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Review

A review on characterizing the metabolite property of anammox sludge by spectroscopy

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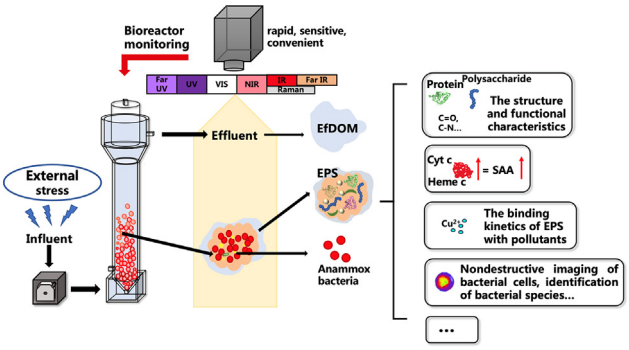
HIGHLIGHTS

- Spectral techniques applied for anammox system was summarized.
- The correlation between anammox sludge activity and its key metabolites was analyzed.
- The feasibility of monitoring anammox reactor by spectral technology is prospected.

GRAPHICAL ABSTRACT



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ABSTRACT

As one of the most promising autotrophic biological nitrogen removal technology, anaerobic ammonia oxidation (anammox) has gained intense attention for the past decades and several full-scale facilities have been implemented worldwide. However, anammox bacteria are easily affected by disturbed external environmental factors, which commonly leads to the fluctuations in reactor performance. The response of anammox sludge to external stress results in changes in components and structural characteristics of intracellular and extracellular polymer substances. Real-time and convenient spectral analysis of anammox sludge metabolites can give early warning of performance deterioration under external stresses, which is of great significance to the stable operation of bioreactor. This review summarized the research progress on characterizing the intracellular and extracellular metabolites of anammox sludge through spectroscopic techniques. The correlation between anammox sludge activity and its key metabolites was analyzed. Also, the limitations and future prospects of applying spectral analytical techniques for anammox bioreactor monitoring were discussed and outlooked. This review may provide valuable information for both scientific study and engineering application of anammox based nitrogen removal technology.

Contents

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1. Introduction

Due to ever-increased human activities such as burning of fossil fuels and excessive use of nitrogen fertilizers, numerous nitrogen has been discharged into our living environment, which causes severe environmental and even climatic issues. Microorganism driven nitrogen conversion process is one of the most economic wastewater treatment technologies and among which anaerobic ammonia oxidation (anammox) process has gained intense attention (Fux et al., 2002). Anammox refers to the process of ammonia oxidation into gaseous nitrogen and nitrate with nitrite as electron acceptor under anoxic condition (Strous et al., 1998; van de Graaf et al., 1995). Compared with the traditional biological denitrification process, anammox process is favored for its advantages of no requirement of external carbon source, high volumetric nitrogen load, low excess sludge yield, etc. (Gao et al., 2014; Jetten et al., 2005; Lackner et al., 2014). At present, more than one hundred full-scale anammox process facilities have been installed at global scale (Ma et al., 2021b). The engineering success of anammox process shed a light on the possible treatment of wastewater with reduced energy and carbon consumption.

However, variations in environmental factors such as temperature, pH, and dissolved oxygen will significantly affect the sludge activity, which may lead to fluctuations in the operation performance of reactor. Moreover, anammox sludge is susceptibly interfered by heavy metals, salts, organic matter, etc., resulting in its growth and metabolism changes (Liu et al., 2018; Tang et al., 2011; Yang et al., 2013; Zhang et al., 2018). Long-term exposure of sludge under adverse environmental conditions would cause a series of changes in the metabolites secretion by anammox sludge and is even possible to cause cell death, thus further prolonging the start-up time of bioreactor. From this point of view, monitoring and analyzing the response characteristics of anammox sludge's metabolites to the external factors variations is of great significance for the stable operation of bioreactor.

Real-time acquiring the bioreactor operation status under external stress is fundamental for its optimization. Multivariate spectroscopy technology has been widely applied in the field of bioreactor monitoring. Compared with other measurement techniques, spectroscopy has the advantages of being rapid, sensitive, convenient, and reducing the usage of chemical reagents (Bourgeois et al., 2001; Fogelman et al., 2006; Wiberg et al., 2003). At present, most studies have adopted spectral technique to analyze the dynamic changes of anammox sludge metabolites, i.e., extracellular polymer substances (EPS) and effluent soluble organic matter, under the external stress.

This paper aims to conduct a literature review on the application of spectral technology for characterizing anammox sludge activity. The types and changes of metabolites characterized by using the spectral technology were summarized. Correlation between anammox sludge activity and its key metabolites was analyzed. At last, the future prospects were outlooked.

2. Metabolism and characteristic products of anammox sludge

2.1. The central metabolic pathway of anammox bacteria

As early in 1997, Van et al. (1997) found that anammox reaction takes NH_3 as the electron donor, NO_2^- as the electron acceptor, hydroxylamine (NH_2OH) and hydrazine (N_2H_4) as important metabolic intermediates, through ^{15}N labeling experiment. Strous et al. (1998) calculated the stoichiometric equation of anammox reaction as follows:

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02\,N_2 + 0.26NO_3^- + 2.03H_2O + 0.066\,CH_2O_{0.5}N_{0.15}$$

Subsequently, Jetten et al. (2001) concluded that nitrite reductase (Nir) reduces NO_2^- to NH_2OH, and then hydrazine hydrolase (HH) catalyzes the condensation of NH_2OH and NH_3 to N_2H_4. However, Strous et al. (2006) analyzed the genome of *Candidatus* Kuenenia stuttgartiensis and found that it lacked reductase genes encoding NO_2^- to NH_2OH. Whereas an oxidoreductase gene encoding NO_2^- to NO was found in the genome of *Candidatus* Kuenenia stuttgartiensis. Therefore, Strous et al. (2006) finally summarized the metabolic pathway of anammox bacteria as the following processes (Fig. 1a).

Firstly, the cytochrome *cd*1 nitrite reductase located on the cytoplasm side of the cell membrane reduces NO_2^- to NO. HH condenses NO and NH_4^+ into N_2H_4. Finally, the hydrazine oxidoreductase (HZO) on the anammoxsome side oxidizes N_2H_4 to N_2 (Jetten, 2009; Kostera et al., 2008). The electrons released during this process are transferred to Nir and HH through cytochrome *c*, ubiquinone, cytochrome *bc*1 complex and other cytochrome *c*. One electron is transferred to Nir and three electrons to HH (Kostera et al., 2008). With electron transport, protons are expelled to the outside of the anammoxsome membrane. The proton gradient formed on both sides of the membrane drives the synthesis of ATP and NADPH, which supports the growth of anammox bacteria (Shimamura et al., 2007; Shimamura et al., 2008). In recent years, it has been found that anammox bacteria have the ability of extracellular electron transfer. Shaw et al. (2020) found that anammox bacteria can transfer electrons to extracellular insoluble electron receptors such as graphene oxide or electrodes. In such system, NH_4^+ was completely oxidized into N_2 by anammox sludge without accumulation of NO_2^- and NO_3^-.

The central metabolism of anammox bacteria is catalyzed by a variety of enzymes and deciphering the crystal structure of protease is crucial for deep

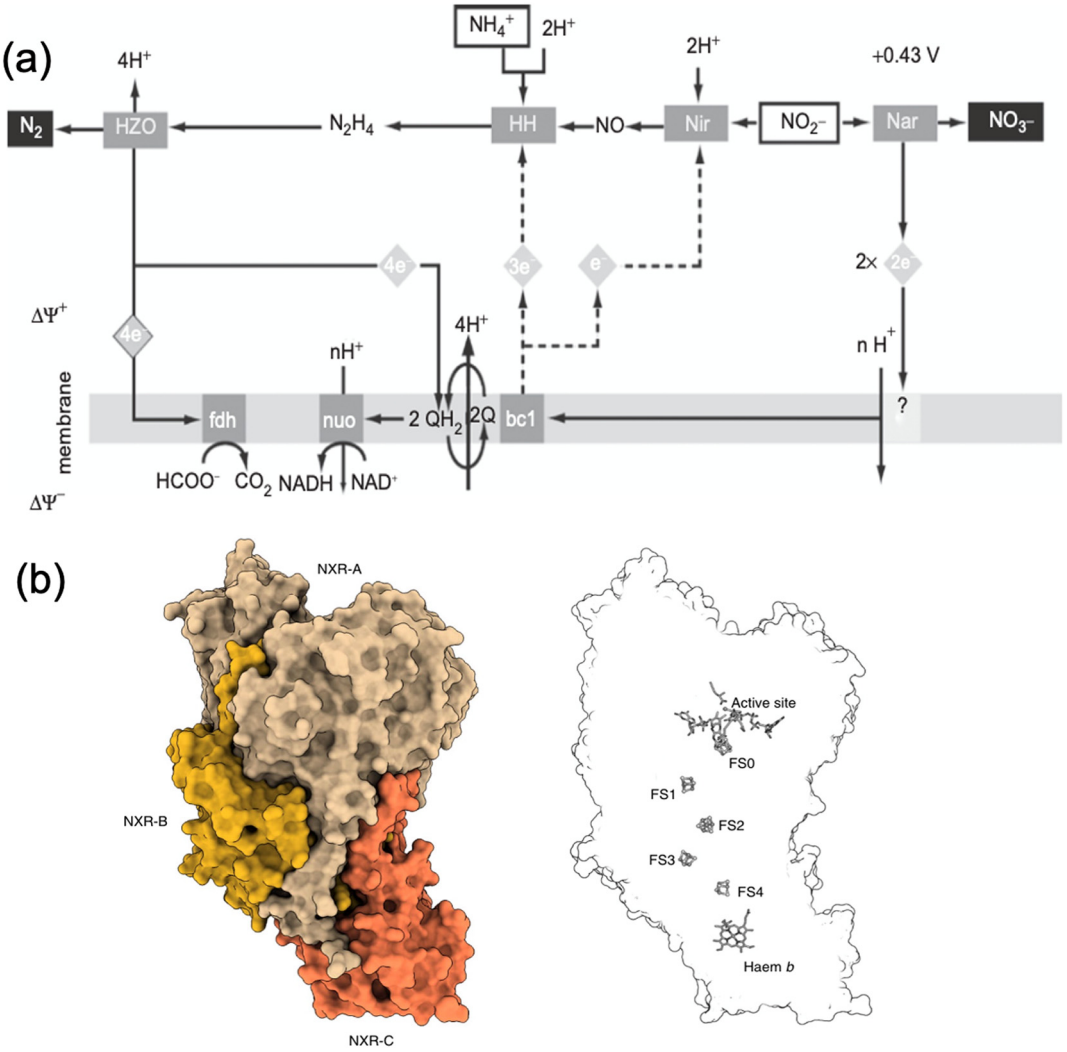

Fig. 1. (a) The metabolic pathways of K. stuttgartiensis and nitrate reductase to generate electrons for the acetyl-CoA pathway (Jetten, 2009). (b) Molecular structure and internal structure of nitrite oxidoreductase (Chicano et al., 2021).

understanding its function. Recently, Chicano et al. (2021) used cryo-electron tomography and helical reconstruction techniques to resolve the nitrite oxidoreductase structure in anammox bacteria that can transform nitrite into nitrate. As shown in Fig. 1b, there is a notch on one side of the nitrite oxidoreductase to fix and then convert nitrite to nitrate. The electrons produced by the reaction are transferred to the other side of the nitrite oxidoreductase, followed by using for cell metabolism.

2.2. Intracellular and extracellular metabolites of anammox sludge

Anammox bacteria excrete a large number of metabolites during nitrogen conversion, and these metabolites can be divided into two categories: intracellular and extracellular components. Extracellular metabolites mainly include soluble microbial products (SMP) and EPS. The production of SMP is related to the consumption of microbial matrix and cell apoptosis, while the EPS secretion is usually affected by changed environmental conditions (Laspidou and Rittmann, 2002; Xu et al., 2013; Zhang et al., 2017).

SMP in anammox bioreactor effluent is commonly a kind of complex organic matter, which is released by microorganisms along with substrate metabolism, cell growth or death (Boero et al., 2001; Jarusutthirak and Amy, 2007). SMP is mainly composed of proteins, polysaccharides and humus, whose composition and content usually depends on microbial activity (Noguera et al., 2010; Rosenberger et al., 2006).

Similar to SMP, EPS is synthesized during substrate metabolism and microbial growth. EPS attaches onto cell surface through complex interactions to form a three-dimensional network, which plays an important role in maintaining the structure and function of microbial aggregates. Proteins and polysaccharides in EPS greatly affect the surface charge, hydrophobicity and spatial structure of sludge. Hou et al. (2015) found that a large number of hydrophobic amino acids and proteins in EPS contributed to the hydrophobic interaction, thereby improving the aggregation ability of anammox sludge.

The intracellular metabolites of anammox sludge include polysaccharides, phosphodiesters, proteins, and riboflavin, among which flavin mononucleotide and flavin adenine dinucleotide are the two common forms of riboflavin in cells (Li et al., 2020a). These two active substances are commonly used as prosthetic groups of oxidoreductases in cell metabolism, such as nicotinamide adenine dinucleotide, dehydrogenase and xanthine oxidase, which are vital electron transporters during cell metabolism (Heikal, 2010).

3. Analysis of anammox sludge metabolites by spectroscopic technique

3.1. Ultraviolet-visible absorption spectrum

3.1.1. Detection of cytochrome c and heme c

Cytochrome *c* is an important component of electron transport chain and heme is the key chromogenic substance of cytochrome *c* (Nelson and Cox, 2005). The ultraviolet-visible absorption spectrum of oxidized or reduced

state of cytochrome *c* is different (Spinazzi et al., 2012). As shown in Fig. 2, the spectrum of reduced cytochrome *c* standard has three specific absorption bands, centered at 549 nm, 520 nm, and 412 nm. The oxidized cytochrome *c* has two specific absorption bands, which are 528 nm and 409 nm, respectively. Compared with the cytochrome *c* standard, anammox sludge has almost the same absorption band (Kang et al., 2020). Due to the exchange of Fe^{2+} and Fe^{3+} in the center of porphyrin ring, cytochrome *c* cyclically changes between the reduced and oxidized state, showing typical changes in visible spectra (Kang et al., 2020).

Kang et al. (2020) used ultraviolet-visible absorption spectrum to investigate the correlation between the activity and color of anammox sludge. They found that the specific absorption peak position of anammox sludge under different conditions is consistent with the cytochrome *c* standard solution, implying that the cytochrome *c* is its primary color source. Hydrazine dehydrogenase contains a large cytochrome *c* electron transport network. The higher the enzyme activity, the more cytochrome *c* in the reduced state. Therefore, the hydrazine dehydrogenase activity is closely related with the color change of sludge. By measuring total cytochrome *c* content and cytochrome *c* synthetase gene abundance, chroma was correlated with anammox bacterial abundance using visible spectrum. The abundance and activity of anammox bacteria can be evaluated according to the correlation equation. Using redundancy analysis, they demonstrated that at the genus level, 69.6% of the total variation of microbial community according to heme c content was positively correlated with the relative abundance of *Candidatus* Kuenenia. Fig. 3 shows the relationship between the color and activity of anammox sludge (Kang et al., 2020). Similarly, heme c is a special cytochrome contained in anammox sludge and its content can also be used to characterize the activity of anammox sludge. Kartal et al. (2012) found that 30% of the total protein in anammox sludge was from heme c. Heme c content is positively related to the sludge color and activity. The concentration of heme c is usually determined by pyridine heme spectrophotometry (Ali et al., 2013). The nitrogenous ligands of heme are replaced by pyridine under alkaline conditions. The characteristic peak of pyridine-cytochrome complex is formed at 557 nm. Heme c content was determined by spectrometric difference (μmol·g^{-1} VSS) between reduced sodium hydrosulfite and oxidized potassium ferricyanide, with an extinction coefficient of 23.97 mM cm^{-1} (Berry and Trumpower, 1987).

At present, a significant positive correlation between heme c content and anammox sludge activity has been confirmed in a variety of studies (Kartal et al., 2012; Kang et al., 2020; Qiao et al., 2013). The increase of intracellular heme c amount can improve the activity of hydrazine dehydrogenase, which is beneficial for the growth of anammox sludge (Kang et al., 2020). From this point of view, the change of heme c content can reflect the metabolic activity of bacteria. Under the external stresses of inorganic salts, high temperature, heavy metals and antibiotics, a declined specific anammox activity and heme c content has been observed (Liu et al., 2008; Ma et al., 2019; Zhang et al., 2015; Zhang et al., 2016). Appropriate increase of Fe^{2+} was beneficial to enhance heme c synthesis and improve hydrazine dehydrogenase activity, thus promoting the growth of anammox sludge (Qiao et al., 2013). However, Xing et al. (2014) proposed that during the preservation of anammox granular sludge, intracellular heme c content would decrease slowly, even no inhibitor was dosed. Therefore, during the storage stage of anammox granular sludge, heme c cannot be directly applied to indicate activity attenuation.

3.1.2. Analysis of binding kinetics

Ultraviolet-visible absorption spectrum can also be applied to study the binding kinetics of anammox sludge EPS with pollutants. The binding constants could be estimated by scanning the ultraviolet-visible absorption spectrum of EPS-pollutant mixture. Li et al. (2020b) reported the binding constant of EPS and Cu^{2+} as 4.68×10^4 L·mol^{-1} according to the double reciprocal formula (Purcell et al., 2000).

$$\frac{1}{A - A0} = \frac{1}{a \cdot K \cdot C} + \frac{1}{a} \quad (1)$$

where A is the absorbance of EPS-Cu^{2+} mixture, A_0 is the absorbance of EPS, a is constant, and C is the concentration of Cu^{2+}. The absorption spectrum of EPS was redshifted with the increase of Cu^{2+} content, indicating the interaction between EPS and Cu^{2+}. pH significantly affects the binding

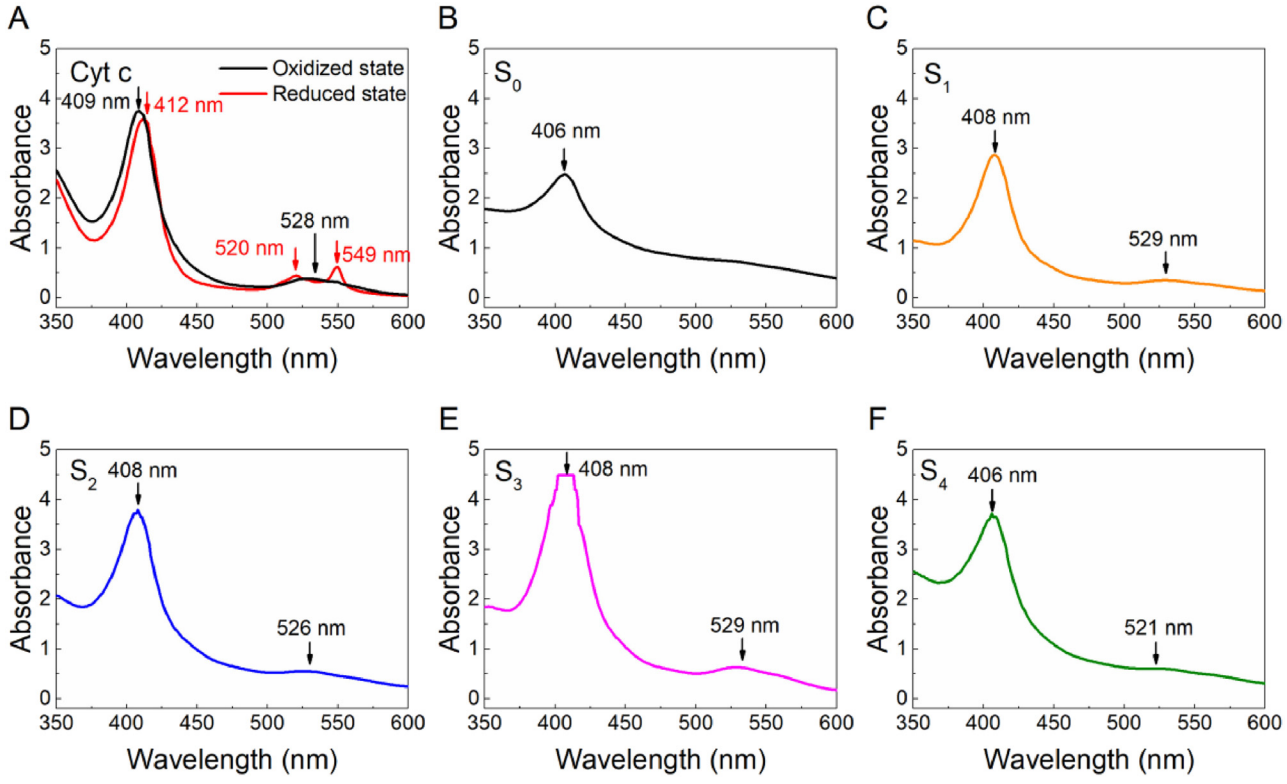

Fig. 2. Visible spectra of standard cytochrome c and crude enzyme of anammox sludge at different nitrogen loading rates (Kang et al., 2020).

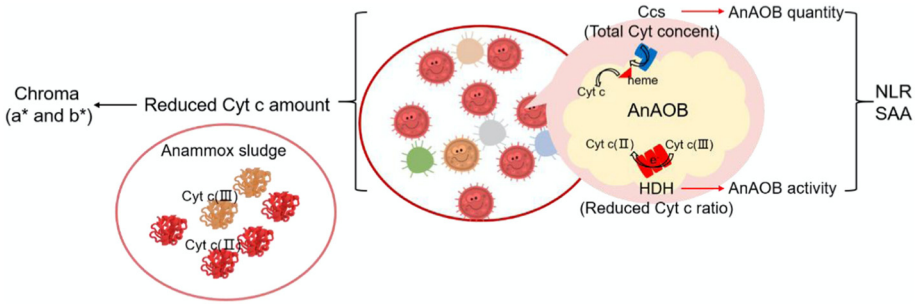

Fig. 3. The relation of anammox sludge chromaticity and specific anammox activity (Kang et al., 2020).

of Cu^{2+} onto EPS (Li et al., 2020b). Similarly, Ma et al. (2021a) explored the binding characteristics of As(III) onto EPS by using ultraviolet-visible absorption spectrum. It was found that with the increase of As(III) concentration, the intensity of the two absorption bands at 210–220 nm and 255–265 nm increased, and there was an obvious red shift phenomenon. The formation of As(III)-EPS complex was identified. The binding constant K was calculated to be 1.97×10^6 L·mol^{-1} through linear regression. Based on the above results, ultraviolet-visible absorption spectrum is a useful tool to obtain the thermodynamics parameters of EPS with pollutants, which is beneficial to provide in-depth information for mechanism analysis.

3.2. Three-dimensional fluorescence excitation-emission matrix spectrum (3D-EEM)

3.2.1. 3D-EEM coupled parallel factor analysis

Microorganism contains natural intracellular and extracellular fluorophore groups, such as proteins, enzymes, coenzymes, cytochrome and other metabolites (Pons et al., 2004). The fluorescence change of reduced nicotinamide adenine dinucleotide has been used to track the living status of microorganisms under the cultivation conditions changes during anaerobic wastewater treatment (Reynolds and Ahmad, 1997; Yang et al., 2015). The EPS secreted by anammox sludge contains a large number of aromatic structures and unsaturated fat chains, most of which are fluorescence substances. Herein, the change of fluorescence spectra can provide information about the structure, functional groups, configuration and heterogeneity of sludge EPS components (Farabegoli et al., 2003).

3D-EEM technology can obtain the fluorescence characteristics of substances by changing the excitation and emission wavelengths simultaneously. This technology has the advantages of high sensitivity, good selectivity and no damage to samples (Hou et al., 2017; Sheng and Yu, 2006). Therefore, it has been widely used in the analysis of organic matter. Since the fluorescence spectra of different components are easy to overlap, it is difficult to obtain reliable information only by the variation of fluorescence intensity at a specific wavelength. Parallel factor analysis can improve spectral resolution by extracting small spectral variation information from highly overlapping signals (Sanchez et al., 2013). Therefore, 3D-EEM combined with parallel factor analysis can be used to characterize dissolved organic matter in the effluent of bioreactor according to the corresponding spectral peak position (Liu et al., 2011). Table 1 shows the corresponding characteristic peak regions of various fluorescent substances.

3.2.2. Application of 3D-EEM technology in anammox system

In earlier studies, researchers conducted the qualitative analysis of fluorescent substances in anammox sludge EPS and reactor effluent. The analysis of *Candidatus Brocadia fulgida* EPS by Kartal et al. (2008) showed that it has two maximum excitation wavelengths of 352 nm and 442 nm and two maximum emission wavelengths of 464 nm and 521 nm. The two fluorescence peaks in Ca. *Brocadia*-dominant anammox sludge EPS are originated from the cellular metabolites coenzyme NADH and riboflavin, respectively. Moreover, the molar concentration of these two fluorophores in Ca. *Brocadia*-dominant anammox sludge EPS is higher than that of nitrifying sludge and Ca. *Jettenia*-dominant (Feng Ying et al., 2017). Three dominated fluorophores in sludge EPS were tryptophan, tryptophan type-proteins and humic acids (Liu et al., 2019). Ruscalleda et al. (2014) analyzed the bioreactor effluent and it was pointed out that the fluorescence component was mainly composed of proteins (excitation peaks less than 240, 280 and 330 nm, and emission peaks at 346 nm) and humic acids (excitation peaks less than 240, 355 and 420 nm, and emission peaks at 464 nm).

Lately, increased studies adopted 3D-EEM technology as an efficient tool for in-depth analysis of the intracellular and extracellular metabolites produced by anammox sludge under different influential conditions. For example, Hou et al. (2017) used 3D-EEM fluorescence spectroscopy to characterize the intracellular substances during the operation of anammox reactor. They found that the substances centered at 420 nm and humic acid were strongly correlated with the nitrogen removal rate of anammox sludge. These two intracellular protein-like peaks are highly correlated with biomass growth rate. Other studies have confirmed that there is a certain correlation between the fluorescence intensity of protein-like substances in effluent and the performance of reactor. For instance, Li et al. (2021) found that with the increase of tetracycline concentration, the fluorescence intensity of protein-like substances in anammox reactor decreased significantly. Adding Fe^{2+} to anammox reactor can promote the growth of anammox bacteria. Wang et al. (2019) found that the main fluorescence components of anammox bioreactor effluent were protein-like and fulvic-like substances, and the protein-like fluorescence intensity increased significantly with the increase of Fe^{2+} concentration. Liu et al. (2015) adopted 3D-EEM spectroscopy to characterize the effluent samples during the start-up of anammox reactor. They found that the main fluorescence groups were protein-like, fulvic-like and humic acid like substances during the start-up stage. Along with the reactor operation, the contents of several fluorescent components gradually decreased, while under the organic matter stress, the protein-like components of anammox reactor effluent increased rapidly. Li et al. (2020a) found that the fluorescence component of protein in anammox reactor effluent was positively correlated with specific anammox activity ($R = 0.976$, $p = 0.024 < 0.05$), through Pearson correlation analysis. Other studies have also showed that effluent fluorescence spectra of dissolved organic matter changes in advance than that of nitrogen concentration (Lu et al., 2017; Ruscalleda et al., 2014; Zhang et al., 2019). The operation status of anammox bioreactor is possibly informed before its complete deterioration by analyzing metabolite changes using spectroscopy, allowing us to take early response measures. Herein, effluent fluorescence substances might be used as a sensitive indicator to track the bioreactor performance.

In addition to semi-quantitative analysis of metabolites, EEM is also efficient in investigating the interaction between anammox sludge and pollutants. Liu et al. (2019) reported that the interaction of tryptophan, tryptophan-like proteins and polysaccharides of granular sludge EPS with that of tetracycline was controlled by hydrophobicity. Zhang et al. (2021) concluded that the increase of salinity altered the surface groups of EPS, thereby leading to a decreased hydrophobicity of anammox granules. Gu (2019) believed that it was the decrease of tyrosine and tryptophan content and the increase of humic acid content that led to the floating of anammox

Table 1
Characterizing the changes of anammox intracellular and extracellular metabolites by 3D-EEM under the stress of external factors.

Reactor type	Nitrogen load	External stress	Typical fluorescence peaks	Ascertained metabolites	Changes		References
UASB		Increased tetracycline concentration (0–20 $mg{\cdot}L^{-1}$)	225/340	Tyrosine-like aromatic protein	The fluorescence intensity decreased with the increase of tetracycline concentration and a new fluorescence peak appeared		(Li et al., 2021)
			275/345	Soluble microbial byproduct-like substances			
		Add tetracycline	220/360	Ascribed to the tyrosine/tryptophan amino acid	Decreased as the concentration of tetracycline increased	Reduced hydrophobicity of microenvironment	(Liu et al., 2019)
			280/380	Tyrosine/tryptophan type-proteins		The change of protein structure	
			320/390	Humic acid			
					When the concentration of tetracycline was greater than 50 $mg{\cdot}L^{-1}$, the fluorescence intensity decreased dramatically to nearly quenching	The binding sites were occupied and the structure of the peptide chains was severely disrupted	
	2.58 kg $N{\cdot}(m^3\ d)^{-1}$	Increased salinity	200–255/335–380	Trypto-phan protein substances	The emission wavelength of peak blueshifted, and the absorption wavelength redshifted and then blueshifted	The redshift and blueshift of the fluorescence peaks indicated that the increase in salinity altered the functional groups of microbial metabolites, which further affected the sludge aggregation	(Zhang et al., 2021)
			200–250/280–335	Tyrosine protein sub-stances	The emission wavelength of peak was blue- shifted, and the absorption wavelength redshifted and then blueshifted		
			250–335/280–380	Humic acid-like substances			
			250–380/380–540	Corresponded to soluble microbial byproducts			
MBR	The nitrogen loading rate gradually reached 0.42 kg $N{\cdot}(m^3\ d)^{-1}$	High substrate concentration influent	225/342	Aromatic (including tryptophan and tyrosine) protein-like substances	The fluorescence intensity increased with the operation of the reactor	Intracellular tryptophan and aromatic protein-like substances were strongly correlated with nitrogen removal rate and mixed liquid volatile suspended solids	(Hou et al., 2017)
			280/347	Tryptophan protein-like substances			
			420/470	Humic acid	Fluorescence intensity decreased significantly	420 nm peak had good ability in reflecting sludge activity and nitrogen removal rate	
			250/470	Humic acid	Fluorescence intensity decreased		
UASB	7.22 kg $N{\cdot}(m^3\ d)^{-1}$	Temperature reduction	290/330	Tryptophan protein-like substances	The fluorescence intensity increased rapidly from 35 °C to 15 °C, and decreases further when the temperature dropped to 10 °C		(Li et al., 2020a)
			350/430	Polycarboxylate-type humic acid-like substances			
			280/350	Tryptophan protein-like	Increased fluorescence intensity		
			270/440	Polyaromatic-type humic acid-like	The fluorescence intensity was relatively stable		
			370/520	Riboflavin- related			

granular sludge through fluorescence spectrum analysis. Guo (2017) came to a similar conclusion, who found that proteins in the anammox sludge EPS play an important role in the formation of microbial aggregates.

3.3. Fourier transform infrared spectroscopy (FTIR)

FTIR can be used to detect specific molecular structures and functional groups of organic matter (Liang et al., 2009). The superposition of the infrared absorption spectrum of group will show the corresponding absorption band in a specific spectral region. These absorption bands can shift due to changes in external conditions or structural changes. Therefore, the changes of groups can be analyzed from the absorption peak size, intensity, shape, and band position. FTIR technology was efficient in analyzing functional groups changes and identifying the types of organic matter in EPS, so as to further explore the influence of heavy metals, salinity and other stimuli on anammox sludge (Chen et al., 2003).

3.3.1. Identification of functional groups

Anammox sludge surface functional groups are mainly composed of negatively charged carboxyl group, hydroxyl group and positively charged amine group (Hou et al., 2015; Liu et al., 2015). Electronegative groups dominate the bacterial aggregation due to the hydrophobic effect and bridging ability of EPS. Wu et al. (2019) found that different concentrations of Ni^{2+} would alter the types of functional groups of bound EPS. The addition of Ni^{2+} resulted in the appearance of NiO signal in the fingerprint region of bound EPS and stretched vibrations of OH and NH bonds of carboxyl and hydrocarbon groups. Huang and Wu (2020) used FTIR to study the effect of rare earth elements on the nitrogen removal performance of anammox and found that the functional groups related to carboxylic acids, proteins and polysaccharides (amide I, II and, III bands) were the main active components in the reaction with La(III). Tang et al. (2021) explored the elemental composition and functional group changes of anammox sludge in the presence of poly-butylene succinate and polyvinyl chloride microplastics. Results showed that the intensity of CH and COC

vibration peaks was increased after the addition of 0.5 $g{\cdot}L^{-1}$ poly-butylene succinate, leading to an increased content of methyl and polysaccharide (Tang et al., 2021; Yang et al., 2020). Gu (2019) employed infrared spectroscopy to investigate the granular sludge floating issue during bioreactor operation. Results showed that the CO and COH contents in the tightly bound EPS of floating particles were increased and the sludge surface was rich in negative charges. Therefore, the granular structure is loose and the stability is reduced, which leads to the floating issue.

3.3.2. Analysis of protein secondary structure

Protein is a major component of anammox sludge EPS and its composition characteristics greatly affect the structure and function of microbial aggregates (Jia et al., 2017; Zhang et al., 2021). The amide I region of infrared spectrum is often used to explore the secondary structure of proteins while the amide III region can distinguish α-helix and random curl structures (Zhang et al., 2021). When the content of α-helix is low and the content of β-sheet and random coils is high, protein molecules tend to have a looser structure. As a result, the hydrophobic groups within the EPS are fully exposed, which endows microorganisms with high hydrophobicity (Zhang et al., 2021). The α-helix/(β-sheet + random coil) ratio is also commonly used to characterize the composition of protein secondary structures (Jia et al., 2017; Zhang et al., 2021).

With the addition of Cu^{2+}, Li et al. (2020b) found that the secondary structure of protein in EPS, such as α-helix, β-sheet, random coil and antiparallel β-sheet/aggregation chain structure, were destroyed, resulting in a loose protein structure. In order to further analyze the discrepancy of protein secondary structure in EPS of anammox sludge with different particle sizes, Yang (2019) performed peak separation analysis on amide I region. Results showed that β-sheet accounts for about 40% of total protein secondary structure content. At the same time, it was found that granules with larger particle size contained a higher hydrophobic protein content, which in-turn promoted the aggregation of anammox particles.

3.4. Two-dimensional correlation spectroscopy (2D-COS)

2D-COS is a spectrum processing method based on statistical principles. It considers the change of spectral intensity under the external disturbance variable t between T_{min} and T_{max}, that is, 2D-COS reflects the change of dynamic spectrum (Pätzold et al., 2008). 2D-COS can be used to determine the order of spectral intensity change according to the generated synchronous and asynchronous correlation spectrum. Synchronous correlation spectrum represents the degree of coordination while asynchronous spectrum represents the sequence of spectral intensities between two dynamic optical signals (Li et al., 2020a).

Li et al. (2020a) used synchronous fluorescence spectrum to analyze the change order of intracellular and extracellular metabolites of anammox sludge under temperature disturbance (Fig. 4). It was found that humic acid components in EPS responded preferentially to temperature changes than that of protein-like components. During the low temperature (10 °C) period, the contents of polysaccharide, phosphate diester, protein and riboflavin in intracellular polymer gradually increased. As for the change of anammox sludge under Cu^{2+} stress, the carboxyl groups of proteins in EPS preferentially respond to Cu^{2+} than that of polysaccharides and hydrocarbons (Li et al., 2020b). Huang and Wu (2020) reported that the response sequence of functional groups in EPS to La(III) was carboxylic acid > polysaccharide > amide III band > amide II band > amide I band.

3.5. Raman spectrum

Raman spectroscopy can obtain structural information by analyzing the structure of different incident light frequencies, molecular vibration and rotational energy levels. Confocal Raman microscopy can perform non-destructive and accurate identification of cell micro-domain structures in three-dimensional space (Cirpus et al., 2005; Pätzold et al., 2006). At present, confocal Raman spectroscopy was applied to analyze the cell structure, characteristic components (such as coenzyme c), and species of anammox bacteria. It was also a powerful tool to study the anammox granular sludge and biofilm structure properties (Hu, 1993; Kirschner et al., 2001).

Anammox bacteria contain a high content of heme protein, while resonance Raman spectra can distinguish the differences between the heme proteins (Desbois, 1994). Heme protein is attached to the heme group by a CXXXH motif. Histidine, two cysteines are bound to heme iron ion and two thioether bonds, respectively. Therefore, distortion of heme groups by surrounding proteins leads to characteristic Raman spectra changes (Pätzold et al., 2006). Confocal resonance Raman microscopy can be used as a new method for nondestructive and rapid identification of bacteria in mixed microbial communities due to the strong characteristic bands of cytochrome *c* (Li et al., 2014). Pätzold et al. (2006) used Raman spectroscopy to analyze the microbial community in the anammox sequencing batch reactor. As shown in Fig. 5, they identified two distinct microbial communities within the same particle. The intensity distribution of the light band at 750 cm^{-1} is the main resonance Raman band of cytochrome *c*. The Raman band at 2900 cm^{-1} was ascribed to CH stretching. Anammox bacteria in the blue box and *Nitrosomonas* in the yellow box could then be identified based on data analysis. Confocal resonance Raman microscopy can be used to in-situ identify anammox granules. Kniggendorf and Meinhardt Wollweber (2011) used this technique to observe anammox granular sludge. They found a symbiosis between *Nitrosomonas* and anammox bacteria in the outer layer of the granules. At the same time, the added polymorph TiO_2 was in the outer layer of granules and showed different toxicity to bacteria. Confocal Raman spectroscopy could provide a unique

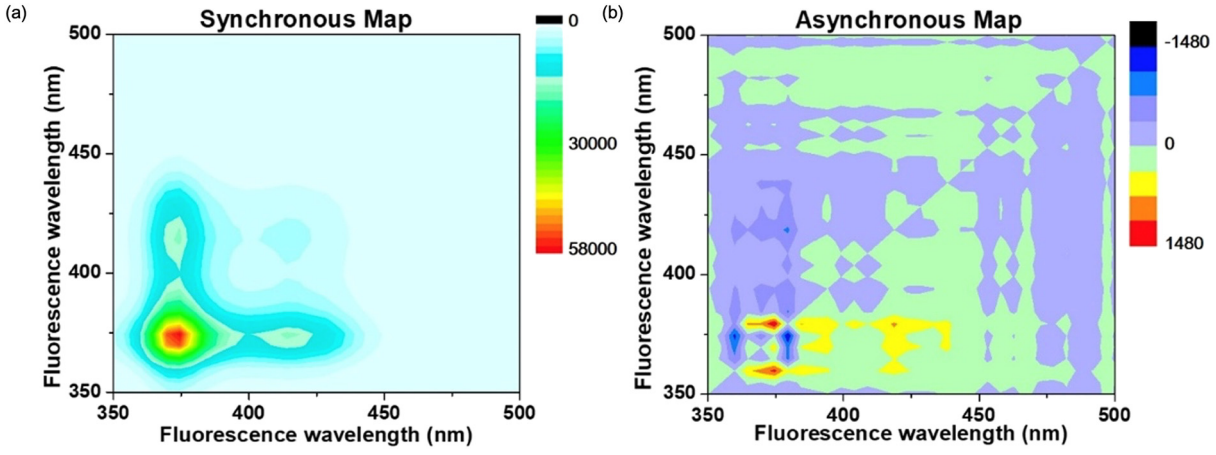

Fig. 4. Two-dimensional correlation spectra generated by EPS synchronous map (a) and asynchronous map (b) fluorescence spectra under temperature change (Li et al., 2020a).

spectral fingerprint without destroying the biofilm, which is useful for microbial analysis.

3.6. Soft X-ray absorption spectrum

Lately, X-ray imaging technology has been developed through the combination of synchrotron radiation X-ray sources. Synchrotron soft X-ray nano-computed tomography is a nondestructive in situ imaging technique. This technique enables a lossless three-dimensional image of the entire hydrated cell without any staining, sectioning, dehydration or embedding pretreatment. Peng et al. (2019a) first used synchrotron soft X-ray nano-computed tomography and the total variation-based simultaneous algebraic reconstruction technique (TV-SART) to conduct nondestructive imaging of intact anammox bacterial cells. The ultrastructure of intact anammox bacteria was analyzed. The linear absorption coefficient of the ultrastructure of anammox bacteria was calculated to quantify its asymmetric structure. On the basis of this study, Peng et al. (2019a) also studied the morphological adaptation response of anammox bacteria to Fe^{2+} and discussed the regulation mechanism of Fe^{2+} on anammox bacteria by using TV-SART algorithm, as shown in Fig. 6. This morphological analysis of anammox bacteria provides an in-depth information for the inhibition of high iron concentration on anammox sludge. At the same time, Peng et al. (2019b) analyzed and measured the morphology and porosity of anammox granules by X-ray tomography imaging (Fig. 7). In this study, Peng et al. (2019b) found that silver nanoparticles increased the pore size and porosity of anammox granules through biological regulation. Therefore, the three-dimensional structure of anammox granules was changed, and the diffusion ability of substrate and iron ion was further enhanced. By using the same technique, Peng et al. (2021) detected a special iron-rich nanoparticle in anammox cell. The local atomic structures of iron-rich nanoparticles were obtained by X-ray absorption fine-structure spectroscopy and proteomics analysis. A metabolic pathway centered on iron-rich nanoparticles was proposed.

4. Discussion

4.1. Sensitivity and limitations of various spectral techniques

For the practical application of spectral technology to anammox system, environmental factors (i.e., pH, DO, temperature) changes, influent quality and quantity shock, as well as inhibitor stress (i.e., heavy metals, antibiotics, organic matter and salinity) changes may affect its reliability and sensitivity, thus affecting the accuracy of detection results.

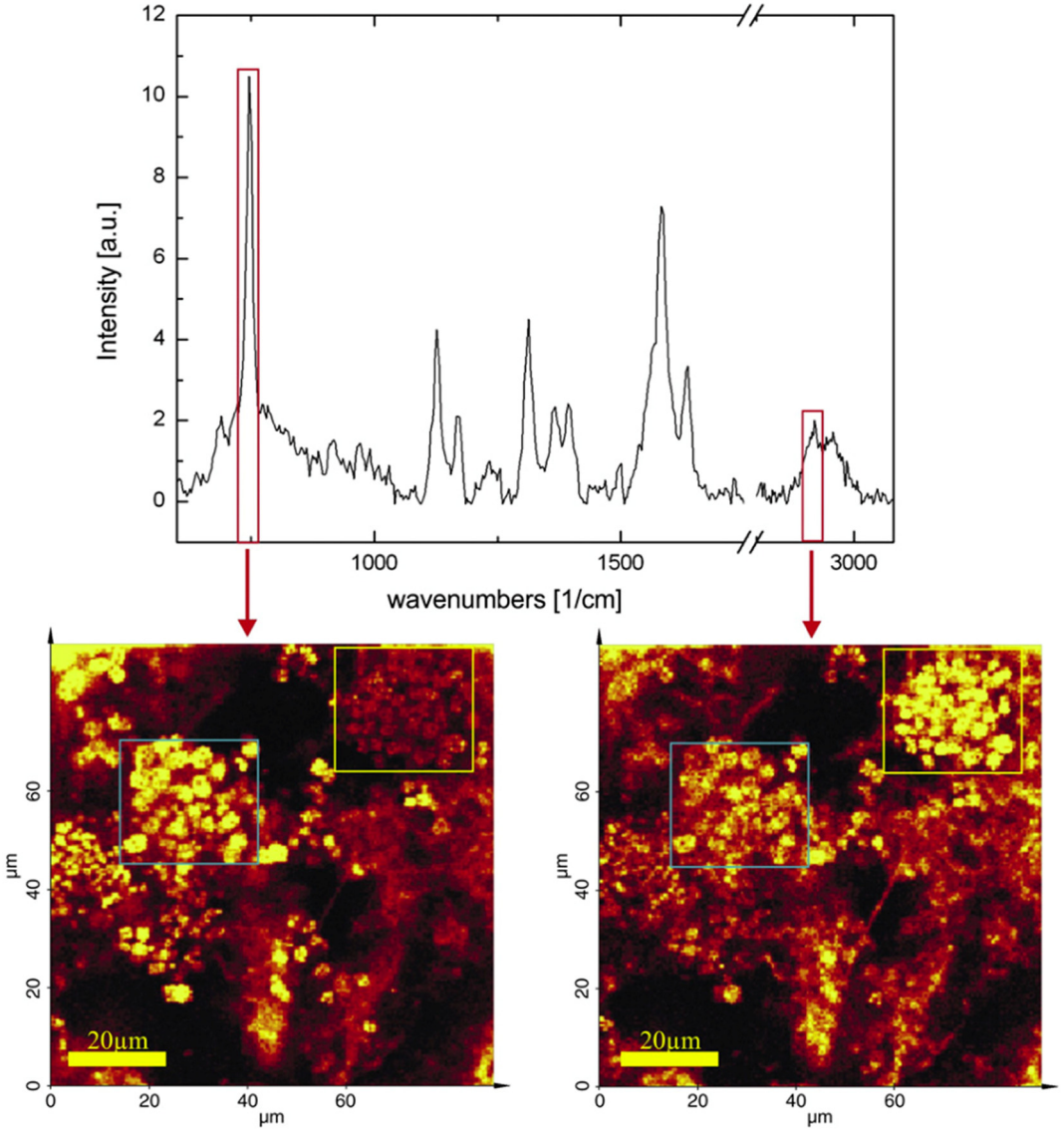

Fig. 5. Raman spectra of anammox granule from a sequencing batch reactor (Pätzold et al., 2006).

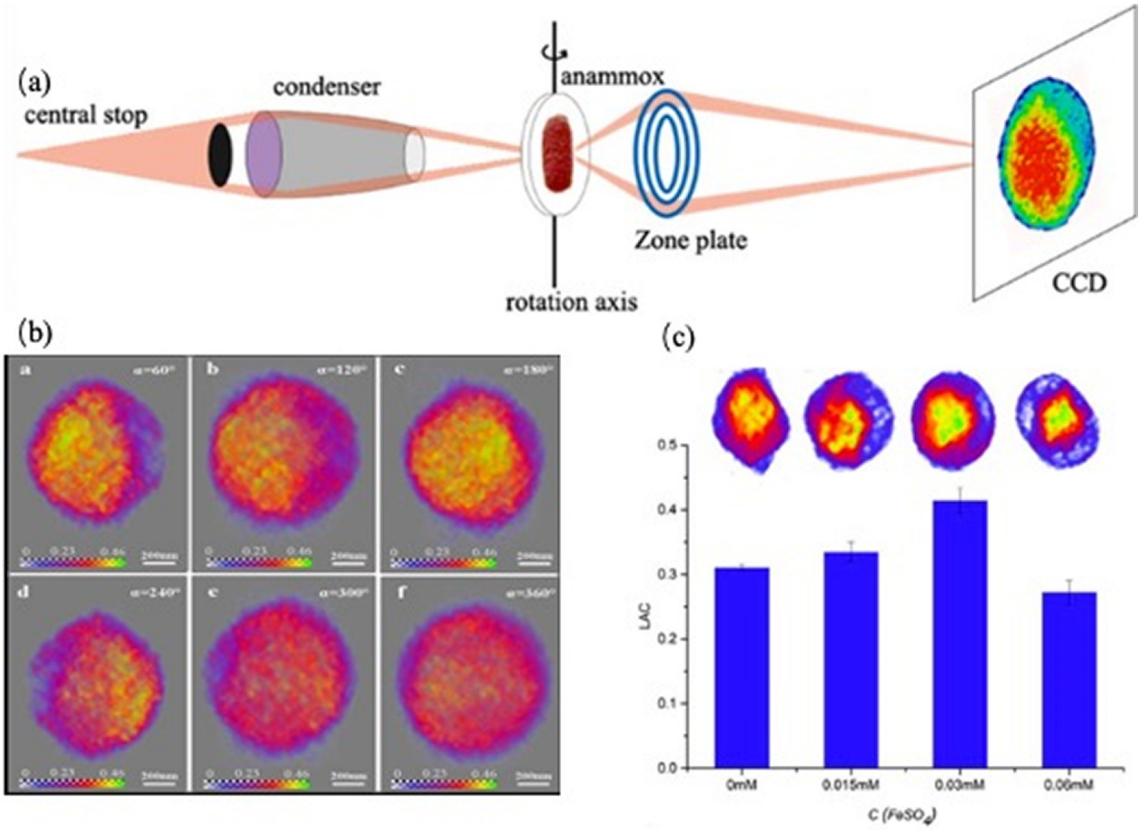

Fig. 6. (a) Schematic of the synchrotron soft X-ray nano-CT imaging process of the anammox cell and (b) the intact anammox cell reconstructed using the TV-SART algorithm. (c) The effect of Fe^{2+} on the morphology of anammox bacteria (Peng et al., 2019a).

Ultraviolet-visible absorption spectrum and EEM are the two efficient tools for qualitative analysis of anammox metabolites. However, the nitrate produced by anammox sludge has a strong absorption interference in the ultraviolet band and the change of temperature will also affect the ultraviolet absorption spectrum (Zielinski et al., 2011). Therefore, Bi et al. (2014) used partial least squares algorithm to establish correction models of ultraviolet-visible absorption spectra in different spectral regions to reduce such adverse impact. Fluorescence spectrum has the advantages of outstanding sensitivity and good selectivity. However, organic interference and fluorescence signal overlap are frequently encountered issues. Although anammox is an autotrophic bioprocess which requires no carbon source, organic is inevitable in the practical wastewater. Herein, during the practical application, the accuracy of fluorescence spectra will be interfered by the organic substances in influent. To figure out the signal overlap issue, parallel factor analysis tool has been widely employed (Lu et al., 2017; Li et al., 2020a; Zhang et al., 2019). However, this tool fails to output accuracy results

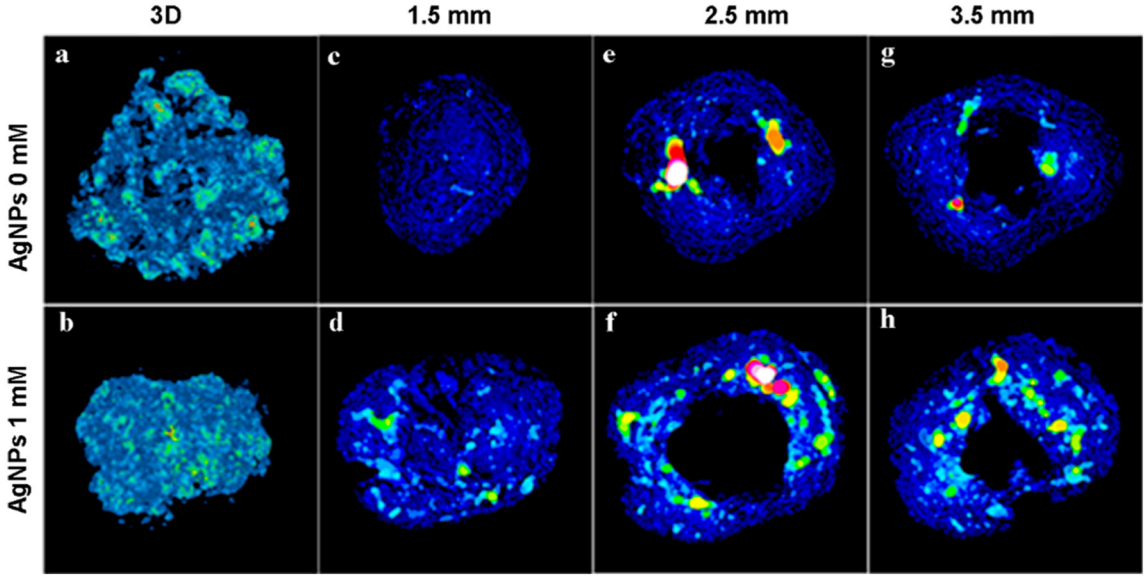

Fig. 7. Three-dimensional reconstruction of pore structure of anammox granules at AgNPs concentrations 0 mM and 1 mM (Peng et al., 2019b).

when decomposing nontrilinear EEM data, resulting in the overestimated number of components. Qian et al. (2019) develops a new method namely prior linear decomposition to solve this problem by introducing prior information into data decomposition. Fluorescence quenching is another issue of EEM technique. Environmental factors (i.e., temperature and pH) and heavy metal ion stress might influence the fluorescent intensity change (Qian et al., 2017; Guan et al., 2017). For the anammox system, a weak alkaline might limits the adverse impact of pH on fluorescence quenching compared to other biological processes, which might be beneficial for obtaining an accurate fluorescence spectrum information.

FTIR, Raman spectrum and soft X-ray absorption spectra are effective to identify and analyze the sludge metabolite chemical structure. Conventional FTIR is less affected by environmental factors, but it is difficult to test opaque and water-bearing samples. Therefore, infrared attenuated total reflection spectroscopy was widely used, benefiting from its no requirement of sample pretreatment and nondestructive processing (Lu et al., 2016). At the same time, because organic metabolite contains a large number of chemical groups and the absorption of each group will overlap. Second order differential, two-dimensional correlation and other data processing techniques can be used to identify the structural changes of such overlapped information (He et al., 2014). Raman spectrum is usually complementary with infrared spectroscopy to obtain molecular structure information due to its low interference with water. However, the strong fluorescence signal of the substance will interfere with the results. The sensitivity and resolution of Raman spectra can be further improved by plasmon-enhanced Raman scattering, surface-enhanced Raman scattering, and tip-enhanced Raman scattering (Bailo and Deckert, 2008; Campion and Kambhampati, 1998; Zhang et al., 2013). Soft X-ray absorption spectrum also has some limitations. It is easy to be affected by mutual element interference and superposition peaks and thus affect the reliability of the results.

4.2. The assembly of multiple spectroscopic techniques for anammox sludge metabolites characterization and analysis

To monitor and characterize the anammox sludge metabolites, the combination of multiple spectroscopic techniques must be employed (Fig. 8). As described above, some quantitative or qualitative results of anammox metabolites can be obtained using a single spectral technique. The combined spectroscopic technique can provide more abundant information for indicating the operation of reactor. Ultraviolet-visible absorption spectrum and EEM can be used to analyze the changes in the structure and characteristics of the intracellular and extracellular metabolites of anammox, providing early warning information for the changes in the water quality of the reactor. For microorganisms, FTIR and Raman spectra can show the living status of microorganisms and the changes of microbial community. Soft X-ray absorption spectroscopy is also a very effective tool for the imaging detection of anammox bacteria.

Comparing to the conventional chemical quantitative analysis, spectra technique requires fewer of chemical reagents and simplifies at procedures. At the same time, the high sensitivity of spectral technology makes it possible to early-warn the anammox bioreactor status. Molecular biological methods can be used to determine the microbial analysis of anammox sludge, which can provide information on the variation of the abundance of anammox bacteria and the expression of related microbial enzymes. It has the advantages of great accuracy and strong specificity. But they require advanced instruments and sophisticated procedures, which are time-consuming. Thus, as an in-situ, real-time analysis tool, spectroscopy can be combined with chemical and molecular biological techniques for quantitative analysis of metabolites. Spectroscopic techniques can also be used as a pre-analytical technique for molecular experiments to roughly analyze changes in the abundance of anammox bacteria. As an auxiliary tool, the combination of these techniques can lead to more accurate and intuitive conclusions.

5. Conclusion and prospect

Multivariate spectral analysis technique is useful to analyze the change characteristics of metabolites of anammox sludge under external factors stress. Thus, valuable information can be obtained and potentially used to guide for the stable operation of reactor. Online monitoring of anammox reactor performance can be achieved by adopting ultraviolet-visible absorption spectrum and 3D-EEM technology to track the component change of metabolites. The operation status of anammox reactor can be quickly reflected so as to provide some reference value for the control strategy of reactor. Confocal Raman spectroscopy and synchrotron radiation soft X-ray nano-CT technology can be used to further analyze the structure of anammox cells under excitation. The characteristics of sludge morphology and bacterial community during the operation of anammox reactor could be characterized from the perspective of structure without damaging cells. With the rapid development of wastewater treatment technology, an in-depth understanding of the metabolic characteristics of anammox

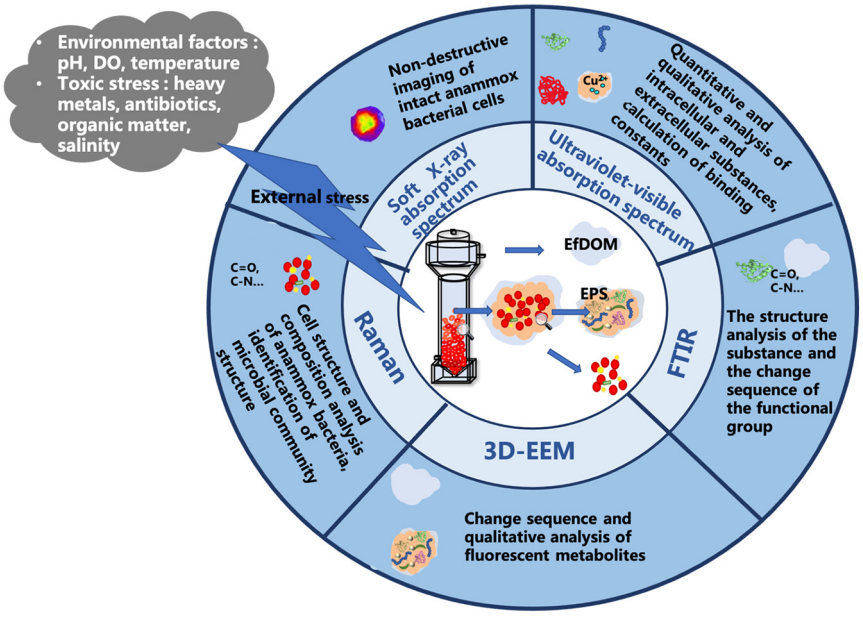

Fig. 8. Comparison of several spectral techniques.

sludge and the establishment of an effective reactor monitoring and analysis method based on this will contribute to the engineering application of this technology. In the future, the following aspects can be further studied.

1) At present, most of the spectral analysis in anammox system focuses on the analysis of the component content and change of EPS and reactor effluent. It provides basic information for anammox reactor from the aspect of microbial metabolic characteristics. However, the effectiveness of spectral analysis under the actual circumstances needs to be evaluated.
2) For a specific sample system, the influence of various non-target factors on the spectrum can be weakened and eliminated, and the spectral information can be extracted, which lays a foundation for the establishment of calibration model and the prediction of unknown sample composition or properties.

CRediT authorship contribution statement

Zhi-Qi Ren: Conceptualization, Visualization, Writing – original draft. **He-Fang Hong:** Data curation, Formal analysis. **Gui-Feng Li:** Data curation. **Xue-Ning Du:** Visualization. **Li-Ge Zhang:** Visualization. **Bao-Cheng Huang:** Supervision, Conceptualization, Writing – review & editing. **Nian-Si Fan:** Writing – review & editing. **Ren-Cun Jin:** Supervision, Conceptualization, Funding acquisition, Writing – review & editing.

Declaration of competing interest

The authors 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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