tetracycline concentration (mg/L)	Additional operational condition ^a	Microorganism	Taxonomy	Function	Abundance	Condition of the dynamic ^b	Reference
250	After stabilized operating for 51 d, 1200 mg/L GAC and 1000 mg/L nZVI were added in addition to tetracycline. ^c	unclassified	genus	acetogenesis	-	20 mg/L tetracycline all conditions GAC and nZVI	[222]
		<i>Syntrophobacteraceae</i>	genus	acetogenesis	-		
		<i>Vallitalea guaymasensis</i>	species	acidogenesis	-		
		<i>Bacteroidetes</i>	phylum	hydrolysis, acidogenesis	-		
		<i>Chloroflexi</i>	phylum	hydrolysis, acidogenesis	+		
		<i>Firmicutes</i>	phylum	hydrolysis, acidogenesis	-		
		<i>Levilinea</i>	genus	acidogenesis, acetogenesis	+		
		<i>Methanobacterium</i>	genus	methanogenesis	-		
		<i>Methanoculleus</i>	genus	methanogenesis	-		
		<i>Methanosarcina</i>	genus	methanogenesis	-		
		<i>Methanotherix</i>	genus	methanogenesis	+		
		<i>Proteobacteria</i>	phylum	hydrolysis, acidogenesis	-		
		<i>Spirochaetes</i>	phylum	hydrolysis, acidogenesis	not influenced		
		<i>Syntrophomonas</i>	genus	acetogenesis	+		
<i>Syntropus</i>	genus	acetogenesis	+				
<i>Thermotogae</i>	phylum	hydrolysis, acidogenesis	+				
10, 30, 50, 80, 100, 150	0.5 g/g VS nZVI was added or not in addition to tetracycline	<i>Anaerolineaceae</i>	family	hydrolysis, acidogenesis, acetogenesis	+	tetracycline	[126]
		<i>Atribacteria</i>	class	acidogenesis	+	tetracycline nZVI	
		<i>Chloroflexi</i>	phylum	hydrolysis, acidogenesis	+	tetracycline	
		<i>Methanobacterium</i>	genus	methanogenesis	-	all conditions	
		<i>Methanotherix</i>	genus	methanogenesis	+	all conditions	
		<i>Syntrophaceae</i>	family	acetogenesis	+	all conditions	
		<i>Syntrophobacterales</i>	order	acetogenesis	+	all conditions	
		<i>Syntrophohabditus</i>	genus	acetogenesis	+	nZVI	
		<i>Christensenellaceae_R_7_group</i>	genus	hydrolysis, acidogenesis	-	N/A ^e	[65]
		<i>Clostridium_sensu_stricto_1</i>	genus	hydrolysis, acidogenesis	+		
<i>Clostridium_sensu_stricto_13</i>	genus	hydrolysis, acidogenesis	+				
<i>norank_f_Caldilineaceae, Rhodobacter</i>	genus	hydrolysis	not influenced				
<i>Proteinclasticum</i>	genus	hydrolysis, acidogenesis	-				
0.5, 50	15 g/L biochar was added or not in addition to tetracycline	<i>Anaerolineaceae</i>	family	hydrolysis, acidogenesis	+	biochar	[169]
		<i>Bacteroidetes</i>	phylum	hydrolysis, acidogenesis	-	all conditions	
		<i>Chloroflexi</i>	phylum	hydrolysis, acidogenesis	+	biochar	
		<i>Firmicutes</i>	phylum	hydrolysis, acidogenesis	+	tetracycline	
		<i>Methanosarcina</i>	genus	methanogenesis	-	biochar not influenced	
		<i>Mathanospirillum</i>	genus	methanogenesis	not influenced	all conditions	
		<i>Methanotherix</i>	genus	methanogenesis	not influenced	all conditions	
		<i>Prolixibacteraceae</i>	class	hydrolysis, acidogenesis	-	all conditions	
		<i>Synergistaceae</i>	family	hydrolysis, acidogenesis	+	biochar	
		<i>Candidatus Methanofastidiosum</i>	genus	methanogenesis	-	all conditions	[176]
0.5	Two operational mode was applied. ^f	<i>Chloroflexi</i>	phylum	hydrolysis, acidogenesis	-	all conditions	
		<i>Firmicutes</i>	phylum	hydrolysis, acidogenesis	+	all conditions	
		<i>Mesotoga</i>	genus	hydrolysis, acidogenesis, acetogenesis	-	all conditions	
		<i>Methanobacterium</i>	genus	methanogenesis	-	CFRs	

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Table 1 (continued)

Tetracycline concentration (mg/L)	Additional operational condition ^d	Microorganism	Taxonomy	Function	Abundance	Condition of the dynamic ^e	Reference
0.5	Two operational mode was applied. ^f 1 g/L PAC was added or not in addition to tetracycline addition. ^c	<i>Methanosarcina</i>	genus	methanogenesis	+	SBRs	[175]
		<i>Methanothrix</i>	genus	methanogenesis	+	SBRs	
		<i>Propionimicrobium</i>	genus	acidogenesis	+	CFRs	
		<i>Syntrophobacter</i>	genus	acetogenesis	-	SBRs	
		<i>Trichococcus</i>	genus	acidogenesis, acetogenesis	+	all conditions	
		<i>Desulfomicrobium</i>	genus	acetogenesis	-	all conditions	
		<i>Enterococcus</i>	genus	acidogenesis, acetogenesis	+	CFRs, PAC	
		<i>Geobacter</i>	genus	acetogenesis	-	SBRs, PAC	
		<i>Methanospirillum</i>	genus	methanogenesis	+	all conditions	
		<i>Methanothrix</i>	genus	methanogenesis	+	all conditions	
50, 400	Operational period: 3 day and 45 days	unclassified <i>Woesearchaeia</i>	class	methanogenesis	-	all conditions	[144]
		<i>Clostridium_sensu_stricto_1</i>	genus	hydrolysis, acidogenesis	+	50 and 400 mg/L tetracycline, 3 days	
		<i>Enterococcus</i>	genus	acidogenesis, acetogenesis	+	50 mg/L tetracycline, 3 days	
		<i>Firmicutes</i>	phylum	hydrolysis, acidogenesis	+	all conditions	
		<i>Lentilactobacillus</i>	genus	acidogenesis	+	400 mg/L tetracycline, 3 days, 45 days	
		<i>Limosilactobacillus</i>	genus	hydrolysis, acidogenesis	-	50 mg/L tetracycline, 3 days	
		<i>Proteobacteria</i>	phylum	hydrolysis, acidogenesis	+	50 mg/L tetracycline, 45 days	
			-	all conditions			

Abbreviations: CFR, continuous-flow reactor; GAC, granular activated carbon; nZVI, nano zero-valent iron; PAC, powdered activated carbon; SBR, sequencing batch reactor; VS, volatile solids.

^a Unless otherwise noted, tetracycline addition was compared to tetracycline-free controls.

^b Abundance shifts under conductive material conditions were determined by comparing treatments with and without conductive materials; both conditions included tetracycline.

^c Tetracycline addition was not included as a comparative condition.

^d Expressed as mg per kg of total suspended solids (mg/kg TSS).

^e Only tetracycline addition was compared to the control group.

^f Two operational modes: continuous-flow reactor and sequencing batch reactor.

phyla *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Chloroflexi*, and *Spirochaetes* dominate in anaerobic digestion systems with complex organic substrates [144,169,193,196,222]. These microbes are responsible for the hydrolysis and acidification of organic substrates, producing VFAs [117,222]. *Firmicutes* can utilize complex organic substrates such as proteins and carbohydrates [159]. Xiong et al. [193] found that the abundance of this phylum was inhibited by 0.15 mg/L tetracycline, even after long-term acclimation. However, at 20 mg/L, the abundance was comparable to that of the tetracycline-free control group. Similarly, Wang et al. [169] observed the enrichment of *Firmicutes* at 50 mg/L tetracycline, with the relative abundance increasing from 9.9 % to 48.9 %. This could be attributed to the strong resistance to antibiotic stress of *Firmicutes* [106,217]. Besides, *Bacteroidetes* and *Proteobacteria* have also been reported as potential hosts of tetracycline resistance genes [106,217].

At the genus level, *Rhodobacter* is a hydrolytic bacterium and has been identified as a dominant genus in anaerobic fermentation of activated sludge [65]. Nevertheless, the relative abundance of *Rhodobacter* was not affected by tetracycline addition or concentrations [65]. Due to the prevalence of tetracycline-resistant genera among hydrolytic and

acidogenic bacteria, these species have been found to be enriched. Specifically, when tetracycline serves as the sole carbon source, the hydrolytic-acidification genus *Bacillus* warrants particular attention due to its reported high resistance to tetracycline stress [148,174]. Likewise, *Klebsiella*, another participant in the hydrolysis and acidification process, could be enriched and has been shown to potentially degrade tetracycline [206,225].

As key members of acidogenesis-related genera, VFA producers may become enriched under tetracycline exposure. For instance, Xiong et al. [193] observed an increase in the relative abundance of the butyrate-producing bacterium *Clostridium_aurantibutyricum* (belonging to the *Clostridium* genus) under tetracycline stress. Additionally, the relative abundances of two propionate-producing bacteria, *Porphyromonas_pogonae* and *Proteiniphilum_acetatigenes*, increased from 8.64 % to 14.21 %, and from 0.16 % to 0.41 %, respectively. Meanwhile, during anaerobic fermentation of waste activated sludge, *Clostridium_sensu_stricto_1* and *Clostridium_sensu_stricto_13* were enriched under 60 mg/kg total suspended solids (TSS) tetracycline pressure, despite declines in *Proteiniclasticum* and *Christensenellaceae_R_7_group* at the genus level [65]. The enrichment of *Clostridium_sensu_stricto_1* with 50 and

400 mg/L tetracycline was also demonstrated by Shang et al. [144]. The proliferation of these microbes under tetracycline stress can lead to VFA accumulation, potentially destabilizing the system.

3.3. Acetogenesis

VFAs are key intermediates in anaerobic digestion, and their concentration is closely linked to system performance [193]. The accumulation of VFAs is an important indicator of tetracycline inhibition of system performance [51].

VFAs accumulated under tetracycline stress are mainly acetate, butyrate and propionate [103,222,65]. Zhang et al. [222] observed that adding 250 mg/L tetracycline resulted in 280 mg/L butyrate remaining from an influent chemical oxygen demand (COD) of 6000 mg/L. Meanwhile, Wang and Wu [176] reported that under 5 mg/L tetracycline, propionate accumulation ranged from 342 to 502 mg COD/L, originating from an influent COD concentration of 2000 mg/L. Remarkably, VFA accumulation in the system could be positively correlated with the tetracycline dosage [30]. For example, Cetecioglu et al. [20] established a tetracycline concentration gradient ranging from 1.65 to 8.5 mg/L, and reported that propionate began to accumulate only at 8.5 mg/L, reaching 750 mg/L with a substrate COD concentration of 4400 mg/L. Meanwhile, with an influent COD concentration of 2500 mg/L, Liu et al. [103] observed that 4 mg/L tetracycline led to a propionate concentration of 154.7 mg/L in the effluent after long-term operation. When the tetracycline dose increased to 8 mg/L, propionate further rose to 603.0 mg/L.

Correspondingly, the metabolic effects of tetracycline on systems utilizing propionate or butyrate as the carbon source were studied. Regarding butyrate utilization, Shang et al. [144] reported that 50 mg/L and 100 mg/L tetracycline had no negative effect on butyrate degradation to acetate, based on short-term experiments. In another similar study on acute impacts, 25 mg/L tetracycline did not hinder butyrate utilization, while propionate removal efficiency decreased by 39.5 % [169]. Likewise, Cetecioglu and Orhon [23] found that 25–1000 mg/L tetracycline restricted the consumption of VFAs, with the total residual concentration increasing alongside tetracycline levels. In contrast, Zhang et al. [212] stated that tetracycline above 50 mg/L could cause propionate and butyrate accumulation in the short term, but the accumulation of butyrate did not correlate positively with tetracycline concentration. Compared to butyrate, propionate degradation is more susceptible to tetracycline inhibition. Propionate degradation is relatively challenging in anaerobic digestion systems due to its high Gibbs free energy [112]. With propionate as the substrate, tetracycline at 4 mg/L and 8 mg/L respectively reduced the 48-hour propionate degradation percentage from 78.21 % to 52.53 % and 41.39 % [103]. Consequently, the immediate effect of tetracycline on VFA utilization may not necessarily depend on the dosage. Since tetracycline is easily adsorbed onto solid phases in the system during short-term exposure [141], evaluating the effect of tetracycline on acetogenesis requires consideration of both operation duration and substrate composition.

Regarding the microbial community structure, in addition to the previously mentioned VFA producers, the enrichment of key acetogenesis participants is also affected by tetracycline stress. *Syntrophorhabdus* is a syntrophic propionate and butyrate oxidizer [73,96]. This genus showed a decline in the relative abundance in a reactor containing 20 mg/L tetracycline, as observed by Xiong et al. [193]. Meanwhile, another VFA-degrader, *Syntrophomonas*, exhibited reduced abundances at low tetracycline concentrations (0.001 and 0.15 mg/L) but was enriched at higher levels (20 mg/L) despite propionate accumulation. Similarly, *Syntrophobacter* was also reported by Wang and Wu [176] to exhibit suppressed enrichment in tetracycline-added reactors as a known participant in propionate oxidation. However, Pan et al. [126] reported that the enrichment of multiple VFA-utilizers, including *Anaerolineaceae* (family), *Syntrophorhabdus*, *Syntrophobacter*, and *Syntrophaceae* (family), was promoted by 10–150 mg/L tetracycline [131,

184]. This may be attributed to the use of granular sludge as inoculum, suggesting that anaerobic granular sludge may have a distinct microbial community structure and show great tolerance to tetracycline stress [184].

Changes in microbial abundance do not necessarily reflect changes in metabolic activity under antibiotic pressure [44]. Liu et al. [103] measured the activity of key metabolic enzymes and found that both 4 mg/L and 8 mg/L tetracycline could reduce dehydrogenase activity in the system. Furthermore, there is syntrophic relationship between acetogens and methanogens, and the hydrogen partial pressure in the system affects the consumption of propionate and butyrate as intermediates [166]. Hence, further investigation into methanogenic responses to tetracycline is needed.

3.4. Methanogenesis

Methanogenesis is the final stage of anaerobic digestion, producing biogas mainly composed of methane and carbon dioxide [68]. In terms of system performance, methane production tends to be more strongly affected by the presence of tetracycline than VFA generation [105]. The inhibition of tetracycline on methane production, both acetoclastic and hydrogenotrophic methanogenesis, has been widely reported, with its negative effect shown to be dose dependent.

Table 2 summarizes reported inhibition concentrations (IC) of tetracycline. IC10, IC20, and IC50 represent tetracycline concentrations required to achieve 10 %, 20 %, and 50 % inhibition, respectively [138, 139]. Delgado-Mirquez et al. [41] found in batch tests using swine wastewater as a substrate that 45.28 mg/L tetracycline resulted in 50 % inhibition of specific methanogenic activity (SMA). When acetate was used as the substrate, although tetracycline showed no effect on acetate consumption, IC50 for SMA was determined to be 37.00 mg/L [41]. This indicated inhibitory effects of tetracycline on acetogenic methanogenesis. However, in an earlier study, Cetecioglu et al. [22] reported an IC50 of 204.4 mg/L for tetracycline when acetate was used as the substrate. Meanwhile, Cetecioglu and Orhon [23] demonstrated that in systems with mixed VFAs (butyrate, propionate, and acetate) as substrates, methane production at a tetracycline concentration of 50 mg/L was only 59 % of that in the control without tetracycline. This could be attributed to the combined inhibition of acetogenic syntrophs and methanogens by tetracycline.

Nevertheless, the IC50 values for tetracycline in Table 2 were based on acute effects. Unlike short-term batch tests, long-term acclimation allows functional microorganisms more time to respond to tetracycline stress, potentially leading to different outcomes. Liu et al. [103] found that 4 mg/L and 8 mg/L tetracycline reduced acetogenic methanogenesis by 61.25 % and 97.58 %, respectively. Using glucose and peptone as mixed carbon sources, Wang and Wu [176] reported that the addition of 5 mg/L tetracycline reduced the maximum methane production rate by 24.5 % in continuous-flow reactors and by 48.8 % in sequencing batch reactors. Meanwhile, Lu et al. [105] observed inhibition of methane production in a reactor continuously fed with 0.25 mg/L tetracycline for 72 days with glucose as the carbon source. The inhibitory effect increased over time, potentially due to tetracycline accumulation in the system. Cetecioglu et al. [20] studied long-term operation of an anaerobic sequencing batch reactor fed with mixed VFAs, glucose, and starch, with tetracycline added at concentrations ranging from 0 to 8.5 mg/L. At 1.65 and 5.7 mg/L, methane production dropped to around 60 %, with no changes observed over time. However, methane production began to steadily decline until reactor failure when tetracycline dosage increased to 8.5 mg/L. In contrast to acute inhibition, although methane production decreased, methane yield per COD removed remained at 0.2 L CH₄/g COD removed during long-term operation [20]. This indicates that tetracycline has limited direct effects on methanogens, and that the inhibition of methane production mainly results from disruption of upstream acetogenesis. Meanwhile, the reduction of methane production by tetracycline is positively

Table 2
Reported inhibitory concentrations (IC10/20/50) of tetracycline in anaerobic digestion.

Inoculum concentration	Carbon source	Carbon source concentration	Parameter	Indicator	Amount (mg/L)	Reference
1 g ds/L	Yeast extract	2.5 g/L	biogas production	IC10	2.1	[55]
				IC20	5.6	
				IC50	37.3	
2 g/L VSS	Acetate	4 g/L	methane production	IC50	204.4	[22]
				N/A ^a	Dog food	
4.5 g VS	microcrystalline cellulose	1 g VS/4.5 g VS inoculum	biogas production	IC10	19	[155]
				IC50	219	
2 g/L VSS	NaAcetate, NaButyrate, NaPropionate	3 g/L of each VFA	methane production	IC50	58.6	[23]
5.4 g VSS/L	Acetic acid	2.2 g/L of acetic acid carbon	methane production	IC50	62.85 ± 8.4	[41]
			SMA	IC50	37.00 ± 4.0	
	Swine wastewater	2.2 g/L of swine wastewater carbon	methane production	IC50	82.76 ± 9.4	
			maximum methane production rate	IC50	45.68 ± 4.4	

Abbreviations: COD, chemical oxygen demand; IC10/20/50, inhibitory concentration resulting in 10 %, 20 %, or 50 % inhibition; SMA, specific methanogenic activity; VFA, volatile fatty acid; VS, volatile solids; VSS, volatile suspended solids.

^a Inoculum concentration was not reported.

^b Expressed as mg/kg.

correlated with its concentration. Additionally, compared to short-term addition, methane production in the above studies was more sensitive to low concentrations of tetracycline during long-term exposure. This may be due to the accumulation of tetracycline or its toxic metabolites, prolonged acidification, or a lack of functional redundancy of methanogens.

ARGs are less prevalent in archaea compared to bacteria, and archaea do not produce ARGs under tetracycline stress. Nevertheless, tetracycline is unlikely to directly affect archaea due to the diversity of their aminoacyl-tRNA synthetases [197,221]. Thus, archaea are generally considered to have better resistance than bacteria when exposed to antibiotics [30]. However, some studies have found that archaea are more sensitive to tetracycline pressure compared to bacteria [6]. Tetracycline at concentrations as low as 0.15 mg/L could affect the community composition of methanogens [193]. *Methanothrix*, a typical acetoclastic methanogen, has been reported as the dominant methanogen under stress conditions [168]. For example, Pan et al. [126] reported that tetracycline concentrations ranging from 10 to 150 mg/L led to an increase in the relative abundance of *Methanothrix*, while the hydrogenotrophic methanogen *Methanobacterium* showed a decline in relative abundance.

Similarly, Wang et al. [169] found that under 0.5 mg/L tetracycline, *Methanothrix* was the dominant methanogen, with its relative abundance further increasing at 50 mg/L. *Methanosarcina*, which is both hydrogenotrophic and acetoclastic, showed an increase in relative abundance at 0.5 mg/L, but its abundance was lower in reactors with 50 mg/L compared to 0.5 mg/L tetracycline. Besides, despite the fact that *Methanobacterium* was also enriched under 0.5 and 50 mg/L tetracycline, its relative abundance remained much lower than that of *Methanothrix*. Generally, acetoclastic methanogenesis accounts for about 75 % of methanogenesis in the system, with the remainder being hydrogenotrophic methanogenesis [41]. The presence of tetracycline in the systems at the milligram per litre level may not influence the metabolic pathway of methanogenesis. Additionally, although *Methanosarcina* has been reported to grow and remain active in acidic systems [43], *Methanothrix* has higher substrate affinity with a slower growth rate compared to *Methanosarcina* [37]. These findings suggest that dynamics in methanogens are largely driven by upstream metabolic shifts, particularly the suppression of acetogenesis. Therefore, tetracycline appears to inhibit methanogenesis mainly through indirect pathways, such as disrupting acetogenesis and causing system acidification, rather than through direct action on methanogens.

3.5. Influencing factors

The impact of tetracycline on anaerobic digestion system performance could be affected by various factors, including inoculum type, substrate, temperature, and co-existing contaminants. Regarding inoculum type, different types of sludge could lead to varied system performance. For instance, granular sludge is generally considered more resistant to antibiotics than flocculent sludge [184]. Du et al. [45] conducted methanogenic activity tests using intact granular sludge with acetate as the substrate. They found that SMA was inhibited by only 15.7 % with 400 mg/L tetracycline. In contrast, when the inoculum was crushed granular sludge, the inhibition increased to 25.5 %. This difference could be attributed to the high microbial diversity, dense structure providing a protected internal micro-environment, and close syntrophic associations within intact granules [151]. Moreover, crushed granules or flocculent sludge may have higher exposure of microorganisms to toxic compounds such as tetracycline [45].

The substrate composition can influence the inhibitory effects of tetracycline on anaerobic digestion. Previous studies have demonstrated that the inhibition of methane production by antibiotics is generally stronger in systems using substrates not previously exposed to antibiotics (food waste), compared to those using substrates inherently containing antibiotics (livestock manure and waste activated sludge) [213]. Under identical antibiotic concentrations, anaerobic digestion systems exposed to external antibiotic addition showed greater inhibition of methane production than systems with slurry containing the same antibiotic levels [72]. This difference may be attributed to the microbial adaptation developed in slurry-based systems [138]. Furthermore, the presence of heavy metal ions may also affect tetracycline toxicity. Tetracycline is a strong metal chelator, capable of forming complexes with metal ions such as iron ion [122]. While iron-tetracycline complexes are considered not to affect the antibiotic activity of tetracycline [60], Tong et al. [163] demonstrated that in the presence of copper (II), the formation of copper-tetracycline complex reduced the toxicity of both copper and tetracycline on the luminescent bacterium, *Vibrio fischeri*. Although limited studies have investigated the combined effects of heavy metals and tetracycline in anaerobic digestion systems, the coexistence of heavy metals in the substrate may potentially influence tetracycline toxicity. Moreover, in systems containing readily degradable compounds such as glucose, enhanced tetracycline biodegradation may alleviate its inhibitory pressure in anaerobic digestion processes (see Section 4.2).

In addition, even within the same system, degradation of different substrate components depends on distinct microbial metabolic

pathways during anaerobic digestion, resulting in varying sensitivities to tetracycline. For example, Cetecioglu and Orhon [23] investigated the effects of tetracycline on the degradation of mixed VFAs (butyrate, propionate, and acetate). At a tetracycline concentration of 25 mg/L, all three VFAs showed inhibited degradation, with residual propionate reaching 32.7 %, while butyrate and acetate exhibited lower residual ratios of 2.9 % and 7.9 %, respectively. When the tetracycline concentration increased to 1000 mg/L, butyrate not only exhibited inhibited degradation but also underwent isomerization. The authors concluded that the substrate nature and composition are direct factors influencing the system performance under tetracycline stress [23].

Since tetracycline is not a competitive inhibitor [41], substrate concentration changes may have little impact on its inhibitory effect. However, low organic loading rates (OLR, $OLR \leq 4.0$ g volatile solids/L/d) were found to cause the accumulation of antibiotics in anaerobic digestion systems, including tetracycline, and promote horizontal gene transfer of ARGs [95]. Thus, system performance could be indirectly affected.

Temperature may indirectly affect the impact of tetracycline by influencing the activity of functional microorganisms and the rate of biological reactions, such as hydrolysis [56,98]. Wang et al. [172] found that thermophilic anaerobic digestion achieved lower tetracycline resistance gene abundances compared to the mesophilic condition. Similar results were observed by Zou et al. [235]. They reported that high temperature (55 °C) could reduce the abundances of resistant microbes. Zhang et al. [214] demonstrated that thermophilic anaerobic digestion exhibited reduced resistance to high antibiotic concentrations compared to mesophilic conditions. Despite the opposing conclusion reported by Tian et al. [162], the effect of temperature on anaerobic digestion under tetracycline pressure warrants further investigation.

Overall, system performance under tetracycline stress is the result of the varied responses of different microbial communities. These responses are influenced by metabolic substrates, tetracycline dosage, multiple operational conditions, and the ecological niches of the microorganisms. Even in cases where tetracycline leads to long-term inhibition or system collapse, recovery of system performance is possible after tetracycline removal. This may be attributed to the tetracycline dosage and the dosing strategy applied. For example, Cetecioglu et al. [20] gradually increased tetracycline concentrations over 143 days in a reactor fed with complex mixed organic substrates. They found that the system experienced a complete loss of COD utilization capacity at 8.5 mg/L tetracycline dosage and continued to deteriorate for 10 days following the cessation of tetracycline addition. However, subsequent SMA tests with VFAs as substrates indicated that methanogenic activity could recover once tetracycline pressure was removed. Although tetracycline is a bacteriostatic agent, elevated concentrations can still result in the death of microorganisms [174]. Therefore, there might be a threshold concentration beyond which tetracycline accumulation causes irreversible effects. Thus, mitigating tetracycline toxicity and identifying the threshold concentration at which system performance remains recoverable warrant further investigation.

3.6. Combined antibiotic effects

The coexistence of multiple antibiotics is common in the environment [30], which can introduce variability in the effects of tetracycline. The presence of mixed antibiotics may change the impact of tetracycline through either synergistic or antagonistic effects.

On the one hand, synergy occurs when combined antibiotics cause greater inhibition than individual tetracycline. Tetracyclines have been reported to exhibit the highest interaction magnitude among all classes of antibiotics [110]. When co-administered with quinolones or penicillins, the toxicity could be increased by 4.8–7.6 folds [110]. Synergy often happens between antibiotics targeting the same cellular process [15]. Although most ribosome-targeting antibiotics are thought to have no strong physical interactions, synergy between tetracyclines

(doxycycline) and macrolides (erythromycin) has been reported [137, 24]. This is attributed to independent binding to the ribosome [137]. Similar effects have been observed in anaerobic digestion systems. For example, Delgadillo-Mirquez et al. [41] observed in short-term tests using swine manure that 45 mg/L tetracycline inhibited the maximum methane yield by 28 %, whereas a mixture of tetracycline, oxytetracycline, and tylosin at the same concentration doubled the inhibition.

Synergy may also occur between antibiotics that target different processes. For instance, antibiotics affecting the cell wall can improve the uptake of others, which can lead to synergy with 30S-targeting antibiotics [137,204]. Although tetracycline mainly enters cells via active transport, synergy between β -lactams and tetracycline has also been reported [15]. Aydin et al. [5] studied the effects of combinations such as tetracycline-erythromycin-sulfamethoxazole, erythromycin-tetracycline, sulfamethoxazole-tetracycline, and erythromycin-sulfamethoxazole on methane production from mixed VFAs. It was found that tetracycline-erythromycin-sulfamethoxazole caused similar inhibition as erythromycin-sulfamethoxazole, but less than the other two groups. This suggests that tetracycline could have synergistic effects with erythromycin and sulfamethoxazole, respectively [190].

On the other hand, antagonistic effects occur when the combined antibiotics cause less inhibition than sole tetracycline. This is explained by functional buffering between drugs targeting different processes, such as DNA and protein synthesis [14,15]. It can also result from interference with intracellular drug concentrations, either by reducing drug uptake or promoting efflux [15]. During anaerobic digestion, Rani et al. [133] found that the simultaneous presence of spectinomycin reduced the acute inhibition of tetracycline on methanogenesis when using activated sludge as the substrate. However, although Chait et al. [24] reported antagonism between tetracyclines (doxycycline) and quinolones (ciprofloxacin), the effects may vary when more than two antibiotics are combined, rather than in simple binary mixtures. Gaballah et al. [51] studied the effects of mixed tetracycline, oxytetracycline, sulfadiazine, and norfloxacin on anaerobic digestion of swine manure. They found that the negative effect on methane production was greater for the combined antibiotics than for the sole tetracycline. These findings demonstrate that the effects of mixed antibiotics depend not only on their types but also on the concentrations and ratios. Moreover, the resistance of microbial communities and substrate characteristics could also influence the final outcomes.

Currently, research on the combined effects of tetracycline and other antibiotics in anaerobic digestion systems remains limited, and the underlying mechanisms are not fully understood. More systematic studies are needed to clarify the toxicity of mixed antibiotics on anaerobic digestion performance and microbes.

4. Tetracycline removal pathways in anaerobic digestion systems

Adsorption and biodegradation are the two main pathways for tetracycline removal in anaerobic digestion systems. Adsorption is the process by which substances accumulate on the surface of an adsorbent from a gas or liquid through physical or chemical bonding and is a relatively economical removal method [58]. Aside from adsorption, biodegradation is another pathway for tetracycline removal. Although tetracycline exhibits the slowest biodegradation rate compared to other tetracycline antibiotics, such as oxytetracycline and chlortetracycline [25], acclimated microorganisms have been shown to possess the ability to biodegrade tetracycline [147]. The removal outcomes of tetracycline in anaerobic digestion systems are summarized in Table 4.

4.1. Adsorption

Tetracycline is generally known to adsorb onto solid phases. In anaerobic digestion systems, anaerobic sludge serves as a major reservoir for tetracycline [122]. The adsorption of tetracycline onto sludge is

Table 3
Reported impacts of tetracycline on anaerobic digestion system performance.

Substrate	Substrate COD (g/L)	Inoculum	Reactor (working volume)	Tetracycline (mg/L)	Operation duration (d)	Temperature (°C)	VFA mainly impacted	VFA accumulation ^a	Methane production inhibited (relative decrease, %) ^b	Methane production parameter	Reference
Mixed VFAs, glucose and starch	2.25	Anaerobic sludge	Anaerobic sequencing batch reactor (1 L)	1.65 (day 78–90); 5.7 (day 91–114); 8.5 (day 115–143)	154	35	Propionate	750 mg/L (day 143)	37.5 (8.5 mg/L tetracycline)	Average specific methane production yield (L/g COD removed)	[20]
Glucose	2	Anaerobic sludge	Anaerobic baffled reactor (21 L)	0.25	90	35 ± 1	Acetate	-	19.8–56.1 (C1); 20.21–72.82 (C2); 16.3–61.76 (C3)	Methane production (L/(g MLSS·d))	[105]
Synthetic wastewater	0.69	Anaerobic sludge	Anaerobic digester (1 L)	20	39	37	Propionate	260 mg/L	28.6 (day 3)	Methane production (mL)	[193]
Short chain fatty acid mixture (butyrate, propionate and acetate)	13.08	Anaerobic sludge	Anaerobic batch reactor (0.06 L)	1, 10, 25, 50, 100, 250, 500, 750, 1000	6	Mesophilic	Acetate, butyrate, propionate	- ^c	10 (1 mg/L tetracycline); 59 (50 mg/L tetracycline); 100 (1000 mg/L tetracycline)	Cumulative methane production (mL)	[23]
Glucose	5	Anaerobic granular sludge	Anaerobic batch reactor (0.1 L)	1, 10, 30, 50, 80, 100, 150	5	37 ± 0.5	-	-	-31.7 to -66.1 ^d	Cumulative methane production (NmL)	[126]
Synthetic swine wastewater	2.5	Anaerobic digestion sludge	Anaerobic reactor (0.4 L)	2, 4 (stage 1); 4, 8 (stage 2)	> 100	35	Propionate	154.7 mg/L (4 mg/L tetracycline); 603 mg/L (8 mg/L tetracycline)	73.3 (8 mg/L tetracycline)	Daily methane production (mL/d)	[103]
Waste activated sludge	8.7	No additional inoculum	Anaerobic fermentation reactor (0.5 L)	60 mg/kg TSS	7	35 ± 2	Propionate	50.2 mg COD/g VSS	-	-	[65]
Swine manure	10.9	Anaerobic sludge	Anaerobic batch reactor (0.09 L)	0.5, 50	24	35 ± 1	-	-	11 (0.5 mg/L tetracycline); 40.2 (50 mg/L tetracycline)	Maximum methane production rate (mL/g substrate/d)	[169]
Propionate	16.7	Anaerobic sludge	Anaerobic batch reactor (-)	25	17	-	Propionate	- ^e	-	-	
Peptone and glucose	1 (days 1–8); 2 (days 9–90)	Anaerobic sludge	CFR (5 L); SBR (5 L)	5	75	35	Propionate	342 mg COD/L (CFR); 502 mg COD/L (SBR)	32.8 (CFR); 13.8 (SBR)	Maximum methane production (mg COD/L)	[176]
Culture medium (broth)	-	Anaerobic sludge	Anaerobic batch reactor (0.16 L)	400	3	37	Acetate, butyrate, propionate	- ^f	-	-	[144]
Activated sludge	-	Anaerobic sludge	Anaerobic reactor (0.15 L)	1, 5, 10	5	35 ± 2	-	-	15.2 (1 mg/L tetracycline); 34.1 (5 mg/L tetracycline); 31.8 (10 mg/L tetracycline)	Cumulative methane production (mL)	[133]

Abbreviations: CFR, continuous-flow reactor; COD, chemical oxygen demand; MLSS, mixed liquor suspended solids; SBR, sequencing batch reactor; VFA, volatile fatty acid.

^a Data were collected at the final stage of operation.

^b Relative decrease (%) for methane production = $([\text{control}] - [\text{with tetracycline}]) / [\text{control}] \times 100\%$.

^c With 1000 mg/L tetracycline, residual butyrate, propionate and acetate were detected as 128.4 %, 128.9 % and 52.4 %, respectively.

^d Methane production in the reactors with tetracycline addition exceeded that of the blank control.

^e The propionate degradation efficiency decreased from 76.3 % in the control group to 36.8 % in tetracycline-added group on day 7.5.

^f Acetate and butyrate proportions decreased by 33.3 % and 68.0 %, respectively, while propionate proportion increased by 71.4 % (based on percentage composition of total VFAs).

Table 4
Overview of tetracycline removal performance in anaerobic digestion systems.

Carbon source	Carbon source concentration	Inoculum type ^a	Tetracycline concentration (mg/L)	HRT (d)	SRT (d)	Operating duration	Temperature (°C)	Initial pH	Removal efficiency	Removal pathway	Enhancement	Reference
Tetracycline	80 mg/L	Anaerobic granular sludge (26.7 g/L)	80	-	-	800 min	35 ± 1	3.0–9.0	91.0 %–93.0 %	Adsorption	-	[90]
mixed VFAs, glucose and starch	2250 mg COD/L	Anaerobic sludge (SS: 4.5 g/L)	1.65, 5.7, 8.5	2.8	50	154 d	35	6.8–7.2	> 50 % (1.65 mg/L); > 90 % (5.7 mg/L); ~ 40 % (8.5 mg/L)	Biodegradation	-	[20]
Tetracycline	90 mg/L	Anaerobic sludge (TVS: 2 g/L)	90	-	-	120 d	35	7	89 %	46 % biodegradation	-	[21]
Swine feces	-	Anaerobic sludge	472.9 (winter), 3016.7 (summer) ^b	-	12	Summer & winter	32	8	> 50 % (summer); < -50 % (winter).	Mainly adsorption	-	[100]
Saccharose	5.35 g/L	Granular sludge (TSS: 14.9 g/L)	250	1	-	85 d	35 ± 1	8.0 ± 0.3	81.50 %	-	GAC/nZVI Pretreatment	[222]
Waste sludge	- ^c	Anaerobic sludge	520.87 ^d	-	-	-	37	-	over 71.2 %	- ^e	-	[219]
Glucose	5000 mg COD/L	Anaerobic granular sludge (VS: 7.6 g/L)	1, 10, 30, 50, 80, 100, 150	-	-	25 d	37 ± 0.5	7	68 %–98 %	Biodegradation	nZVI	[126]
Glucose and tetracycline (first two cycles); tetracycline (last two cycles) ^f	1000 mg/L (glucose); 100 mg/L (tetracycline)	Anaerobic sludge	100	5	-	20 d	37	N/A	60 %–80.7 % (with glucose); 58.6 %–99.0 % (without glucose)	Adsorption and biodegradation ^g	Fe ₃ O ₄	[225]
Glucose	3000 ± 200 mg COD/L	Anaerobic sludge (SS: 5 g/L)	0.3	-	-	120 h	25	7.5 ± 0.1	Fully removal	Mainly adsorption	-	[31]
Waste sludge	8.7 g COD/L	Waste activated sludge (VSS: 6.9 g/L)	60 ± 5.1 mg/kg TSS	-	-	7 d	35 ± 2	9	14.80 %	-	-	[65]
Swine manure	10.9 g COD/L	Anaerobic sludge (VS: 0.4 g/L)	0.5, 50	-	-	24 d	35 ± 1	7.5 ± 0.1 (inoculum), 7.2 ± 0.1 (swine manure)	20.8 %–79.7 %	Biodegradation	Biochar	[169]
Peptone and glucose	1000 mg COD/L (days 1–8) and 2000 mg COD/L (days 9–90)	Anaerobic sludge (VSS: 3–4 g/L)	5	2	25	90 d	35	-	45.6 %–46.1 %	82.0 %–88.9 % biodegradation	-	[176]
Peptone and glucose	6000 mg COD/L	Anaerobic sludge (VSS: 5.3 g/L)	5	2	-	75 d	35	-	54.2 %–87.5 %	77.6 %–90.5 % biodegradation	PAC	[175]
Culture medium (broth)	-	Anaerobic sludge with deactivated methanogens	10, 50, 200, 400	-	-	12 d	37	> 5.5	77 %–87 % (day 3); > 90 % (day 12)	Mainly biodegradation	-	[144]

Abbreviations: COD, chemical oxygen demand; SS, suspended solids; TSS, total suspended solids; TVS, total volatile solids; VS, volatile solids; VSS, volatile suspended solids; nZVI, nano zero-valent iron; PAC, powdered activated carbon.

^a Inoculum concentrations are shown in parentheses where reported.

^b Expressed as µg/kg.

^c Soluble chemical oxygen demand of raw waste sludge: 267 mg/L.

^d Expressed as µg/kg.

^e < 33.6 % thermal decomposition for thermal hydrolysis pretreatment-anaerobic digestion; no removal pathway was specified for other conditions.

^f Each cycle lasted 5 days.

^g Tetracycline was first adsorbed by Fe₃O₄ (and/or inoculum sludge), then degraded by microorganisms.

influenced by its physicochemical properties, such as solubility, pK_a , adsorption coefficient (K_d), and octanol-water partition coefficient ($\log K_{ow}$), as well as sludge properties, including composition and surface charge [64]. In biological wastewater treatment systems, K_d represents the partitioning of tetracycline between sludge and the aqueous phase, indicating its adsorption capacity [113]. Wu et al. [182] conducted adsorption experiments using sewage sludge and found a K_d value of 7315 ± 317 mL/g for tetracycline, which was higher than that of sulfamethazine (7.5 ± 0.3 mL/g) and 2.7 times greater than that of ciprofloxacin (2753 ± 6 mL/g). This finding showed the relatively high adsorption affinity of tetracycline for sewage sludge. Meanwhile, tetracycline has high water solubility (0.041 mol/L), and its $\log K_{ow}$ value of 1.25 suggests weak hydrophobicity [39], indicating that the hydrophobic effect could not be the primary mechanism for tetracycline adsorption onto sludge. Tetracycline is an amphoteric molecule with multiple ionizable functional groups, characterized by three pK_a values of 3.3, 7.68, and 9.3 [141]. The affinity of different ionic forms for adsorption onto sludge follows the order: cations \gg zwitterions $>$ anions [88]. In anaerobic digestion systems, tetracycline predominantly exists as a zwitterion at pH 6.5–7.2 [4], and its adsorption onto sludge is primarily driven by electrostatic attraction [104].

For anaerobic digestion systems exposed to tetracycline in the short term, adsorption is the primary removal pathway. For example, Li et al. [90] reported that anaerobic granular sludge removed 91.5–93.0 % of tetracycline through adsorption in batch experiments. In the study by Wang and Wu [176], near-complete tetracycline removal was achieved during the initial operation phase (first five days), which was primarily attributed to adsorption onto sludge. He et al. [65] added 60 mg/kg TSS tetracycline to an anaerobic fermentation system with waste activated sludge and found that 85.2 % of the added tetracycline was detected in the sludge after 7 days of fermentation. Meanwhile, the adsorption of tetracycline onto sludge can occur rapidly. Cheng et al. [31] observed that anaerobic sludge, whether sterilized or not, adsorbed over 90 % of tetracycline within the first 30 min of contact. Moreover, Zhao et al. [225] found that more than 50 % of tetracycline removal occurred within the initial 10 h after influent addition. This indicated that rapid adsorption onto anaerobic sludge is potentially the main removal mechanism at the beginning of the reaction cycle. In systems exposed to tetracycline over the long term, although prolonged pressure could select functional microorganisms and reduce the contribution of adsorption to removal, the role of adsorption in tetracycline removal remains significant. Cetecioglu et al. [21] observed an overall tetracycline removal percentage of 89 % in a methanogenic system using tetracycline as the sole carbon source, with 43 % potentially removed through adsorption. Liu et al. [100] conducted field monitoring of an industrial-scale upflow anaerobic sludge bed reactor treating swine feces. It was found that tetracycline accumulated in the solid phase during winter, while the system achieved tetracycline removal in summer with more than 40 % of the total tetracycline in the substrate adsorbed onto biosolids [100].

In anaerobic systems, besides operational duration (sludge acclimation), factors such as cations, pH, inoculum type, and temperature can also influence the adsorption capacity of sludge for tetracycline. Adsorption kinetics analysis indicated that the adsorption of tetracycline onto sludge over time followed a pseudo-second-order kinetic model [88]. This suggests that chemical adsorption plays a dominant role in the process, potentially driven by chemical reactions or electron sharing/-transfer between functional groups on the sludge surface and tetracycline [220]. Electrostatic interactions, hydrogen bonding, cation exchange, and the cation bridging mechanism are key contributors to the adsorption process. The three pK_a values of tetracycline indicate its ability to form charged structures under different pH conditions, making the adsorption process pH-dependent. For instance, through adsorption experiments at varying pH levels, Lou et al. [104] revealed that the maximum adsorption capacity of suspended organic matter for tetracycline decreased as pH increased from 3.0 to 7.5. This meant that

environmental pH could influence electrostatic interactions and, consequently, adsorption efficiency. Specifically, under acidic or neutral conditions, tetracycline undergoes protonation [179]. In such conditions, tetracycline in the aqueous phase is electrostatically attracted to negatively charged sludge surfaces. Additionally, the quaternary ammonium functional group on tetracycline competes with divalent cations like Ca^{2+} and Mg^{2+} , replacing metal cations on sludge surfaces, thereby enhancing adsorption [88]. Thus, cations in the system can affect the adsorption process. In addition to ion exchange, cations can form complexes with tetracycline that may bind to the sludge surface through a cation bridging mechanism [141,97]. However, under alkaline conditions, these complexes may fail to bridge with the adsorbent, leading to a negative impact of ionic strength on the adsorption process [122,141,210]. Furthermore, under acidic conditions, hydrogen bonding between tetracycline and sludge also serves as an important adsorption mechanism [104]. Moreover, in alkaline environments, tetracycline molecules become deprotonated, resulting in anionic forms. The resulting electrostatic repulsion between the negatively charged tetracycline and the negatively charged sludge surface leads to reduced adsorption capacity.

Operating temperature can influence adsorption. Lou et al. [104] conducted adsorption experiments with suspended organics from swine wastewater at 10 °C, 20 °C, and 30 °C. They found that maximum adsorption capacity decreased from 4.11 $\mu\text{g/g}$ at 10 °C to 1.52 $\mu\text{g/g}$ at 30 °C. This trend might be explained by the reduced van der Waals forces [122]. Therefore, although chemical adsorption is considered the primary mechanism for tetracycline adsorption onto sludge, physical adsorption may also play a role. Furthermore, the inverse relationship between adsorption capacity and temperature indicated that tetracycline adsorption could be an exothermic reaction. Subsequent thermodynamic calculations also confirmed it as a spontaneous and exothermic process [104]. Wang et al. [170] reached similar conclusions. However, Li et al. [90] conducted adsorption experiments using anaerobic granular sludge and observed that the process had a negative ΔG value but a positive ΔH value. This revealed that tetracycline adsorption by anaerobic granular sludge could be spontaneous but endothermic. This discrepancy may be related to the type of sludge used as an adsorbent.

Different inocula may lead to varying tetracycline adsorption outcomes. Wang et al. [170] reported that sludge under aerobic conditions achieved better tetracycline adsorption compared to anoxic conditions. Suspended activated sludge may also exhibit higher adsorption capacity than granular sludge [122], possibly attributed to differences in contact efficiency with tetracycline and sludge surface properties. Extracellular polymeric substances (EPS) secreted by microbial cells play a crucial role in tetracycline adsorption onto sludge. EPS can protect cells from harmful conditions when toxic substances are present [67]. The main components of EPS are polysaccharides (PS) and proteins (PN), and the ratio of PN to PS can affect properties such as surface charge [198,232]. During interactions with tetracycline, proteins are considered the main active component in EPS [152,195]. Tetracycline forms complexes with EPS proteins through electrostatic interactions, involving functional groups such as hydroxyl, carboxyl, and amino groups [152]. Additionally, granular sludge was observed to show higher EPS and PN content compared to flocculent structure sludge [102]. Therefore, different inocula may achieve varied tetracycline adsorption results due to differences in EPS composition and concentration.

Furthermore, the initial concentrations of inoculum and tetracycline are important. Higher concentrations could increase contact efficiency between sludge and tetracycline in the system, potentially affecting adsorption efficiency. Correspondingly, reducing sludge retention time (SRT) could decrease the sludge concentration, which in turn might affect the rate and efficiency of tetracycline adsorption [81].

4.2. Biodegradation

In tetracycline biotransformation processes, functional

microorganisms synthesize and secrete enzymes that act on specific active sites to reduce tetracycline bioactivity [146]. Different functional groups are primary targets for degradation enzymes, and modifications to these groups can affect the activity and stability of tetracycline molecules. For example, demethylation of the dimethylamino group at C4 is considered as an essential step in biodegradation of tetracycline [158]. The enzymes involved in tetracycline biodegradation are mainly oxidoreductases [109]. Functional enzymes include both specific and non-specific enzymes. Non-specific enzymes target particular groups, such as deaminases and demethylases, and could degrade tetracycline in the system by disrupting its structure. For specific enzymes, enzymatic inactivation is one of the tetracycline resistance mechanisms, involving the tetX. tetX encodes flavin-dependent monooxygenase. This enzyme adds a hydroxyl group at the C11a position, thereby inactivating tetracycline [28]. However, due to oxygen limitations in anaerobic systems, this pathway is difficult to achieve during anaerobic digestion [201].

For anaerobic digestion systems, Shang et al. [144] recently identified the biodegradation pathway of tetracycline under anaerobic fermentation conditions through batch experiments by analyzing biodegradation intermediates. Tetracycline was degraded through four pathways involving the removal of various functional groups, including the dimethylamino group at C4, the carbonyl group at C1, the amino group at C4, the hydroxyl group at C3, and the amide group at C2. It also underwent hydroxylation at C11a and/or C2. These processes converted tetracycline into smaller molecules such as ISO-TP370 and TP415, which are further broken down into smaller compounds [144].

Previous research on tetracycline biodegradation mechanisms mainly focused on pure microbial strains, mostly under aerobic conditions [145,146,150]. Although tetracycline was once thought to be biodegradable only under aerobic conditions [147], it has been confirmed that mixed microbial communities in anaerobic digestion systems are eligible to biodegrade tetracycline. Zhang and Li [219] investigated the antibiotic removal in anaerobic digestion systems with different sludge pretreatments. It was observed that tetracycline removal efficiencies exceeded 71.2 % in all groups, and biodegradation was the primary removal pathway when compared to thermal decomposition [219]. Additionally, the contribution of biodegradation can be assessed by measuring tetracycline concentrations in both liquid and solid phases followed by mass balance calculations. Wang and Wu [176] found that in reactors subjected to long-term exposure to 5 mg/L tetracycline, biodegradation accounted for 82.0–88.9 % of the overall tetracycline removal. Under the same conditions, a biodegradation contribution rate ranging from 77.6 % to 86.3 % was further reported [175]. Similarly, Cetecioglu et al. [20] determined tetracycline removal pathways in anaerobic sequencing batch reactors with starch, glucose, and mixed VFAs as substrates. The results indicated that tetracycline was consistently removed through biodegradation as its concentration increased from 1.65 mg/L to 8.5 mg/L. In a subsequent experiment, it was further demonstrated that 90 mg/L tetracycline could serve as the sole carbon source for methane production, with 46 % of the tetracycline being removed via biodegradation [21]. The difference in tetracycline removal efficiency might be attributed to the presence of easily biodegradable substrates in the system. Due to the complex structure of tetracycline and its low concentration, co-metabolism with primary substrates is a key pathway for tetracycline removal in biological wastewater/waste treatment systems [27]. Long-term operation can be conducted to achieve tetracycline biodegradation after acclimation [21], while tetracycline cannot be utilized as the sole carbon source under short-term operation [27]. Easily biodegradable substrates could provide the necessary energy for microbial activities such as enzyme synthesis, mass transfer, and stress responses [57]. Specifically, during tetracycline biodegradation, the positive effect of coexisting substrates on functional enzymes may explain the higher tetracycline removal efficiency under co-metabolism conditions [209,215]. Moreover, easily degradable substrates could promote nicotinamide adenine dinucleotide (NADH) production, which helps maintain cellular stability in the

presence of antimicrobial compounds [71]. Besides, the presence of additional carbon sources may also enhance system biodiversity and increase the variation of metabolic pathways.

In addition to the substrate type, tetracycline biodegradation is also influenced by factors such as pH, tetracycline concentration, SRT, hydraulic retention time (HRT), and temperature. Due to the strong correlation between pH and molecular structure, tetracycline is more readily degraded under mildly acidic or highly alkaline conditions compared to other environments [79]. Additionally, pH could influence enzyme activity and reaction rates, thereby affecting the efficiency of tetracycline biodegradation [145]. Another factor that may influence enzyme activity is temperature [119]. Different operational temperatures can also affect microbial activity and community structure [119]. Compared to the mesophilic condition, thermophilic anaerobic digestion has been found to enhance the removal of various pharmaceuticals, including tetracycline antibiotics, by promoting biodegradation [214, 224]. However, further research is needed to better understand the anaerobic biodegradation of tetracycline. The initial concentration of tetracycline is another factor that could impact biodegradation efficiency. For example, in a short-term (3-day) experiment, when initial concentrations were 10, 50, 200, and 400 mg/L, tetracycline removal dominated by biodegradation in anaerobic fermentation systems was observed at efficiencies of 77 %, 83 %, 86 %, and 87 %, respectively [144]. This suggested the enhanced biodegradation efficiency with increased tetracycline concentrations. As for long-term acclimation, tetracycline concentration is believed to influence the selection of dominant microbial communities [188]. In the study by Cetecioglu et al. [20], the biodegradation efficiency rose from approximately 50 % to over 90 % as the influent tetracycline concentration increased from 1.65 mg/L to 5.7 mg/L. However, when the concentration further increased to 8.5 mg/L, the whole system faced collapse, and tetracycline removal efficiency gradually reduced. Therefore, the toxicity of tetracycline to anaerobic digestion systems at concentrations beyond a certain threshold should not be overlooked. SRT is a key variable in biological treatment systems for wastewater and waste containing antibiotics. Longer SRT could positively influence microbial community diversity and contribute to the higher biomass concentration [116]. Meanwhile, extended SRT could create favorable conditions for the growth of microorganisms capable of degrading trace organic compounds, which are commonly slow growing [116,122,205]. Furthermore, HRT could be another factor that influences the biodegradation of tetracycline by affecting both the reacting time and the tetracycline loading rate [122].

Current research on functional microbes involved in tetracycline biodegradation in anaerobic digestion systems focuses on mixed microbial communities. The enrichment of *Trichococcus* in the presence of tetracycline antibiotics, suggested its potential involvement in tetracycline biodegradation [176]. Meanwhile, Wang et al. [175] proposed that *Desulfomicrobium* might also contribute to tetracycline removal based on correlation analysis. Furthermore, the genus *Pandoraea* has been identified as a potential key contributor in tetracycline removal due to its relative abundance exceeding 70 % in anaerobic systems where tetracycline biodegradation occurred [225]. *Pandoraea* has been reported to include strains capable of degrading tetracycline [185,186]. In addition, after acclimation with 100 mg/L tetracycline as the sole carbon source, *Klebsiella*, *Pseudomonas*, *Escherichia*, *Azonexus*, and *Desulfovibrio* were found to be the dominant genera with the ability to degrade complex organic pollutants. This suggested their potential function in anaerobic tetracycline biodegradation [225]. Similarly, *Klebsiella* strains SQY5 and TR5 have also been shown to possess tetracycline degradation capabilities [145,206]. Although studies on the tetracycline metabolic pathway in single strains, such as *Klebsiella* sp. SQY5, were conducted under aerobic conditions [145], some key enzyme genes involved in tetracycline biodegradation are not oxygen-dependent. For example, alcohol dehydrogenase (EutG) functions independently of oxygen. Therefore,

the genus *Klebsiella* may also degrade tetracycline in anaerobic digestion systems. However, due to the limited research on tetracycline degraders in anaerobic digestion systems, further exploration is needed to validate and expand these findings.

Tetracycline often undergoes hydrolysis or epimerization, and forms products such as 4-epi-tetracycline and ISO-TC that are influenced by environmental conditions [144,61]. Intermediate products with modified functional groups, such as the demethylation of the C4 dimethylamino group on ISO-TC, can initiate further biotransformation [144,87]. Meanwhile, these products may retain partial antibacterial activity, potentially contributing to environmental and ecological risks [183], while biodegradation can offer the possibility of completely eliminating tetracycline toxicity. Nevertheless, while ring-opening processes can effectively eliminate tetracycline toxicity, functional group modifications alone may fail to disrupt the stable tetracyclic structure, potentially resulting in residual bioactivity [158]. Structural modifications of tetracycline can be classified as 'permissible,' which do not affect bioactivity (e.g., changes at C4a, C5, C6, C7, C8, and C9), and 'inviolable,' which cause activity reduction (e.g., changes at C1, C2, C3, C4, C10, C11, C12, and C12a) [61]. In anaerobic digestion systems, even with existence of those capable of biodegrading tetracycline, pollutant removal efficiency could still be inhibited by the accumulation of degradation products [222]. Although such inhibition was reported to be alleviated through long-term acclimation, further research is needed to evaluate the toxicity of tetracycline biodegradation products in anaerobic digestion systems and to ensure their complete removal.

5. Enhancement of system performance through mitigation of tetracycline inhibition

The antibacterial activity of tetracycline negatively impacts the pollutant removal efficiency of anaerobic digestion systems. Therefore, optimizing system performance under tetracycline stress while achieving efficient removal of tetracycline as a trace pollutant has become a critical challenge. Currently, conductive material addition has been applied to improve system functionality and microbial activity. The following subsections summarize the typical enhancement mechanisms of conductive materials, highlighting the potential to improve anaerobic digestion performance under tetracycline stress and to facilitate tetracycline removal.

5.1. Carbon-based materials

5.1.1. Activated carbon

In tetracycline-contaminated systems, activated carbon has been studied as an effective adsorbent due to its high surface area and porosity [1,94]. For example, Choi et al. [32] investigated the adsorption capacity of powdered activated carbon (PAC) for seven commonly used tetracycline antibiotics, including tetracycline. The results showed that nearly complete adsorption (96%–100%) was achieved in deionized water with 0.7 mg/L PAC addition at a tetracycline concentration of 10 µg/L. It should be noted that the adsorption of organic micropollutants by activated carbon can be affected by the presence of dissolved organic matter. Dissolved organic matter may compete for surface adsorption sites and block pores, thereby decreasing the effective adsorption capacity for tetracycline [115]. Rivera-Utrilla et al. [136] reported that the adsorption capacity of commercial activated carbon for tetracycline decreased from 60 mg/g in ultrapure water to 26 mg/g in wastewater. Similarly, Choi et al. [32] observed that with 10 µg/L tetracycline, 1 mg/L PAC showed around 20% lower adsorption in water with dissolved organic carbon than in deionized water. Nevertheless, a substantial removal efficiency close to 80% was still achieved. Additionally, in biological wastewater treatment systems, microbial attachment to activated carbon can alter the surface properties and inhibit tetracycline adsorption [135,136]. With the presence of microbes, the adsorbate-adsorbent relative affinity for tetracycline

decreased by nearly 80%, yet the adsorption capacity remained at 353.3 mg/g [136]. Therefore, even though dissolved organic matter competition and microbial attachment can negatively affect the theoretical adsorption capacity, activated carbon still retains considerable adsorption potential and can be employed as an external adsorbent to enhance tetracycline removal. Meanwhile, the reduction of tetracycline in the liquid phase could alleviate tetracycline inhibition on microorganisms and improve system stability [154].

In addition, activated carbon positively influences microbial activity in anaerobic digestion systems, primarily in two aspects. First, redox reactions based on interspecies electron transfer are key metabolic pathways for microorganisms in anaerobic digestion systems. Compared to biochar, activated carbon exhibits higher electrical conductivity [200,91]. This makes it a facilitator of interspecies electron transfer, promoting the establishment of DIET. Yang et al. [200] found that the addition of 15 g/L granular activated carbon (GAC) improved sludge conductivity by 12.6-fold in an anaerobic digestion system with a sludge volatile suspended solids concentration of 21.3 g/L. Enhanced conductivity approached that of conductive pili. Meanwhile, activated carbon can promote the enrichment of DIET participants. For instance, Ma et al. [107] observed the appearance of *Geobacter* in reactors supplemented with PAC after long-term operation, while it was absent in the control group. Similarly, Yang et al. [203] reported that the relative abundance of *Geobacter* on GAC-attached microbial communities was 14.3 times that in the control sludge, and *Methanotherox* was the most abundant methanogen. The establishment of DIET can effectively enhance methane production rates. Lee et al. [86] conducted methane production tests using suspended sludge and GAC-attached sludge and suggested that the latter exhibited approximately 2.7 times higher methane production capacity.

Second, activated carbon provides attachment sites for microorganisms, facilitating microbial growth and enrichment while promoting the formation of biopolymer clusters or biofilms [107,187,7]. With enhancement of microbial activity, the formation of compact biomass structure may offer a mechanism for microbial communities to resist tetracycline toxicity. Beyond DIET-related microorganisms, activated carbon can also enrich key functional microorganisms in anaerobic digestion processes, such as syntrophic propionate-oxidizing bacteria of *Syntrophobacteraceae* [200]. Ma et al. [107] reported impacts of PAC on microbial community structure, and revealed that, in addition to DIET-associated microbes, PAC stimulated the growth of various syntrophic VFA degraders, including genera *Gelria* and *Syntrophomonas* [107]. One study has reported that 20 g/L of GAC successfully increased the maximum methane production rate in an anaerobic digestion system treating pharmaceutical wastewater by 61.8% [40]. In anaerobic digestion systems treating tetracycline-containing wastewater, the presence of 1 g/L PAC was found to promote the enrichment of propionate-oxidizing bacteria, including *Smithella*, *Syntrophobacter*, *Syntrophomonas*, and *Syntrophorhabdus*, in both continuous-flow and sequencing batch reactors [175]. In terms of system performance, PAC increased the maximum methane production rate by 13.8%–15.6% and enhanced the propionate degradation rate by 4.4%–21.4% [175]. Activated carbon may improve system performance under tetracycline stress by enhancing the degradation of intermediate metabolites and promoting the synergistic activity of functional microbes.

Moreover, activated carbon has been shown to promote the removal of pharmaceuticals in anaerobic digestion systems. Using the luminescence intensity of *Luminescence bacillus* T3 as an indicator, Dai et al. [40] observed that GAC addition reduced pharmaceutical toxicity to 76.1% of its original level. Further analysis revealed that GAC enhanced the degradation of pharmaceutical intermediates. Meanwhile, Zhao et al. [223] reported that 5 g/L activated carbon increased the removal efficiency of sulfamethoxazole in long-term anaerobic systems from 49.1% to 92.3%. For tetracycline removal, the addition of 1 g/L PAC has been shown to enhance removal efficiency by 18.7%–21.4% [175]. This suggests that activated carbon may function as an electron shuttle,

enhancing tetracycline biodegradation. By combining its effects on microbial activity and the enrichment of functional microorganisms, activated carbon holds potential for improving pollutant removal efficiency in anaerobic digestion systems.

5.1.2. Biochar

Biochar is another commonly used carbon-based conductive material for optimizing anaerobic systems treating antibiotic-containing wastewater/waste. Biochar can also act as both an adsorbent for tetracycline and an enhancing strategy for system performance [49]. However, compared to activated carbon, biochar is produced at lower temperatures, resulting in a smaller specific surface area [49]. Li et al. [91] observed that GAC exhibited a more porous structure than biochar derived from sawdust. Correspondingly, in the presence of toxic pollutants, the maximum adsorption capacity of biochar was only about half that of GAC [91]. Similarly, Fakioglu et al. [50] combined ozone pretreatment with GAC and biochar as adsorbents to treat wastewater with mixed antibiotics, and the results showed that only GAC could achieve complete adsorption of nearly all refractory antibiotics and/or by-products. Thus, while the adsorption capacity of biochar varies depending on the feedstock material [49], the ability to absorb antibiotics and other refractory pollutants is generally less effective compared to that of activated carbon when used as an adsorbent. Nevertheless, biochar remains a feasible adsorbent for tetracycline removal. For instance, Kim et al. [80] evaluated the adsorption capacity of biochar prepared from maple leaves at different temperatures, achieving adsorption capacities of tetracycline ranging from 40.3 to 361.0 mg/g.

Meanwhile, lower production temperatures of biochar also result in lower electrical conductivity compared to activated carbon. Gabhi et al. [54] demonstrated that the conductivity of wood biochar was positively correlated with pyrolysis temperature. This research highlighted the importance of carbonization and graphitization in enhancing the conductivity of carbon-based materials. For anaerobic digestion systems, sludge mixed with GAC exhibited electrical conductivity up to 8.8 times higher than that of sludge mixed with biochar [91]. This suggests that biochar showed a reduced ability than activated carbon to act as a conductor and facilitate electron exchange. However, the lower conductivity of biochar does not necessarily imply weaker enhancement capabilities compared to activated carbon. Sun et al. [156] highlighted two electron transfer mechanisms mediated by pyrogenic carbon matrices: direct electron transfer through the carbon matrix and the charging and discharging cycles of surface functional groups. The former occurs only when the pyrolysis temperature exceeds 600°C. Additionally, fourier transform infrared analysis revealed that almost all featured vibrations of surface functional groups on pyrogenic carbon disappeared when the pyrolysis temperature exceeded 700°C (700–800°C) [156]. Consequently, the incomplete combustion of organic matter allows biochar to retain abundant surface functional groups, whereas oxygen-containing functional groups on activated carbon surfaces are difficult to preserve under high temperatures [156,91]. These functional groups, such as quinone/hydroquinone, can accept and transfer electrons, enabling biochar to perform as a redox mediator to enhance electron transfer and metabolic activity in anaerobic digestion systems [154,82]. Shen et al. [149] investigated the use of biochar produced at pyrolysis temperatures of 300°C, 500°C, and 700°C to improve anaerobic digestion systems treating waste activated sludge and showed that although biochar prepared at 700°C exhibited higher electrical conductivity than that of biochar prepared at 300°C, the latter achieved higher methane production. This finding suggested that enhancement is primarily associated with the redox activity of biochar rather than the conductivity [149].

In addition, like activated carbon, biochar could also facilitate the enrichment of key functional microorganisms in anaerobic digestion systems, including *Geobacter* and VFA-oxidizing bacteria such as *Syntrophorhabdus*, *Syntrophomonas*, and *Smithella* [226,91]. According to Huggins et al. [74], despite its smaller specific surface area, the

macropores on the biochar surface were observed to range from 1 to 40 µm, larger than those of GAC (less than 1 µm). These macropores could potentially offer a more favorable environment for microbial growth and system stability when compared to activated carbon. Moreover, the functional groups may enhance the immobilization of extracellular enzymes, thereby increasing enzyme activity and promoting the consumption of substrates and pollutants [127,202].

For treating tetracycline-containing wastewater, biochar addition has been shown to enhance both methane production and tetracycline biodegradation. For instance, Wang et al. [169] added 15 g/L of biochar to anaerobic digestion systems with different tetracycline concentrations, and demonstrated that with 0.5 mg/L tetracycline, biochar increased cumulative methane production. At a higher concentration of 25 mg/L, although methane production was not improved, biochar enhanced propionate degradation efficiency from 36.8 % to 54.9 % within 7.5 days. Furthermore, under 0.5 and 50 mg/L tetracycline conditions, biochar improved tetracycline biodegradation amounts from 0.32 mg/L and 10.4 mg/L to 0.40 mg/L and 26.9 mg/L, respectively [169]. Therefore, biochar is a viable strategy for enhancing the treatment of tetracycline-containing wastewater.

5.2. Iron-based materials

Iron-based conductive materials are the most commonly used metal-based materials for enhancing anaerobic digestion, with extensive research focusing on zero-valent iron (ZVI)/nanoscale ZVI (nZVI) and magnetite (Fe₃O₄). Like carbon-based conductive materials, the mechanisms of metal-based materials can be categorized into three main categories: (i) serving as adsorbents to facilitate tetracycline adsorption and removal; (ii) acting as mediators to enhance electron transfer within the system; and (iii) improving microbial activity.

Iron-based materials have been reported as effective solid-phase electron shuttles for the adsorption of tetracycline. For instance, ZVI could provide abundant active sites on its surface, facilitating tetracycline adsorption. In addition, Yan et al. [199] confirmed the formation of iron oxides on ZVI surfaces under acidic conditions using X-ray diffraction analysis. This finding suggested that electrostatic interactions or the formation of stable complexes between iron ions/ferrous ions and the functional groups in tetracycline molecules might be critical mechanisms for its adsorption by ZVI. These mechanisms may also account for the strong adsorption capacity of magnetite for tetracycline. Hanay and Türk [62] demonstrated that a nZVI concentration of only 0.05 g/L achieved a 71 % adsorption efficiency for 60 mg/L tetracycline at pH 6. Moreover, nZVI was found to adsorb not only tetracycline but also its transformation byproducts, such as epi-tetracycline [62]. For magnetite, Zhao et al. [225] observed that the addition of magnetite increased tetracycline adsorption onto the solid phase. Specifically, increasing magnetite concentrations from 0 to 1 g/L enhanced the adsorption efficiency from 3.9 % to 38.1 %. Under biotic conditions, the residual tetracycline in the solid phase decreased as magnetite concentrations increased. This illustrated that when magnetite was used as an adsorbent in anaerobic digestion systems, tetracycline was initially adsorbed by Fe₃O₄ and/or sludge, followed by biodegradation [225].

5.2.1. ZVI/nZVI

The enhancement effects of ZVI on anaerobic digestion have been widely studied. ZVI can directly improve DIET in the systems. Zhong et al. [229] reported that adding 1 g/L ZVI with a particle size of 4 µm increased the electrical conductivity of mixed sludge to 6.2 times that of the original sludge. After EPS removal, the conductivity dropped to 44.6 % of its initial value but remained 3.1 times higher than the control group. This indicated that ZVI could not only promote the establishment of microbe-mediated DIET structures, such as conductive pili, but also act as a physical conductive bridge to support DIET. On the other hand, ZVI exhibits strong reductive properties, with a low standard oxidation

potential ($E^{\circ} = -0.44$ V) in solution [154]. Therefore, the addition of ZVI to anaerobic digestion systems could reduce the redox potential [123]. Wang et al. [171] demonstrated that adjusting the oxidation-reduction potential (ORP) under specific pH conditions could regulate the metabolic pathways in the system. It was further reported that low ORP could reduce the proportion of propionate fermentation, indirectly preventing propionate accumulation [171]. Besides, ZVI can serve as an electron donor, providing extra electrons to the system. These electrons combine with protons to alleviate acidification and supply direct substrates for hydrogenotrophic methanogens, thereby enhancing methane production. Meanwhile, the ferrous ions released by ZVI are critical trace elements for microbial activity, which can improve the activity of both microorganisms and enzymes [11,173].

Furthermore, the production of multivalent cations by ZVI has been found to promote EPS secretion [234,76,93]. This could further enhance the formation of microbial protective barriers under tetracycline stress and induce biofilm formation, contributing to system stability. The electroactive components in EPS may also facilitate DIET [93]. Elevated EPS concentrations could influence the adsorption capacity of the solid phase for tetracycline in anaerobic digestion systems. However, high doses of nZVI may negatively impact system performance due to its high activity and strong reductive properties, which can damage cell membranes [11,191]. Li et al. [93] found that 50 mM nZVI increased the EPS concentration to 1.6 times that of the control group, while VFAs accumulated to 2.3 times the control level. Further analysis revealed that excessive EPS concentrations could inhibit hydrogen diffusion, thereby reducing system performance. Thus, selecting an appropriate nZVI dosage is essential when utilizing it as an enhancement strategy.

Moreover, ZVI could regulate microbial community structure through the effects on electron transfer and hydrogen partial pressure. This adjustment was considered independent of tetracycline concentration in systems exposed to tetracycline [126]. In anaerobic digestion systems, ZVI has been frequently reported to optimize the enrichment of hydrogenotrophic methanogens [211,83,93]. However, acetoclastic methanogens (*Methanotrix* and *Methanosarcina*) have also been observed as dominant methanogens in ZVI-amended systems [126,229,234]. This shift may be a consequence of different concentrations of ZVI or nZVI. For example, Zhu et al. [234] observed that *Methanoculleus* accounted for 54.7 % of the microbial community in reactors supplemented with 5 g/L ZVI, whereas *Methanotrix* and *Methanosarcina* became predominant when ZVI concentrations were increased to 10 and 20 g/L. Li et al. [93] reported that in a system with 50 mM nZVI, hydrogen transfer between species was the primary metabolic pathway rather than DIET. Additionally, ZVI could promote the abundances of VFA degraders. For instance, Meng et al. [111] observed that the addition of 5 g/L ZVI to a system using propionate as the sole carbon source increased the relative abundance of propionate-utilizing bacteria from 11.3 % (control) to 52.9 %. Meanwhile, Pan et al. [126] reported an increased relative abundance of *Syntrophorhabdus*, *Syntrophobacter*, and *Syntrophaceae* with nZVI supplementation.

In anaerobic digestion systems under tetracycline stress, ZVI has been found to optimize system performance. Pan et al. [126] investigated the effects of 3.8 g/L nZVI across varying tetracycline concentrations. The results showed that nZVI enhanced methane production in all systems, with the strongest improvement observed at the highest tetracycline concentration (150 mg/L). Moreover, Liu et al. [101] reported that in an anaerobic membrane bioreactor containing 0.1 mg/L tetracycline alongside mixed antibiotics, 2.6 g/L nZVI positively impacted membrane fouling reduction. Specifically, nZVI could mitigate the increase in transmembrane pressure and the decline in membrane flux. Additionally, the decreased distribution of tetracycline in biosolids suggested that nZVI might facilitate tetracycline biodegradation.

5.2.2. Magnetite (Fe_3O_4)

Due to its ease of synthesis, low cost, and non-toxic properties, magnetite is one of the most extensively studied and promising DIET

promoters [19]. Compared to ZVI, magnetite is considered more stable in anaerobic digestion systems [154]. Unlike ZVI, the enhancement effect of magnetite in anaerobic digestion systems could be primarily attributed to DIET. For example, Cruz Viggli et al. [38] observed that in reactors with magnetite, propionate degradation was less influenced by hydrogen partial pressure compared to reactors without magnetite. Subsequent hydrogen-based methanogenesis experiments showed that magnetite had no effect on the maximum methane production rate. This finding further confirmed that magnetite primarily enhanced DIET rather than interspecies hydrogen transfer in the system.

Attributable to its high conductivity, magnetite can serve as a direct conductive pathway to facilitate DIET [11,167]. Xing et al. [192] reported that the electrical conductivity of anaerobic sludge with 10 g/L magnetite was approximately 2.7 times higher than that of sludge without magnetite. Similarly, Jin et al. [77] found that 20 mM magnetite increased sludge conductivity from 8.7 μ S/cm to 25.6 μ S/cm. Another mechanism by which magnetite enhances DIET is the stimulation of gene expression related to electron transfer. Liu et al. [99] monitored the relative transcript abundances of key genes and found that magnetite supplementation promoted DIET by stimulating the expression of *pilA*, which encodes the structural pilin protein. Additionally, magnetite restored the function of the OmcS-deficient *Geobacter sulfurreducens* strain, demonstrating its potential to substitute for OmcS in DIET. However, their study also indicated that magnetite could not compensate for the loss of conductive pili, particularly for long-distance electron transfer. Similarly, Xiao et al. [189] reported that 0.35 g Fe/L magnetite nanoparticles upregulated c-type cytochrome genes in *Geobacter metallireducens*. Meanwhile, the expression of genes encoding NADH-quinone oxidoreductase, an auxiliary enzyme in the electron transport chain, was also enhanced by magnetite supplementation.

In addition to directly promoting the establishment of DIET, magnetite could enhance anaerobic digestion by influencing the microbial community. The redox cycling of Fe(II)/Fe(III) could facilitate microbial metabolism [10]. Magnetite has been found to enrich iron-reducing bacteria [9]. These bacteria utilize Fe(III) as an electron acceptor to degrade complex organic compounds through dissimilatory iron reduction [8]. This process may improve the removal of recalcitrant organics and enhance system stability under stress conditions. However, with this mechanism, Fe(III) in magnetite could compete with methanogens for electron donors. For example, Zhao et al. [227] investigated the effects of magnetite on different stages of anaerobic digestion using waste-activated sludge as the substrate, and indicated that magnetite competed for electrons with CH_3S-CoM , thereby inhibiting methanogenesis. However, the enhancement of hydrolysis and acidogenesis offset this inhibition, ultimately increasing methane production by 29.9 %.

In magnetite-added anaerobic digestion systems, *Methanotrix* has been identified as the dominant methanogen involved in DIET [192,78,93]. Additionally, other than iron-reducing bacteria, magnetite has been reported to enhance the abundance of VFA degraders. For instance, in the study by Xing et al. [192], the relative abundances of syntrophic propionate-oxidizing bacteria *Syntrophobacter* and *Smithella* reached 2.2 times and 4.8 times that of the control, respectively. Similarly, the enrichment of these bacteria with magnetite supplementation was also reported by Baek et al. [12].

Magnetite has been shown to improve system performance in the treatment of tetracycline-containing wastewater. For example, Zhao et al. [225] reported that magnetite enhanced COD removal efficiency and methane production under 100 mg/L tetracycline stress. Additionally, in magnetite-amended reactors, tetracycline served as the sole carbon source, facilitating both adsorption and biodegradation.

5.3. Composite materials

The use of composite materials can enhance the performance of

conductive materials in anaerobic digestion systems. Single iron-based materials often agglomerate, reducing surface active sites and hindering performance [108,153,208]. Carbon-based materials, with their high adsorption capacity, large surface area, and porous structure, can serve as effective biocompatible carriers to prevent agglomeration [153, 164]. This combination also increases electrical conductivity, promoting more efficient electron transfer [153].

The interaction between iron- and carbon-based materials has shown synergistic effects in treating pharmaceutical wastewater [40]. Composite materials can simultaneously improve COD removal and methane production while enhancing antibiotic and intermediate removal, ultimately reducing biological toxicity. Zhou et al. [230] synthesized ferroferric oxide nanoparticle assisted powdered activated carbon (FONP-PAC) through chemical co-precipitation to study tetracycline adsorption. The results indicated a maximum adsorption capacity of 215.5 mg/g at a 1:1 FONP/PAC mass ratio. This demonstrated the effectiveness of the composite material as a tetracycline adsorbent. Compared to sole PAC, FONP-PAC exhibited superior regeneration ability and magnetic separation properties, suggesting its potential for practical applications. Similarly, Zhang et al. [220] observed that Fe₃O₄-modified poplar wood biochar (MPBC) achieved optimal tetracycline adsorption under low ionic strength and neutral conditions. After four adsorption-desorption cycles, MPBC could retain 84.3 % of its initial adsorption capacity. In anaerobic digestion reactors, Zhang et al. [222] introduced GAC/nZVI mediators, with respective concentrations of 1200 mg/L and 1000 mg/L, into a tetracycline-containing wastewater system. The addition of GAC/nZVI increased COD removal efficiency by 12.1 % and the methane production rate by 73.0 %. Microbial community analysis revealed that GAC/nZVI promoted the enrichment of *Treponema*, a genus involved in complex compound degradation, potentially enhancing tetracycline degradation and system resilience. Additionally, acetogens such as *Syntrophomonas* and *Syntrophus*, along with *Methanotrix*-dominated methanogens, increased in abundance after GAC/nZVI addition. Therefore, composite materials have promising applications in anaerobic digestion systems treating tetracycline-containing wastewater.

6. Future perspectives

With the adoption of One Health approach, the management of tetracycline and other antibiotics in waste/wastewater should incorporate ecological safety in addition to energy recovery. This calls for an integrated Environment-Energy-Health perspective (Fig. 4). Anaerobic digestion has shown promise in treating tetracycline-containing wastewater, as well as renewable energy recovery. However, several critical research gaps remain, particularly in the areas of microbial ecology and functional gene dynamics to antibiotic stress. The following aspects should be addressed in future studies.

(1) Inhibition of tetracycline and microbial response strategies

Current studies on tetracycline inhibition in anaerobic digestion systems are mainly reactor-focused and limited to macro-level parameters such as methane production and COD removal efficiency. However, the responses of complex microbial communities under tetracycline stress deserve further mechanistic exploration. At the community level, microbial responses to antibiotic stress may be better understood with reference to ecological frameworks. When tetracycline serves as a disturbance factor, microbial responses can show structured responses, ultimately driven by niche adaptation [84]. Ecological theories, such as stress response models, may offer novel insights into microbial adaptation under tetracycline stress.

On the genetic level, variations in functional genes, especially those involved in antibiotic resistance, metabolism, and electron transfer, can reveal integrated microbial responses to tetracycline exposure. Microbial responses involve trade-offs between resistance, growth, and other functions under tetracycline pressure [134,233]. Therefore, future research should focus on mapping such trade-off processes between various functions, identifying optimal resource allocation strategies under antibiotic pressures, and establishing predictive models of microbial response to guide engineering operation in anaerobic digestion systems.

In addition, tetracycline rarely occurs individually in practical waste matrices. Co-existing antibiotics may produce synergistic or antagonistic effects, thereby altering microbial stress responses and community dynamics. However, current research on the interactions between different

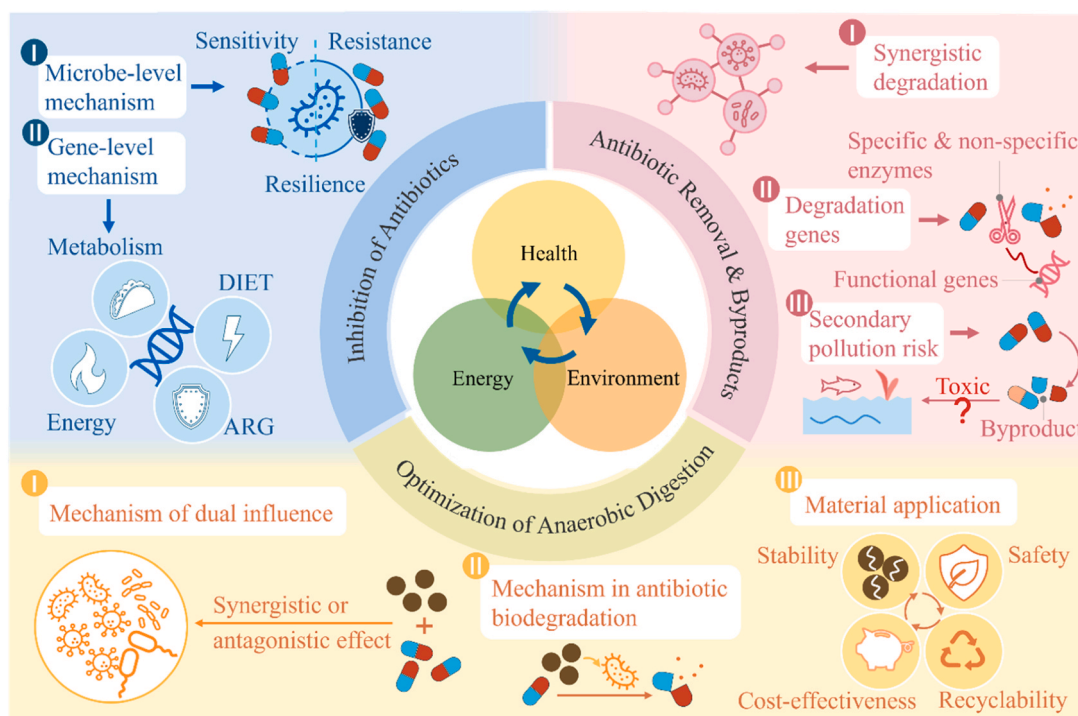


Fig. 4. Future research directions for tetracycline-contaminated anaerobic digestion systems.

antibiotics, as well as microbial responses under multi-antibiotic stress, remains limited. Future studies should address this gap through multi-factor experiments and ecological analyses.

(2) Degradation of antibiotics and functional redundancy in microbial communities

Current research on tetracycline degradation has largely focused on single strains, with limited attention to the complex microbial interactions in anaerobic digestion ecosystems. Many functional genes and enzyme systems potentially involved in tetracycline degradation remain unexplored. Non-specific enzymes and functional redundancy can compensate for inhibited degraders, enhancing system resilience. Therefore, future focus should be given to the integrating pathway prediction and functional gene annotation to identify non-specific degraders and genes.

In addition, the ecotoxicity and environmental risks of intermediate metabolites produced during tetracycline biodegradation remain insufficiently studied. Existing research has primarily focused on the reduction of tetracycline concentrations, while neglecting the potential for byproducts to retain antibiotic activity. Future studies should test the toxicity of tetracycline breakdown products, not just track the loss of the parent drug. Meanwhile, toxicity assessment models can be considered. By linking pathway analysis with computer-based toxicity prediction, early identification of toxic intermediates and optimization strategy guidance may be achieved.

(3) Optimizing strategies for anaerobic digestion performance

The application of conductive materials has shown considerable potential for enhancing anaerobic digestion by facilitating interspecies electron transfer, improving substrate degradation, and alleviating the inhibitory effects of tetracycline. Their effects work through two connected levels: the physico-chemical layer (conductivity, redox, adsorption) and the microbial-ecological layer (community restructuring, functional gene shift). Future studies should integrate multi-omics approaches to reveal material-microbe interactions under antibiotic stress. A deeper understanding of this coupling between material properties and ecological regulation is essential for mechanism-oriented design in practical applications.

Meanwhile, most existing studies have focused on single materials. However, comparative evaluations of different conductive materials under consistent experimental conditions are still lacking. Systematic comparisons of different conductive materials under standardized conditions are needed to assess the relative advantages, limitations, and potential risks of various materials.

Moreover, excessive dosing of some materials (e.g., nZVI) may impair microbial activity. Future research should focus on balancing performance improvement with stability, cost-effectiveness, and environmental sustainability of conductive materials in practical applications. Developing an optimization framework that integrates treatment performance with environmental adaptability will be essential to achieve synergistic mitigation of tetracycline toxicity while enhancing energy recovery.

CRediT authorship contribution statement

Bang Du: Writing – review & editing, Methodology, Formal analysis.
Yuyin Wang: Writing – original draft, Methodology, Investigation, Formal analysis.
Guangxue Wu: Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Guangxue Wu reports financial support was provided by Sustainable Energy Authority of Ireland. Yuyin Wang reports financial support was provided by China Scholarship Council. Guangxue Wu reports financial support was provided by Galway University Foundation CLG. If there are

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

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.jhazmat.2025.139378](https://doi.org/10.1016/j.jhazmat.2025.139378).

Data availability

Data will be made available on request.

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