Politecnico di Milano

SCHOOL OF INDUSTRIAL AND INFORMATION ENGINEERING

Master of Science – Energy Engineering

Extraction, recovery and characterization of extracellular polymeric substances (EPS) of anammox and anaerobic granular sludge

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Abstract

Extracellular polymeric substances (EPS) are chains of polysaccharides secreted by bacterial cells. EPS protect bacteria from external factors such as environmental conditions and toxic substances; ensure cell stability; enhance adsorption and adhesion properties; represent a source of carbon and energy. EPS are present in granular sludge both in anaerobic ammonium oxidation (anammox) and anaerobic processes, two broadly adopted wastewater treatment techniques for nitrogen removal. The main objectives are to analyse the differences among anammox granules, to unravel their composition, to compare anammox and anaerobic granules and EPS, and to correlate the performances of the anammox reactor with the EPS quality.

During my work at the laboratory of environmental engineering (laboratorio di ingegneria ambientale – LIA) of the Department of Civil and Environmental Engineering (DICA) of Politecnico di Milano, EPS both from anammox and anaerobic granular sludge have been extracted, analysed and compared. In particular, the work was conducted on five anammox samples and two anaerobic. The anammox were collected monthly from July to December from a pilot-scale reactor of a textile industry located in Como (Lombardy) while anaerobic granules come from two different wastewater treatment plants (WWTP) dealing with effluents from a brewery and a paper industry. First of all, granular sludge has been studied, both quantitively and qualitatively. Secondly, EPS was extracted using the heating-Na_2CO_3 based method; polysaccharides (PS) and protein (PN) fractions of EPS and its yield have been calculated; UltraViolet-Visible Spectroscopy (UV-vis) and polymeric fraction characterisation were performed.

It was observed that anammox granules are more reddish, smaller and less robust than anaerobic granules. Moreover, the EPS yield of anammox granules is above the anaerobic granules average, and it is characterised by a higher amount of PN and PS. On the other hand, in anammox samples collected from July to December, a decreasing PN/PS trend is visible, which might indicate a reactor malfunctioning.

Sommario

Le sostanze polimeriche extracellulari (EPS) sono polimeri naturali secreti da microorganismi, fra cui i batteri, nell'ambiente circostante. Le EPS proteggono gli organismi da condizioni ambientali avverse come la presenza di sostanze tossiche; garantiscono la stabilità cellulare; migliorano le proprietà di adsorbimento e adesione; rappresentano una fonte di carbonio ed energia. Le EPS sono presenti nei fanghi granulari sia nell'ossidazione anaerobica dell'ammonio (anammox) che nei processi anaerobici, due tecniche di trattamento delle acque reflue ampiamente adottate per la rimozione dell'azoto. L'obiettivo del lavoro è triplice: capire le caratteristiche delle EPS anammox, scoprirne la composizione, evidenziare le differenze fra le EPS dei fanghi granulari anammox e anarobici e correlare la qualità e il rendimento degli EPS anammox con le performance del reattore.

Durante il mio lavoro presso il Laboratorio di Ingegneria Ambientale (LIA) del Dipartimento di Ingegneria Civile e Ambientale (DICA) del Politecnico di Milano, sono stati estratti, analizzati e confrontati EPS sia da fanghi granulari anammox che anaerobici. In particolare, il lavoro è stato condotto su cinque campioni di anammox e su due anaerobici. Gli anammox sono stati raccolti mensilmente da luglio a dicembre da un reattore pilota di un'industria tessile nella zona di Como (Lombardia) mentre i granuli anaerobici provengono da due diversi impianti di trattamento delle acque reflue (WWTP) che trattano rispettivamente gli scarti di un birrificio e di un'industria cartaria. In primo luogo, è stato studiato il fango granulare, sia quantitativamente che qualitativamente. In seguito, abbiamo estratto le EPS utilizzando il metodo riscaldamento-Na_2CO_3 e calcolato le frazioni di polisaccaridi (PS) e proteine (PN) nelle EPS e la sua resa. Infine abbiamo effettuato la spettroscopia ultravioletta-visibile (UV-vis) e la caratterizzazione della frazione polimerica.

È stato concluso che i granuli di anammox sono più rossastri, più piccoli e meno robusti dei granuli anaerobici. Inoltre, la resa in EPS dei granuli di anammox è superiore alla media dei granuli anaerobici ed è caratterizzata da una maggiore quantità di PN e PS.

D'altra parte, nei campioni di anammox, è visibile una tendenza negativa da luglio a dicembre del rapporto PN/PS, che potrebbe indicare un malfunzionamento del reattore.

Keywords

Granular sludge

Extracellular polymeric substances

Anammox bacteria

Anammox process

Anerobic processes

Extraction/recovery

Reactors

Parole chiave

Fanghi granulari

Sostanze polimeriche extracellulari

Batteri anammox

Processo anammox

Processo anaerobico

Estrazione/recupero

Reattori

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Chapter 1 – Introduction and scope

1.1 Background

Microbial cells secrete in the surrounding environment a slime-like and gel-like matter called extracellular polymeric substances (EPS) (Flemming & Wingender, 2010). EPS forms a three-dimensional scaffold for the microbial aggregates and involves several vital functions: adhesion to surfaces, aggregation of bacterial cells, structure stabilisation, the formation of a protective barrier that provides resistance against the harmful environment, retention of water, sorption of exogenous organic compounds for the nutrients accumulation and acquisition (Comte et al., 2006). Mainly, EPS is composed by proteins (PN) and polysaccharides (PS), as well as small amounts of nucleic acid, lipids and humic-like substances (HS-like) (D'Abzac et al., 2012). EPS can be found in bacteria, fungi, algae, and archaea (Feng et al., 2019) in different amounts.

Anaerobic ammonium oxidation (or anammox) and anaerobic granular sludge are two different types of sludge rich of bacteria secreting EPS. Anammox granules are red and rich in cytochrome c while anaerobic granular sludge is black, probably thanks to FeS content that contributes to this colour (Lim & Kim, 2014). Several environments are suitable for anammox and anaerobic granules growth. For the anammox process the up-flow anaerobic sludge (UASB) reactor, up-flow stationary fixed film (USFF) reactor, the anaerobic sequencing batch reactor (SBR) (Jin et al., 2008) and moving bed biofilm reactor (MBBR) (Lotti et al., 2014). While for anaerobic process the most common types are up-flow anaerobic sludge blanket (UASB), the expanded granular sludge bed (EGSB) and static granular bed reactor (SGBR) (Lim & Kim, 2014).

1.2 Problem statement

Several external factors influence EPS compositions and characteristics. Mainly they can be divided into three macro-categories (Shi et al., 2017):

- feed characteristics, as salinity and substrate type
- operational parameters, like temperature and operating conditions
- exogenous substances

Some parameters affect only anammox granules, as the hydraulic retention time in the reactor (Liu & Tay, 2008) while other tend to influence only anaerobic granules activity as the nitrogen load (S. Wang et al., 2020). Therefore, granules and EPS quality are not only influenced by the extraction method but also by the reactor status.

1.3 Research objectives

This dissertation aims at four different goals:

- Understand and evaluate the differences among different samples of anammox granular sludge EPS extracted from the same reactor at different periods
- Unravel the compositions of extractable EPS from different granules
- Identify the differences in physicochemical properties between anammox and anaerobic EPS
- Correlate anammox granule and EPS characteristics with reactor performances

Chapter 2 – State of the art

2.1 Extracellular polymeric substances (EPS)

Microorganisms secrete aggregates of polymers in the environment, known as extracellular polymeric substances or EPS (Flemming & Wingender, 2010). EPS are characterised by high molecular weight and are present both inside and outside the cells of microbial aggregates (Figure 1). EPS are present in bacteria, in fungi, algae, and archaea (Feng et al., 2019). EPS is a gel-like, highly hydrated sticky substance, which forms the cell-matrix and acts as a protective shield for microorganisms. Indeed, EPS protects bacterial cells from different environmental adverse conditions as extreme dry ambience, predation and antibiotics effects (Flemming & Wingender, 2010).

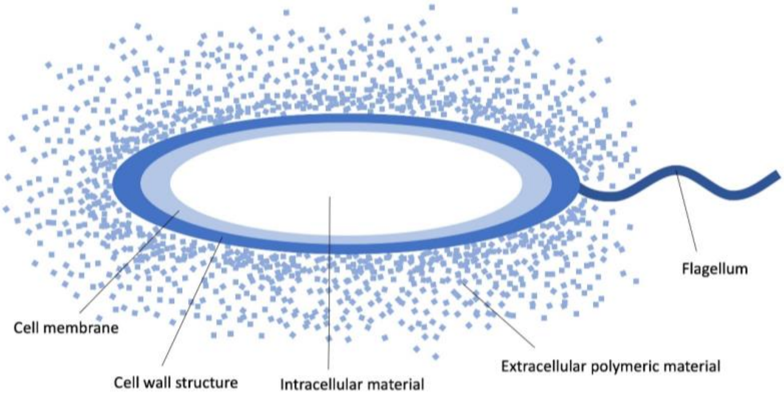


Figure 1 Bacterial cell and secreted EPS representation (adapted from Sudip Samal 2010)

Generally, EPS components are polysaccharides (chains of carbohydrates molecules), proteins, humic substances (HS), lipids and nucleic acids; other minor components are uronic acids and some inorganics. Furthermore, functional groups like carboxyl, phosphoric and hydroxyl exist in EPS (Tian et al., 2019). The EPS composition vary widely and the recognition of EPS components is mainly dependent upon the extraction methods. EPS-producing bacteria are found in several diverse environments. They have complex physiological roles depending on the specific habitat, and many studies show that microbial communities and their metabolisms are significantly affected by distinct substrates in wastewater (B. Bin Wang et al., 2018). However, the predominant contents in extractable EPS are proteins (PN) and polysaccharides (PS), present in different ratios; another organic component largely present in EPS is humic substances (HS) (Lotti et al., 2019).

As is shown in Figure 2, two EPS forms exist, i.e., soluble EPS (soluble macromolecules, colloids, and slimes) and bound EPS (sheaths, capsular polymers, condensed gels, loosely bound polymers, and attached organic materials) depending on their localisation and roles in microbial metabolism (Comte et al., 2010). Soluble EPS are weakly bounded or dissolved into the solution and are extractable by simple centrifugation. Bound EPS are distinguished by strong bonds with cells and require additional extraction treatments. Soluble EPS disperse in the cell surrounding environment as a not organised or amorphous slime, and they do not contact directly with the cell. On the other hand, bound EPS may be loosely attached to the cell surface as peripheral capsules (Comte et al., 2006). In literature, there are two subcategories of bound EPS depicted by a two-layer model, the inner layer of polymers or tightly bound EPS (TB-EPS) and the outer layer which consists of loosely bound EPS (LB-EPS), it is a slime stratum without a distinct edge. Usually, the TB-EPS content is higher in microbial aggregates.

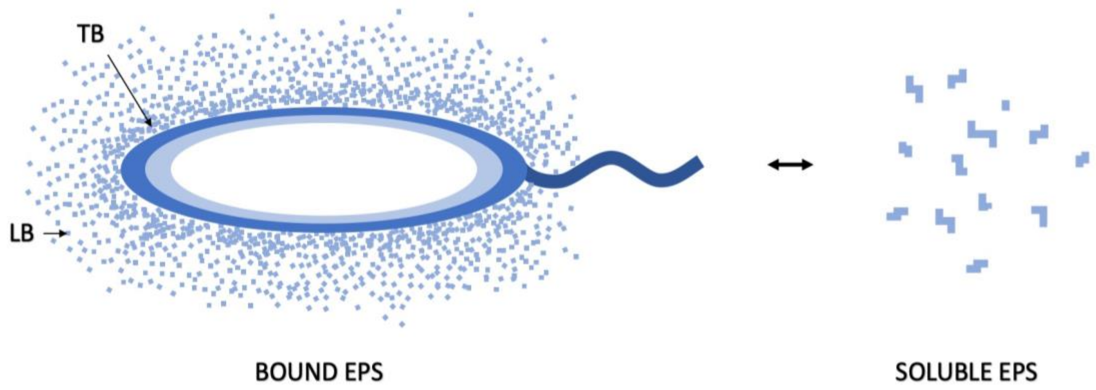


Figure 2 Bound and soluble EPS representation (adapted from Sheng et al. 2010). TB: Tightly-bound EPS; LB: Loosely-bound EPS.

The EPS features considerably influence microbial aggregate properties, such as adsorption ability, mass transfer or surface quality, and they also accelerate the aggregate formation through binding cells (Sheng et al., 2010). The main characteristics are the structure, adsorption abilities, flocculability, biodegradability and hydrophilicity/hydrophobicity, surface charge, settleability (Wang et al., 2018). Thus, the EPS study helps to improve our comprehension of biological wastewater treatments and allows enhancing the system efficiency through operational parameters optimisation.

2.2 EPS functions and general characteristics

The essential functions of EPS are adhesion to surfaces, aggregation of bacterial cells in flocs, stabilisation of the floc structure, formation of a protective barrier that provides resistance to biocides or other harmful effects, retention of water, sorption of exogenous organic compounds for the accumulation of nutrients from the environment, and enzymatic activities, such as digestion of exogenous macromolecules for nutriment acquisition, favouring the cells digestion of metal nutrients (Comte et al., 2006).

Mechanical Stability: The EPS constitutes and maintains the microbial aggregate structure (as films, mats flocs, sludge or biofilms), thanks to the binding between EPS and divalent cations, such as Ca^{2+} and Mg^{2+}, and other intermolecular reactions (Feng et al., 2019). In microorganisms accumulated in biofilms, the organism accounts for more or less 10% of the dry mass while the matrix accounts for the rest. The surrounding shield is a self-produced immediate environment of hydrated EPS that forms the scaffold for the three-dimensional biofilm structure. Also, the matrix provides mechanical stability to the biofilms, thanks to adhesion and cohesion properties, and it immobilises cells and keeps them close, allowing interactions, (Flemming & Wingender, 2010).

Surface adhesion: EPS are responsible for bacterial cell adhesion onto solid surfaces; the stickiness increases as the absolute values of zeta potential decreases (electrokinetic potential in colloidal dispersion), and the electrostatic interaction suppresses cell adhesion efficiency (Tsuneda et al. 2003).

Adsorption properties: EPS can absorb organic matter, such as aromatics, aliphatics in proteins, and hydrophobic regions in carbohydrates. Absorption is possible since EPSs and organic pollutants have opposite charges, respectively negative and positive, so they attract each other (Sheng et al., 2010). Similarly, EPS help the cells tolerate heavy metals (as chromium). It was observed an increase of EPS material production to cope with metal stress (Comte et al., 2006). Due to their high binding capacity, probably due to many functional groups in EPSs as carboxyl and hydroxyl, EPS can complex with metals (B. Bin Wang et al., 2018).

Biodegradability-Nutrient source: due to EPS biodegradability properties, it can be a source of carbon and energy. Indeed, bacteria in biological treatment reactors can degrade carbohydrates and proteins and utilise them for their metabolic activity. The small molecular substances produced by degradation represent a vital resource for cell growth, principally in conditions of nutrient shortage (Sheng et al., 2010). For example, the EPS that form the biofilm matrix functions as an external digestive system, sequestrating dissolved nutrients from the liquid phase and employing them as useful resources (Flemming & Wingender, 2010).

Hydrophilicity/hydrophobicity: EPS molecules present hydrophilic and hydrophobic areas which indicate that EPS are amphoteric: it can react either as an acid either as a base. Moreover, since statistical analyses showed a correlation between the ability to degrade organic pollutants and sludge surface hydrophobicity, the formation of hydrophobic groups is beneficial for organic pollutant adsorption (Cao et al., 2017).

2.3 Anaerobic ammonium (anammox) process

A well-established biochemical process for wastewater treatment is the anaerobic ammonium oxidation (or anammox) for nitrogen removal. This process was firstly observed in 1992; before its introduction denitrification was considered the only natural sink of reactive nitrogen. There are several advantages to conventional denitrification processes: 1) energy and cost savings thanks to no oxygen addition: operational costs savings are estimated to be higher than 40% (Lotti et al., 2014); 2) no demand for external carbon source and aeration; 3) the production of excess sludge in wastewater treatment is reduced (Chen et al., 2020); 4) the production of methane-rich biogas that entails partial recovery of chemical energy (Lotti et al., 2014). These characteristics make the anammox process very competitive compared with conventional biological processes.

The removal process is articulated in two main phases: partial nitritation operated by aerobic ammonium bacteria and nitrogen removal through nitrogen gas production by anammox bacteria.

Partial nitritation chemical reaction features ammonium (NH_4^+) as an electron donor for denitrification with nitrite (NO_2^-) (Jin et al., 2008):

$$NH_4^+ + 1.32NO_2^- + 0.066\,HCO_3^- \rightarrow 1.02N_2 + 2.03H_2O + 0.066\,CH_2O_{1.5}N_{0,15} + 0.26NO_3^-$$

Based on the overall stoichiometry, for each mole of ammonium ion removed, 0.111 moles of nitrite ions are generated. Therefore, there is a theoretical maximum limit of 89% on the removal efficiency. In practice, this limit might be exceeded with the activity of organic biodegradable matter and heterotrophs bacteria (Lotti et al., 2014).

During partial nitritation, half of the ammonium is oxidised to nitrite. The second phase is nitrogen removal through the production and release of dinitrogen (N_2) into the atmosphere (Lotti et al., 2014). The removal process can be arranged in two separate reactors (partial nitritation is achieved separately from anammox) or in one single reactor system (Jin et al., 2008).

Nowadays the anammox based process is installed in 90 plants for treating wastewater of high nitrogen concentration digestate and for dealing with effluents of several industries as food, tanning, semiconductors, yeast production, distilleries and wine production (Lotti et al., 2014). Other studies also revealed the potential application for treating the wastewater from pharmaceutical industries, petrochemical plants zootechnical digestate and digestate contaminated by human excrements (Lotti et al., 2014).

Although the anammox based process is spreading and it entails significant technical improvements for wastewater treatment plants, a large portion of treatment installations continues to use traditional removal processes. Indeed, three problems limit the anammox-based process application: the low growth rate of anammox bacteria; the scarce resistance of this living organism to environmental change, and the limit of maximum nitrogen removal efficiency (NRE) due to a low cell yield (Guo et al., 2020; Ganesan & Vadivelu, 2020). Besides, some kinds of wastewater not only contain nitrogen but are also rich in phosphorus to be removed (Guo et al., 2020). The intrinsic low growth rate of these bacteria slows the start-up of anammox based process; a solution might be to accelerate the process feeding a large amount of anammox sludge. Moreover, according to Lotti et al. (2014) under favourable conditions, the daily growth rate is equal to 0.21 d^{-1} at 30 °C (with respect to 2.05 and 12.0 d^{-1} at 30 °C for aerobic ammonia oxidising and heterotroph bacteria, respectively) and it might potentially increase further.

On the other hand, bacteria inhibition frequently occurs in the case of non-optimal conditions, and it causes nitrite accumulation, and it becomes hard to recover the sludge; a useful tactic is to wash the granules (Guo et al., 2020).

Anammox granules are distinguished from anaerobic ones for their original red colour as is visible from Figure 3. They can be found in many wastewater treatment plants and different natural habitats. For example, in the Black Sea, it was estimated that anammox bacteria activity contributed to at least 30% of the total nitrogen turnover (Kuenen, 2008). Also, the anammox process is responsible for a part of global nitrogen renewal (Kuenen, 2008), so it has excellent potential for undesired ammonium removal from wastewater.

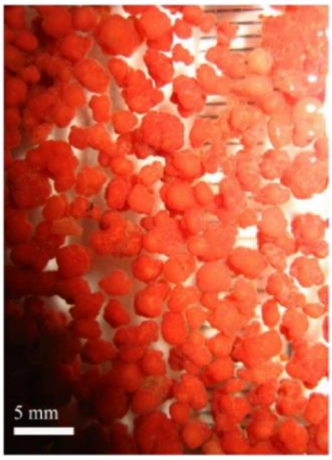


Figure 3 Anammox bacteria

There are different types of bioreactors to perform the anammox process:

- up-flow anaerobic sludge (UASB) reactor (Figure 4a)
- up-flow stationary fixed film (USFF) reactor (Figure 4b)
- anaerobic sequencing batch reactor (ASBR) (Figure 4c) (Jin et al., 2008)

Jin et al., 2008 compared the stability of the anammox process of these three different types of reactors. It is a significant performance index that measures the variations due to changes in operational parameters. Also, Jin et al. (2008) evaluated reactors response to varying substrate concentration and to flow rate shocks (i.e. hydraulic shocks) against steady-state conditions. The aim was to understand if a slight disturbance in inlet conditions results in considerable alterations of the effluent substrate concentration. The main conclusions are: 1) all anammox reactors are more tolerant of a hydraulic shock than substrate concentration one; 2) the stability was ranked as UASB reactor > USFF reactor > ASBR, hence the most stable bioreactor is the UASB type (Jin et al., 2008). Another reactor type suitable for the anammox process is the moving bed biofilm reactor (MBBR) (Lotti et al., 2014).

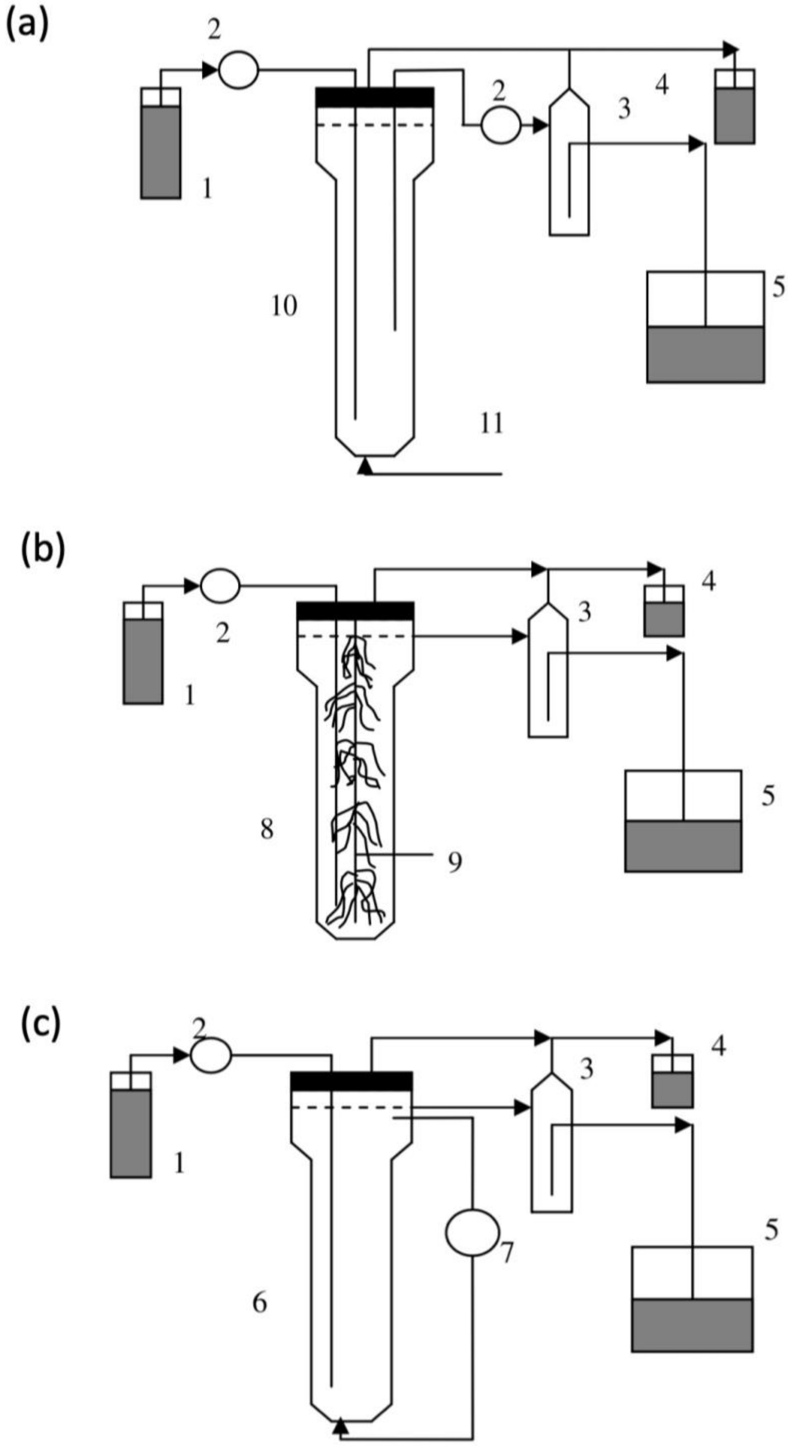


Figure 4 (a) Schematic diagram UASB reactor; (b) USFF reactor; (c) ASBR (Jin et al., 2008). *(1) Feeding tank, (2) Peristaltic pump, (3) Gas-solid-liquid separator, (4) Gas outlet, (5) Effluent collection tank, (6) UASB reactor (7) Recycling pump, (8) USFF reactor (9) Soft packing material. (10) ASBR reactor (11) Argon inlet from* (Jin et al., 2008)

2.4 EPS extraction from anammox granules

It is crucial to optimise the extraction of EPS to maximise the reusable content, following a circular economy strategy. Waste anammox granular sludge is particularly suitable for EPS extraction and recovery, thanks to the high EPS content (Feng et al., 2019).

EPS yield composition and properties mostly depend on the extraction methods of the substance. Choosing the best EPS extraction method is very challenging since it should effectively isolate the functional EPS from the samples while not affecting their structure and causing cell lysis. Isolation techniques include physical, chemical treatment or a combination. The first category includes centrifugation, sonication, grinding and heating, among others. While, the chemical extractions consists of PH adjusting, ethylenediaminetetraacetic acid (ETDA), cation exchanges resins (CER), and alkaline or aldehydic reagents (Feng et al., 2019). To evaluate the effectiveness of the extraction methods, some parameters are considered: EPS yield (the amount of EPS extracted), PN and PS concentrations, and PN/PS ratio. After the extraction, extracellular polymers must be recovered to be reused. The extraction and recovery protocols can be considered significant if the yield is high, PN and PS concentration are relevant and if they are not time-consuming and friendly-operated. Several studies analysed and compared numerous extraction methods, among these Feng et al., 2019 focused on seven different techniques to separate as much EPS as possible: sonication, Heating-Na_2CO_3, CER, EDTA, Formamide-NaOH, Formaldehyde-NaOH, and H_2SO_4 (acid). In Feng et al., 2019 study, tests were performed on existing anammox granules extraction procedures, which performance indicators found in the literature are summarised in Table 1: EPS yield ranged from 33-594 mg/g VSS. In most studies, PN/PS ratio is over 1.0, indicating PN contents are higher than PS. Based on these results of yield and PN and PS content, the methods can be ordered for their recovery efficiencies: Heating-Na_2CO_3 > Formamide-NaOH > Formaldehyde-NaOH > CER > EDTA > acid > sonication. It is essential to underline the relatively high yield of proteins and polysaccharides and the highest ESP quality obtained with the heating method (Feng et al., 2019).

Table 1 Summary of extractable EPS contents in anammox granular sludge (Feng et al., 2019)

Methods	Mechanism	PN [mg/g VSS]	PS [mg/g VSS]	PN/PS [g/g]	EPS yield	T[°C]
EDTA	Ethylenediaminetetraacetic acid: 0.4 g of freeze-dried anammox granules + 20 ml 0.9%NaCl water + 20 ml at 4% ETDA centrifuged at 400 rpm for 3 hours.	164 ± 9 298 ± 9 -	72 ± 2 112 ± 3 -	2,3 2,7 -	236 410 210	35 35 30
CER	Cation Exchange Resins: 0.4 g of freeze-dried anammox granules + 40 ml 0.9%NaCl water + 70 g/g VSS resin centrifuged at 400 rpm for 3 hours.	43 ± 7 56 ± 3 75 ± 9 64-83 64-307 21 ± 9	83 ± 8 71 ± 7 36 ± 4 151-210 151-287 10 ± 0.3	0.5 0.8 2.1 0.3-0.5 0.4-0,11 2.6 ± 1.4	126 128 111 215-271 215-594 90 ± 48	37 35 35 35 35 35
Heating Na_2CO_3	0.4 g of freeze-dried anammox granules + 40 ml 0.9%NaCl water diluted with for 0.5% with Na_2CO_3 at 80°C for 30 minutes.	140-250 17-188 34-109 82-122 210-122 - 35	13 4-32 9-31 25-30 32-61 - 10	7-12 3-46.3 3.4-3-9 3.3-4.9 3.2-4.4 8.8 3.5	163-257 21-193 43-140 - - 150 45	35 30 25,35 4 35 20 30
Sonication - Formaldeide-NaOH	0.4 g of freeze-dried anammox granules + 40 ml 0.9%NaCl water with pulsed sonication applied on ice with a power density of 6 W/mL for 1 minute	13-114	7-26	1.5-2.5	33-181	35

2.5 Anaerobic granular sludge

Anaerobic granular sludge found its main application in industrial wastewater treatment plants (for example downstream pulp and paper production plants) characterised by biodegradable chemical oxygen demand (COD) concentration, higher than 5 and up to 60-80 g COD/L. The aim of the anaerobic process is to remove hydrophobic organic pollutants. Its development started in the late 1960s. Compared to an aerobic process, it implies lower energy consumptions and a smaller amount of nutrients. However, it has several drawbacks such as vulnerability, sensitivity, odour issues, long start-ups, and post-treatments to meet discharge standards for environmental protection.

Granulation process is not clearly understood since it is complex, and it depends on several physicochemical aspects such as the species of organisms and the growth/decay rate of organisms in granules (Lim and Kim 2014). As assumed, granulation happens in four steps: first, the transportation of a cell to an uncolonised inert material or another cell; second, the initial reversible adsorption into the substratum by physicochemical forces; third, an irreversible adhesion of cells onto the substratum by microbial appendages or polymers attaching the cells to the substratum; and fourth, the multiplication of cells and the development of granules (Lim & Kim, 2014). Cells are attracted and establish an irreversible adhesion when van der Waals forces are predominant. However, it remains unclear if bacteria adhere first or if they secrete EPS first.

An increase of ash content results in a density increase, but it has no significant effect on the granule strength (Lim & Kim, 2014). The anaerobic granular sludge is black, and some researchers stated that FeS content contributes to this colour. The EPS forms and maintains the granules. The surface charge of particles is commonly negative, therefore positive charges or polymers are necessary to form stable microorganisms. Usually, the amount of EPS is smaller in thermophilic conditions rather than in the mesophilic environment (Lim & Kim, 2014).

There are various types of anaerobic reactors; the most typical ones are the up-flow anaerobic sludge blanket (UASB), the expanded granular sludge bed (EGSB) and static granular bed reactor (SGBR). The most widespread type is the UASB reactor (Figure 5a). With a quite simple configuration, it does not require any specific mixing device. It is provided with a set of gas-liquid-solid separators (GSS) to divide the granules, allowing to separate the gas easily. Usually, it is suitable for high-strength organic wastewaters with an organic loading rate (OLR) between 10 and 15 kg/m3*d (Lim & Kim, 2014). The main advantages of this technology are excellent performances, low energy consumption, low sludge production and the production of a valuable by-product - methane. However, it is deficient with long start-up periods, decreased performances at low loadings, and there are strict standards for effluent quality.

On the other hand, EGSB (Figure 5b) is suitable for high OLRs, up to 30 kg/m3*d, and its characterised by a high up-flow velocity (ranges 0.5 m/h to 1.0 m/h) which allows an intensified mixing. The EGSB is also capable of treating low strength wastewaters (with small concentrations). Even though EGSB is part of the family of the UASB reactors, and it has similar efficiencies, they have some differences such as geometry, operational parameters, and application. In addition to the different OLR, EGSB has a more extended height (5.5-6.5 m vs 12-18 m).

The third reactor type is the SGBR (Figure 5c). The most significant advantage of this reactor is the simple configuration, supplementary mixing system and recirculating pumps are not required. It can treat low to mid-strength wastewaters. However, a fast solids removal rate is necessary to avoid clogging when the granules concentration is high. Also, SGBR can operate at ambient temperature or mesophilic temperature (around 35°C) and has a broad OLR range from 0.63 to 9.72 kg/m3*d. Considering the COD removal efficiency, the SGBR showed better performances than the UASB, which is the most common type thanks to its competitive capital and operating costs (Lim & Kim, 2014).

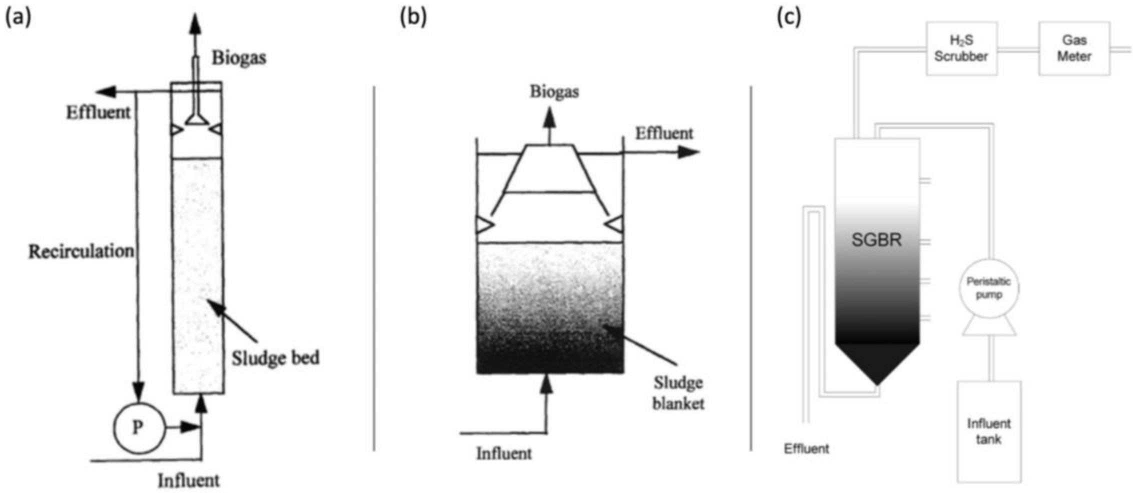


Figure 5 Schematic diagram of (a) UASB (b) EGSB and (c) SGBR reactors (Lim & Kim, 2014)

2.6 EPS extraction from anaerobic granular sludge

As for the anammox granules, different extraction methods and sludge origins lead to different yields and compositions. Collection data, reported in Table 2, are from a study that considered three physical methods (centrifugation, heating, and sonication) and one chemical extraction method (sodium dodecyl sulfate, SDS) (Cao et al., 2017). The heating method is characterised by the highest PN concentration, HS-like substances and PS concentration and PN/PS ratio. Sonication and SDS can be considered effective methods too, while centrifugation technique reports the lowest performances.

Table 2 Characteristics of EPS extracted from anaerobic granular sludge (Cao et al., 2017)

Extraction Method	**EPS type**	**Inial pH**	**Dry weight (g/L)**	**Volatile dry weight (g/L)**	**Biochemical composition (mg/g EPS untreated, dry weight**			**PN/PS (g/g)**
					PN	HS-like	PS	
Centrifugation	LB	7,9	0,7 ± 0,1	0,2 ± 0,1	5 ± 1	104 ± 5	31 ± 2	0,161
Heating	LB+TB	8,4	4,3 ± 0,3	3,5 ± 0,6	185 ± 10	288 ± 8	133 ± 7	1,391
Sonication	LB+TB	7,2	1,0 ± 0,3	0,4 ± 0,1	60 ± 3	98 ± 6	50 ± 2	1,200
SDS	LB+TB	6,8	0,7 ± 0,1	0,2 ± 0,1	58 ± 2	118 ± 5	56 ± 2	1,036

Moreover, the anaerobic sludge extraction technique affects the proton/metal-binding properties of the polymeric substances. This ability allows the EPS to protect the granule structure adsorbing toxic pollutants present in wastewaters such as metallic elements (ME) (d'Abzac et al., 2010). The biosorption of ME is the result of several steps: ion-exchange reactions, complexation, adsorption and precipitation (d'Abzac et al., 2010). This process is due to ionisable functional groups present in the EPS molecules, for example, carboxyl, hydroxyl or amino groups that participate in metal binding. The presence of these functional groups depends on the EPS composition. Therefore, a comparison among different extraction methods was performed to test the binding capacity of EPS and ME, such as lead and cadmium. d'Abzac et al. (2010) analysed nine widespread extraction techniques: centrifugation as a control method, four physical ones (sonication, CER, CER + sonication and heating) and four chemical ones (formaldehyde + heating, formaldehyde + NaOH, ethylenediaminetetraacetic acid (EDTA) and ethanol). From this study, it was clear that chemical procedures induce sample contamination of the samples that modifies the binding EPS properties. For example, the EDTA method leads to the overestimation of metals and protons binding capacities. On the other hand, physical methods can be considered less detrimental procedures and have to be preferred even though they provoke unwanted macromolecules disruption that may affect the proton and metal-binding properties (d'Abzac et al., 2010).

2.7 External factors influencing EPS

External parameters strongly influence EPS secretion from biomass. Therefore it is important to underline and understand the link between environmental conditions and characteristics and yield of EPS extracted from anaerobic (Figure 6a) and anammox sludge (Figure 6b). Those parameters, can be classified into three macro-categories: 1) influent characteristics, 2) operational parameters and 3) exogenous substances (Shi et al., 2017). Figure 6 schematises the classification of environmental influences. Also, it is crucial to underline the power of the protein and polysaccharides ratio (PN/PS) an index of the reactor status both for anaerobic and anammox reactors (Chen et al., 2009). In particular, a high PN/PS is linked with high hydrophobicity and granules compactness (Chen et al., 2009).

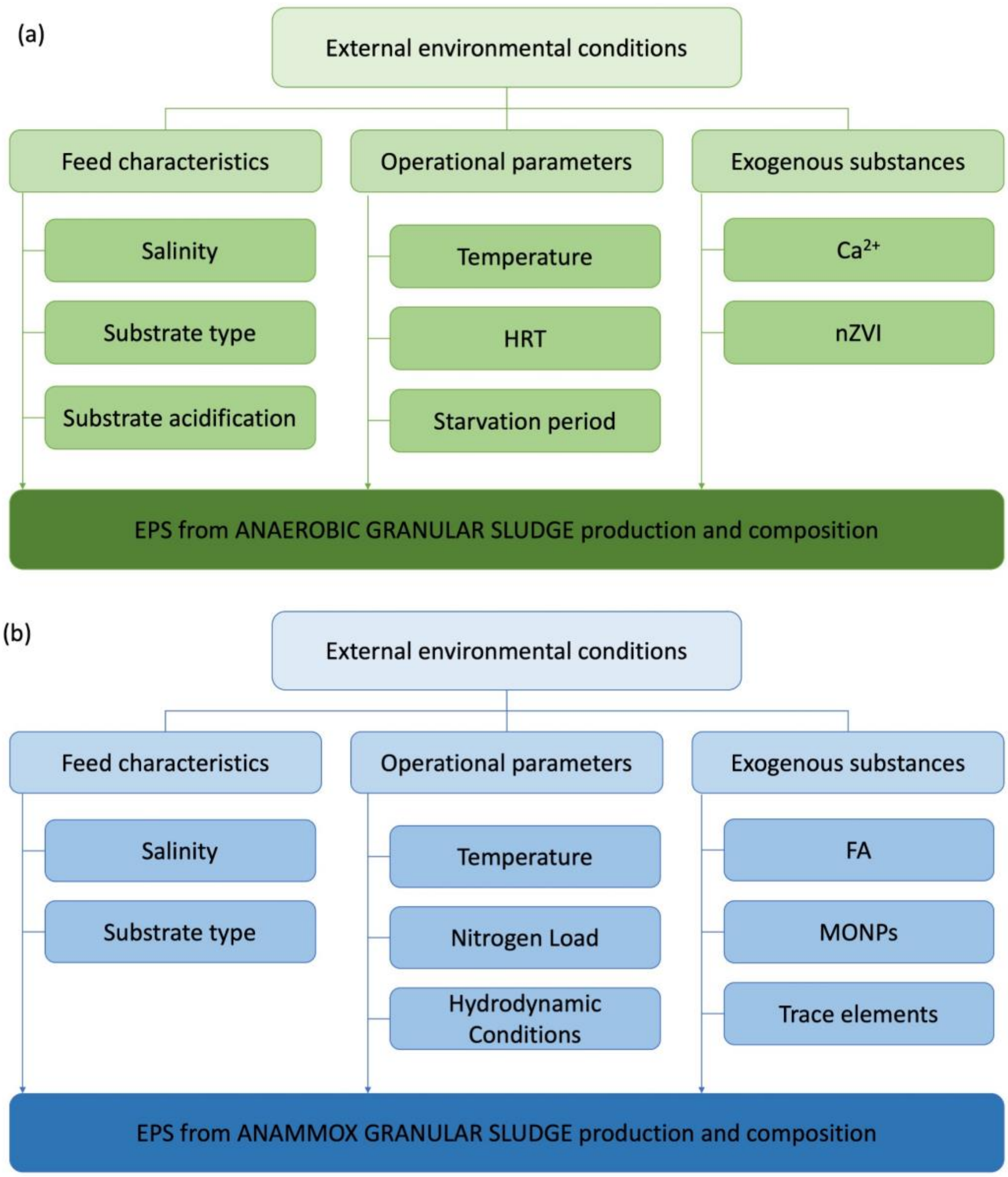


Figure 6 (a) Classification of different factors affecting AnGS EPS production and composition adapted from Shi et al. (2017); (b) Classification of different factors affecting anammox EPS production and composition adapted from Shi et al. (2017)

2.7.1 Anaerobic Bacteria

Feed characteristics

- Salinity

Salinity strongly affects EPS from anaerobic granules production and composition: a higher salinity corresponds to an increased protein fraction and total EPS amount (S. B. Ismail et al., 2010).

- Substrate type

Carbon source and carbon/nitrogen (C/N) ratio influence the production and the composition of insoluble EPS from anaerobic granular sludge. Miqueleto et al. (2010) evaluated their contribution in an anaerobic sequencing batch biofilm reactor (ASBBR). The highest EPS production (23.6 mg EPS/g carbon) was observed with an easily degradable substrate, glucose, and a C/N ratio of 13.6 gC/gN. When the reactor was fed with volatile fatty (a mixture of acetic, propionic, and butyric), the EPS production was lower than with glucose feeding. An intermediate result was obtained with soybean oil feeding (13.3 mg EPS/g carbon), and the lowest EPS production was measured with protein-based wastewater (1.4 mg EPS/g carbon) (Miqueleto et al., 2010). EPS production was affected not only by the carbon source but also by the C/N ratio. Generally, a high ratio between carbon and the limiting nutrient, nitrogen, favours the polysaccharide production and accordingly positively influences the total amount of EPS (Miqueleto et al., 2010). Probably because, when nitrogen is insufficient for protein synthesis (high C/N ratio condition), bacteria may modify the cellular growth path towards EPS production (Miqueleto et al., 2010).

- Substrate acidification

Ismail et al. 2010 compared the operation of four anaerobic granular sludge UASB reactors fed with different substrates. The UASB reactors were fed either with a partly acidified substrate (PAS) composed of acetate medium or fully acidified substrate (FAS) consisting of acetate, gelatine, and starch medium. The results showed a higher EPS content, and more robust and more prominent granules, under PAS conditions (S. B. Ismail et al., 2010).

Operational Parameters

- Temperature

Anaerobic digesters can operate in different temperature conditions: mesophilic conditions with a range of 25°C-40°C; thermophilic conditions with a temperature higher than 45°C (Lim & Kim, 2014). A link between the extractable EPS from anaerobic sludge and the operating temperature was discovered, showing that the volume of EPS in a thermophilic anaerobic digester is usually smaller than that operated under mesophilic conditions (Lim & Kim, 2014). Furthermore, the amount of protein and polysaccharides was enhanced by mesophilic conditions while the lipid content by thermophilic conditions (Schmidt & Ahring, 1994).

- HRT

The hydraulic retention time (HRT) is the average time that a compound remains in the bioreactor; it affects both aerobic and anaerobic granulation process and, in particular, short HRT promotes granulation (Liu & Tay, 2008). Regarding ESP concentration, HRT in the anaerobic process was found to affect TB-EPS PN concentration at low TOC (104 ± 13 mg/L), while it does not influence it when the TOC concentration increases (266 ± 13 mg/L) (Iorhemen et al., 2019).

- Starvation Period

In literature, the effect of starvation on both aerobic and anaerobic granules was widely explored. The long starvation phase leads to low wastewater treatment efficiency and insufficient treatment capacity. However, it has a positive effect on microbial granulation as it promotes granules formation and stability (Liu & Tay, 2008). Anyhow, the starvation period is strongly interrelated to the selection pressure, substrate concentration, and substrate type, so it is difficult to isolate its contribution. On the other hand, longer starvation time enhances the production of EPS reach in PN (Rusanowska et al., 2019).

Exogenous substances

- Ca^{2+}

Calcium ions are essential for cell-cell aggregation, contribute to bacterial adhesion to surface and enhance anaerobic granulation process (S. B. Ismail et al., 2010). Also, Ca^{2+} has a high affinity for EPS from anaerobic granules (S. B. Ismail et al., 2010) and influence their secretion, promoting the production and also improving sludge digestion performances during the anaerobic digestion. The addition of Ca^{2+}alters the functional groups of EPS and the microstructure of granular sludge (Ma et al., 2020). Ma et al. (2020) noticed a significant effect of Ca^{2+}addition (Figure 7): the EPS secretion notably increased and in particular, PN and PS production were favoured (Ma et al., 2020).

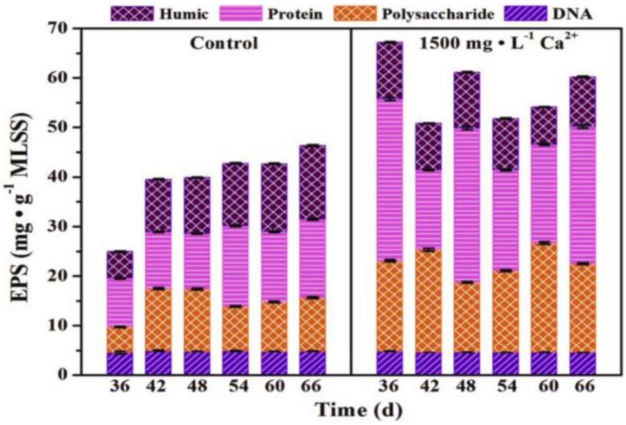


Figure 7 EPS concentration at 36 d, 42 d, 48 d, 54 d, 60 d, and 66 d in R_0 (control) and R_2 (1500 mgL^{-1} Ca^{2+}) reactors* (Ma et al., 2020)

- nZVI

Nanoscale zero-valent iron (nZVI) is sometimes used for wastewater treatment. He et al., (2020) studied the mutual influence between EPS and nZVI dosage. On one side, the presence of EPS reduced H_2 generation from nZVI, and it inhibits the methanogenesis process. On the other hand, the presence of nZVI causes a significant reduction in EPS concentration, coherently with the coagulant properties of nZVI. As illustrated in Figure 8, the addition of 1 g/L of nZVI into water containing 102.6 mg C/L of EPS produced a decrease after one day of proteins, polysaccharides, and humic substances by 15.4%, 26.7%, and 14.5%, respectively. Over the following 20 days, the concentration gradually decreased to 65%, 53%, and 80% respectively. Probably, the components concentration reduction is due to the reaction between nZVI and EPS or the adsorption of EPS onto the iron oxide surface (He et al., 2020).

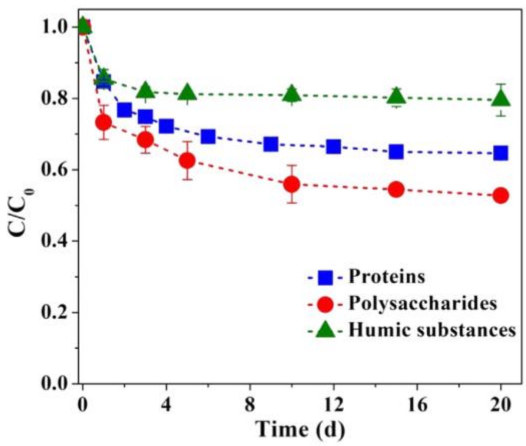


Figure 8 Variation in EPS concentration after the addition of 1 g/L nZVI (He et al., 2020)

2.7.2 Anammox Bacteria

Feed characteristics

- Salinity

Salinity has an impact on the granulation process. In particular, it affects the production and composition of EPS from anammox granular sludge. Salinity modifies bacterial surface properties, functional and structural groups, metal ion composition, surface charge, hydrophobicity, and aggregate space structure (Fang et al., 2018). According to Fang et al. (2018) an increase in salinity stimulates PS secretion, leads to a decrease of PN and therefore alters the EPS composition. Consequently, the variation in concentration reduces cell hydrophobicity and negatively affects the aggregation of anammox granular sludge.

- Substrate type

Moreover, a recent study from Ganesan & Vadivelu (2020) analysed the influence of substrate type and storage conditions on anammox bacteria and their EPS yield. Starvation has adverse effects on the bacteria, but different storage conditions can diminish them: the presence of ammonium, nitrite, and hydrazine. All these situations were compared with the base case with no substrate.

The results showed that the substrate with the lowest reduction of both protein and carbohydrate concentrations was the one with hydrazine. At the same time, nitrite presence led to the highest reduction in EPS concentration during starvation and recovery (the phase immediately after the starvation period). Also, it was proved the inability of anammox bacteria to produce EPS in the absence of a sufficient energy source in the reactors (Ganesan & Vadivelu, 2020). Supplementing with hydrazine produces relatively higher energy that helps to maintain EPS concentration at healthy levels.

Operational Parameters

- Temperature

Also for anammox process temperature is a crucial factor for microbial metabolism and reactor performance (H. Chen et al., 2020), the ideal temperature for anammox process is considered mesophilic, ranging from 30°C to 37°C (H. Chen et al., 2020). However, it is significant to understand the EPS response to temperature variations and, in particular, to temporary temperature shocks. Chen et al. (2020) examined the effect of temperature shocks (15,25, and 55°C) on EPS: a sharp increase in quantity was registered, especially after the 55°C shock (duration time 4 hours) when the EPS content reached five times greater concentration. Hence, anammox bacteria secrete a higher number of EPS when a sudden temperature shock occurs.

- Nitrogen Load

Increasing the influent nitrogen load in the anammox process has a positive impact on both sludge particle size and EPS content (S. Wang et al., 2020). While a higher nitrogen load actively promotes PN concentration, the PS content does not change significantly at different loads, and it always remains at a lower concentration than PN (S. Wang et al., 2020). Also, the total nitrogen concentration (TNC) corresponded to average EPS concentration (S. Ismail et al., 2019).

- Hydrodynamic conditions

Hydrodynamic conditions affect the microbial structure, biofilm formation, and sludge granulation. Therefore, under high hydrodynamic stress due to hydrodynamic wash, anammox granules characteristics are altered. In particular, EPS content decreases with the increasing degree of hydrodynamic wash (S. Zhang et al., 2020)

Exogenous substances

- FA

Free ammonia (FA, NH_3) concentration stimulates EPS production since microbial cells tend to protect themselves from damages due to unfavourable environments secreting more extracellular substances (X. Li et al., 2020).

- MONPs

Metal oxide nanoparticles (MONPs) impact on anammox process needs to be clear as their use in consumer products is increasing. MONPs are, for example, titanium oxide (TiO_2), ceria dioxide (CeO_2), aluminium oxide (Al_2O_3), or silicon dioxide (SiO_2). EPS content increases as the MONP level rises. Mainly, the PN concentration is affected by MONPSs, while PS levels remain relatively stable. As a consequence, the PN/PS ratio is altered (Z. Z. Zhang et al., 2018). Probably, the reason for the more copious EPS secretion is a protective response against environmental stresses (Zhang et al., 2018).

On the other hand, Zinc oxide (ZnO) and silver nanoparticles inhibit anammox granules and cause a decrease in both proteins and polysaccharides in EPS (Zhao et al., 2019). As the ZnO concentration increases, the EPS concentration and capacity to protect the microorganism of anammox granules decreases as Zhao, Zhang, & Zuo (2019) demonstrated (Figure 9).

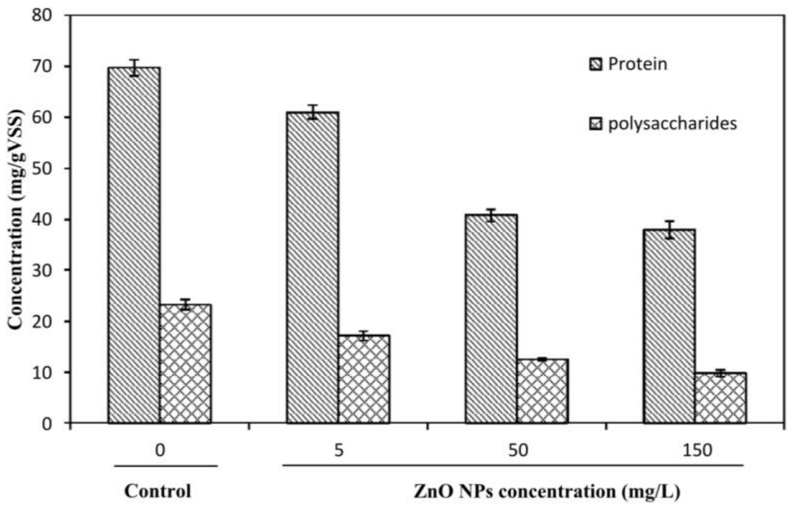


Figure 9 Variation of the content of PN and PS in the EPS of anammox granules (Zhao et al., 2019)

- Trace Elements

Trace elements as manganese (Mn^{2+}), zinc (Zn^{2+}), and copper (Cu^{2+}) affect the anammox process and the production of EPS from anaerobic anammox bacteria. Shot-term (1 day) addition encourages the anammox activity and NRE while a long-term (90 days) exposure alters the microbial structure (H. Li et al., 2018).

Table 3 shows the results of Huayu Li et al., 2018 study on the affection of trace metals on PN and PS concentration, total EPS amount, and PN/PS ratio. After 90 days of exposure, the EPS content has slightly increased in Control, Mn, and Cu treatments, while it has sharply increased with Zn addition. On the other hand, the PN/PS ratio significantly changed only in Mn treatment with a drastic decrease in the ratio due to an increase of PS content.

Table 3 EPS variation under short- and long-term exposure to trace elements (H. Li et al., 2018)

Treatment	Time (d)	PN (mg/g SS)	PS (mg/g SS)	EPS (mg/g SS)	PN/PS
Control	1	76.48 ± 2.32	56.76 ± 1.82	133.24 ± 2.95	1.35
	90	79.48 ± 4.64	59.70 ± 1.89	139.18 ± 5.01	1.33
Mn	1	76.01 ± 3.85	56.65 ± 2.65	132.67 ± 4.68	1.34
	90	76.67 ± 3.13	62.11 ± 2.61	138.77 ± 4.07	1.23
Zn	1	76.66 ± 3.72	58.56 ± 2.32	135.22 ± 4.39	1.31
	90	93.57 ± 3.14	69.07 ± 2.64	162.64 ± 4.10	1.35
Cu	1	77.27 ± 2.96	57.16 ± 2.21	134.43 ± 3.70	1.35
	90	81.14 ± 2.31	59.47 ± 1.46	140.61 ± 2.73	1.36

2.8 EPS potential applications

The circular economy is a new economic novel, that overcomes linear economy, and aims at waste recovery and valuation, so it brings both an environmental and economic advantage. It involves strategies for sustainable development, environmental preservation, zero-waste and clean production, waste regeneration and reuse, environmental quality and economic well-being (Secco et al., 2020).

EPS extracted from waste sludge is a resource that can be employed in various fields with multiple functions instead of being thrown away as circular economy strategy suggests. Indeed, the cost of handling and dispose of the waste sludge represents up to 50% of wastewater treatment costs (Kim et al., 2020).

Aiming for this goal, after EPS extraction and biomaterials recovery, there are several potential applications as a coating agent and fire retardant (Kim et al., 2020; Lai et al., 2018). Also, aerobic granular sludge hydrogel-forming capacity implies some potential EPS industrial utilisation as textile printing and paper coating (Karakas et al., 2020). Moreover, it could be applied in the production of thickeners and gelation agents in the food industry and drug-delivery systems and also it has already been commercialised as a product for the construction engineering market, as an improvement for curing of cement (http://www.ngcm.nl/curing-compound.html) (Karakas et al., 2020).

These several industrial solutions, pave the way for further use of "wastes" from wastewater treatment plants by material improvement, attributing added-value to sludge, reducing the mass of refuse to be handled and so stimulating the desirable shift from wastewater treatment to wastewater biorefinery.

2.8.1 Flame retardant materials

EPS both from flocs and from granules have been tested as bio-based flame retardant materials, and EPS coated fabrics result to achieve self-extinguishment thanks to char formation (Kim et al., 2020). Indeed, these biopolymers can be a resource for developing high-performance bio flame retardant materials. Another advantage would be the reduction of consumptions of conventional flame retardants treated with halogenated (e.g. bromine and chlorine-based agents). Nowadays, halogenated flame retardants cover almost 31% of the market even though they have been associated with a wide range of harmful effects in humans and the environment due to the emissions of toxic organic pollutants as dioxins and furans (Kim et al., 2020). Also, Kim et al. (2020) tested flax fabric covered with EPS recovered from aerobic granular sludge and activated sludge. A Bunsen vertical burning test was performed for twelve seconds applying an ignition flame on flax without treatment, coated with EPS flocs from activated sludge and EPS granules from aerobic granular sludge, as is shown in Figure 10.

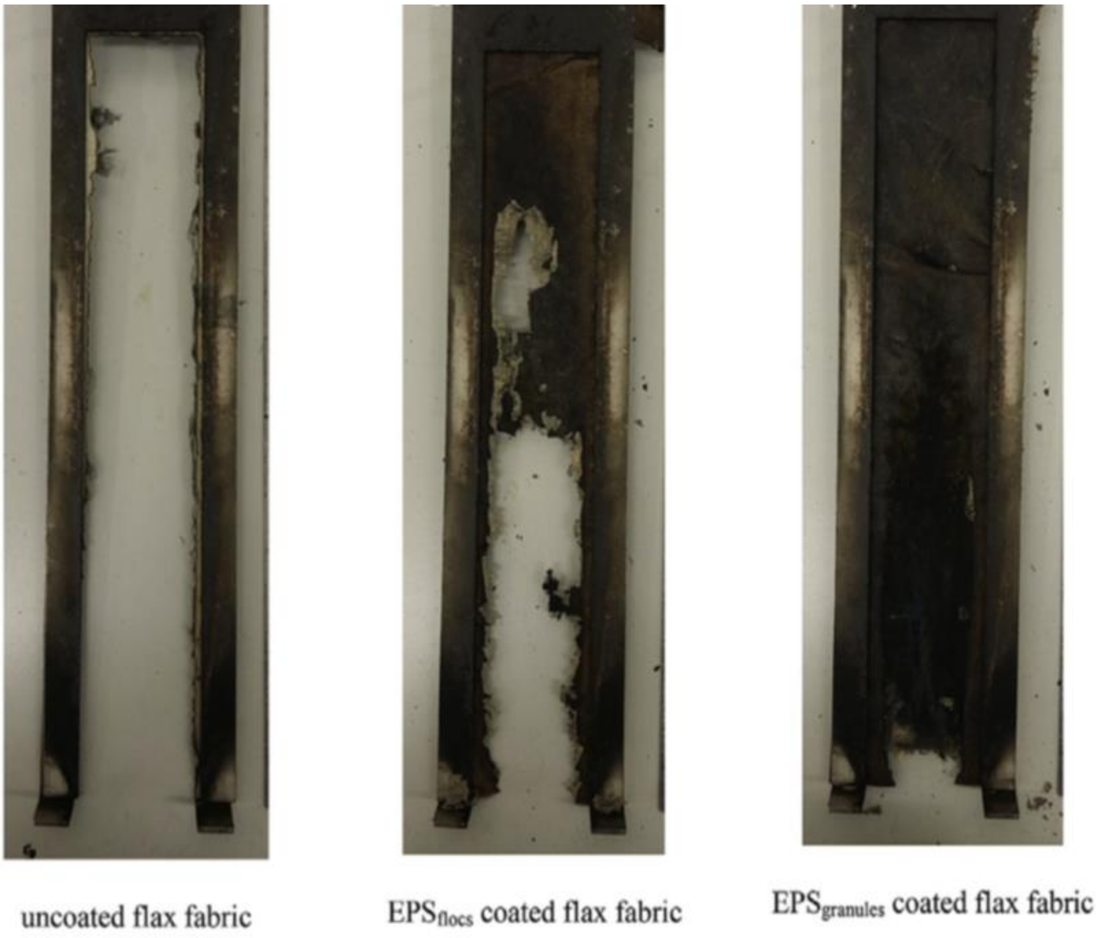


Figure 10 Comparison between uncoated flax fabric, EPS flocs coated flax fabric and EPS granules coated flax fabric after burning for twelve seconds, respectively (Kim et al. 2020)

The result of Kim et al. (2020) study is that both EPS from aerobic and activated granular sludge and have self-extinguishing properties and are both suitable for coatings; however, aerobic EPS is a better candidate since it showed the better performances.

2.8.2 ALE

Alginate like exopolysaccharides (ALE) is a fraction of EPS from granular sludge that resembles standard sodium alginate (Sepúlveda-Maradones et al., 2019). ALEs are polymer of sugar and proteins and can be used in agriculture and horticulture, paper industry, medical and construction industry (Leeuwen et al., 2018). Fibres can be used for tissues production, and gels can be recovered as glue for the production of fertiliser pellets, and foam can be used for fire-resistant board production (Leeuwen et al., 2018). Even though ALEs are present also in flocculent sludge, the amount in granular sludge is twice as much (160 ± 4 mg/g VSS and 72 ± 6 mg/g VSS, respectively). The extractable

ALE is about 15-25% of the organic fraction in aerobic granular sludge (Y. Lin et al., 2010) and it is easy to retrieve; indeed, it represents more than 50% of the turnover in WWTP (Scandolara et al., 2020).

2.8.2.1 Curing of cement

The final step of cement-based materials production is curing, the process of controlling the amount of moisture loss from the surface of the materials. The curing step is essential for several reasons: it provides continuous cement hydration; it prevents cracking due to drying shrinkage; it decreases voids formation; it guarantees water depercoliation of capillary pores (Zlopasa et al., 2014). Water-based bio-polymers can be employed to prepare eco-friendly durable cement-based materials. Anaerobic granular sludge derived biopolymers, in particular alginate-like exopolysaccharides (ALE) a fraction of EPS from granular sludge, are adopted as an improvement for curing cement (Karakas et al., 2020) thanks to their hydrophilic properties and their similarity with alginates. Moreover, using EPS avoids the need for workers on-site to keep concrete moist, which is a costly technique (Zlopasa et al., 2014).

1.8.2.2 Gelification

ALE has a unique gel-forming property and a viscoelasticity capacity that are essential for gel stability. Thanks to this gelation property ALE are responsible for the formation of EPS-based hydrogels. Indeed, EPS forms stable ionic hydrogel beads without the addition of any cross-linker (Karakas et al., 2020) and hydrogel binding capacity emerged as a function of EPS (Lotti et al., 2019). ALE mechanical properties are enhanced by the guluronic acid presence (Meng et al., 2019). A hydrogel is a network of polymer chains with a remarkable hydrophilic capacity, high absorption properties, and it can contain over 90% water. These gels have several uses mainly in bioindustry as in tissue engineering, breast implants, contact lenses, drug delivery systems, and others.

2.8.2.3 Inducement of struvite formation

Struvite or ammonium magnesium phosphate ($NH_4MgPO_4 \cdot 6H_2O$) is a soft mineral. In wastewater treatment plants struvite can be a problem as it provokes inefficiencies and might cause pipes or other equipment clogging. It was found that EPS from aerobic granular sludge is capable of inducing struvite-like structure formation and accumulation (Y. M. Lin et al., 2012). Whether it is K-struvite or ammonium struvite depends on the availability of potassium and ammonium ions in bulk; their ammonium adsorption capacity was 0.36 mg/g (at an initial concentration of 10.4 mg N/L), which is more or less 60% of the adsorption capacity of granular sludge at the same equilibrium ammonium concentration. Struvite-like precipitates have not only the capacity of absorbing ammonium but also the ability to remove phosphorus that becomes available, and struvite can be utilised as a fertiliser (Leeuwen et al., 2018; Y. M. Lin et al., 2012).

2.8.2.4 Biosorbent for dyes and phosphorous removal

It is urgent to find innovative cleaning solutions to avoid water contamination due to dye-containing effluents. Dyes are used mainly in textile, leather, paper, pulp, cosmetic, drug, plastic, electroplating, and printing industry. These products can negatively affect the photosynthesis and could be toxic. Unfortunately, it has been estimated that annually are produced more or less 7×10^5 to 1×10^6 tons of dyes, around 20% of this amount is released in the environment (Ladnorg et al., 2019). It was demonstrated that ALE could be applied as a modern and innovative bio-sorbent for dye methylene blue removal from aqueous solutions. Owing to its adsorption ability, ALE can reach up to 69% dye removal a bit below the one of alginate 78% (Ladnorg et al., 2019). Several factors affect the adsorption effectiveness: pH even though not in a significant way; dosage absorbent, an increase of ALE leads to an increase of methylene blue up to a maximum concentration (≃117 g/L); temperature, the optimum temperature for adsorption is 35°C; initial dye concentration has a positive effect on the adsorption rate until a saturation level (Ladnorg et al., 2019).

EPS has a high adsorption potential against heavy metals (J. Li et al., 2019), ALE, for example, can be employed as biosorbent is for phosphorus removal from liquid samples. Phosphorus demand is increasing as it is commonly adopted in animal feed, detergents, beverages, fertiliser production, chemical pesticides, and water processing. Also, phosphate deposits and reserves around the world are lacking, and as a scarce resource, it appears on the European Critical Raw Materials list (Leeuwen et al., 2018). Thanks to its effectiveness, bio-based adsorption ALE might be a promising technique for phosphorus removal and recovery (Dall et al., 2020). ALE beads demonstrated the potential to remove phosphorus (49.54 ± 2.23%) from liquid samples better than commercial seaweed alginate (36.78 ± 2.10%). The amount of phosphorus removed depends on several factors: pH, temperature and initial concentration. ALE regeneration has also been tested, to increase the life cycle of beads; it should be combined with acid and alkali solutions (Dall et al., 2020).

Further studies for textile wastewater treatment could focus on the possibility of an integration of the adsorption process and other biological processes for carbon and nitrogen removal (Ladnorg et al., 2019).

2.8.3 Paper coating

A distinctive feature of aerobic granular sludge EPS is that it has an amphiphilic character, which means it has both hydrophilic and lipophilic properties, so the molecule has both polar, water-soluble groups and nonpolar, water-insoluble groups. The amphiphilic property was measured by using Material Adhesion to Hydrocarbons (MATH) test, which revealed a high hydrocarbon affinity of EPS (Y. M. Lin et al., 2015). Therefore, EPS can strongly bind with water, to thicken or jellify liquids (Lotti et al., 2019). Thanks to these peculiarities, EPS has perfect film-forming properties: it can create a water-resistant continuous and homogenous film on hydrophilic surfaces as paper (Y. M. Lin et al., 2015; Nancharaiah & Kiran Kumar Reddy, 2018). When used as a paper coating agent, EPS also showed a grease resistance effect (Lotti et al., 2019).

Adopting EPS based coating agents could be a valid eco-friendly alternative to commonly adopted coatings, so water and oil-grease permeability on paper have been tested and analysed. Lotti et al. (2019) examined two different aqueous solutions: carboxymethyl cellulose (CMC) (Finnfix CMC by CP Kelko, Atlanta GA, USA) only (10% g/g-solution) and a composite of CMC and EPS (10% CMC and 4% EPS g/g -solution, respectively). The paper sheets coated only with CMC were used as control. Water absorbance potential was calculated using the Cobb technique (UNI EN ISO 535:2014 method) while the opposition to grease, oil and, waxes was evaluated by measuring permeability (turpentine oil method, ISO 16532-1:2010) and surface repellency (ISO 16532-2:2010 method).

CMC+EPS coating resulted in reducing the water absorbance by 30% compared to the control sample; this implies that EPS contributes to decreasing water absorbance (Lotti et al., 2019). While (Y. M. Lin et al., 2015) concluded that a 5% (w/v) polysaccharide-based biomaterial water solution provides the same water-resistant property to paper as the commercial coating product - 10 % (w/v) alkenyl succinic anhydride (ASA) – does. For what regards grease resistance, the degree of surface repellency was increased by EPS coating. Paper cellulosic fibres absorb EPS that fills the pores of the paper. Therefore, EPS acts as a barrier to water and grease penetration (Lotti et al., 2019).

2.8.4 Heavy metals toxicity reduction

EPS plays a fundamental role as biodetoxification agent against heavy metals; it helps to resist to metals as lead (Pb) one of the most common toxic elements in the environment. EPS is correlated with organisms tolerance to survive to toxicity; for example, it works to resist to Pb toxicity. This ability is enhanced by EPS metal-binding ability (J. Li et al., 2019).

2.8.5 Fabrication of EPS based membranes

EPS from anaerobic granular sludge resulted in being effective in regulating anion and cation concentrations and toxicity. Thanks to ion exchange properties, EPS can be applied to form a selective membrane towards the transport of monovalent ions. Sudmalis et al. (2020) tested a novel approach, combining EPS with an inert binder (PVDF_HFP) to form a membrane that acted as a cation exchange membrane (CEM). The EPS layers resulted in being productive with an 80% efficiency (concerning CEM) in the transport of potassium (K^+) over sodium (Na^+)ions (Sudmalis et al., 2020). However, the membranes of this study are yet of insufficient stability for commercial application and need to be further investigated.

Chapter 3 – Material and Methods

3.1 Sampling information

In this study, both anammox granular sludge and anaerobic sludge were used for the analysis (Table 4). Anammox granules were taken from a pilot-scale reactor of a textile industry located in Como at different periods from July 2019 until the end of the year. The reactor has a total volume of 12 m^3, a working volume of 9 m^3 and the volume exchanged in every cycle is 4 m^3. Two different biomass were adopted: one from the beginning July until the 10th of October, with a biomass concentration factor of 9.94 g VSS/L; the second one, was introduced on the 11th of October, with a concentration factor of 13.09 g VSS/L. Also, the experiments were conducted on two different types of anaerobic granules from two different industrial WWTP, treating the effluents of, respectively, a brewery and a paper industry.

Table 4 Granular sludge samples information: name, origin and collection date

Type	Sample Name	Abbreviation	Collection Date
Anammox GS	Initial	B0	09-07-2019
	Control (Cultivated in the lab)	B0-1	17-07-2019
	Batch 1	B1	06-08-2019
	Batch 2	B2	16-09-2019
	Batch 3	B3	17-10-2019
	Batch 4	B4	12-11-2019
	Batch 5	B5	09-12-2019
Anaerobic GS	Anaerobic GS from Brewery	AnGS-Brewery	26-01-2020
	Anaerobic GS from paper industry	AnGS-Paper	01-02-2020

3.2 Materials and equipment

The work was conducted at the laboratory of environmental engineering (laboratorio di ingegneria ambientale – LIA) of the Department of Civil and Environmental Engineering (DICA) at Politecnico di Milano. For performing the analysis, different materials (Table 5) and reagents (Table 6) were employed.

Table 5 Instruments information

Instruments	Brand	Model	Type	Country
Centrifuge	Thermo Scientific	IEC CL10	Max 6500 rpm	Germany
Water Bath	Falc	WB U4	Max 120 °C	Italy
Magnetic Stirrer	VELP Scientifica	-	Multistirrer/microstirrer	Europe
Filter 3 kDa, 30 kDa	Merck	Amicon - Ultra Centrifugal filters - 4 ml	3 kDa NMWL and 30 kDa	Germany
UV/Vis/NIR Spectrophotometer	HACH	LANGE DR. 6000	-	Italy
Quartz Cuvette	Hellma	100-OS	10 µm	Germany
Disposable Cuvettes	BRAND	1.5 ml semi-micro	12,5x12,5x45 mm	Germany

Table 6 Reagents information

Reagents	Main component	Form	Colour	Brand	State
Glucose	Glucose Anhydrous	Crystalline	White	Farmitalia Carlo Erba	Italy
Phenol	Hydroxybenzene	Crystalline	Colourless	SIGMA-ALDRICH	United Kingdom
Sulfuric Acid	95.0-97.0% concentrated	Liquid	Colourless	SIGMA-ALDRICH	Germany
Bicinchoninic Acid solution		Liquid	Colourless	SIGMA-ALDRICH	USA
Protein Standard	Bovine Serum Albumin – BSA 0.1 mg/ml BSA in 0.15 M NaCl with 0.05% sodium azide as a preservative	Liquid	Colourless	SIGMA-ALDRICH	USA
Copper sulphate pentahydrate	$CuSO_4*5H_2O$	Crystalline	Blue	E. Merk, Darmstadt	Germany

3.3 Chemical analysis

Total solids (TS) is the material left in a sample vessel after evaporation and subsequent oven drying at 105°C (APHA, AWWA, & WEF, 2017). TS includes both total suspended solids and total dissolved solids, which are physically separated via filtration. Volatile solids (VS) are the total, suspended or dissolved solids lost from a sample after ignition at 550$_o$C for 2h (APHA et al., 2017). Figure 11 schematises the experimental analysis.

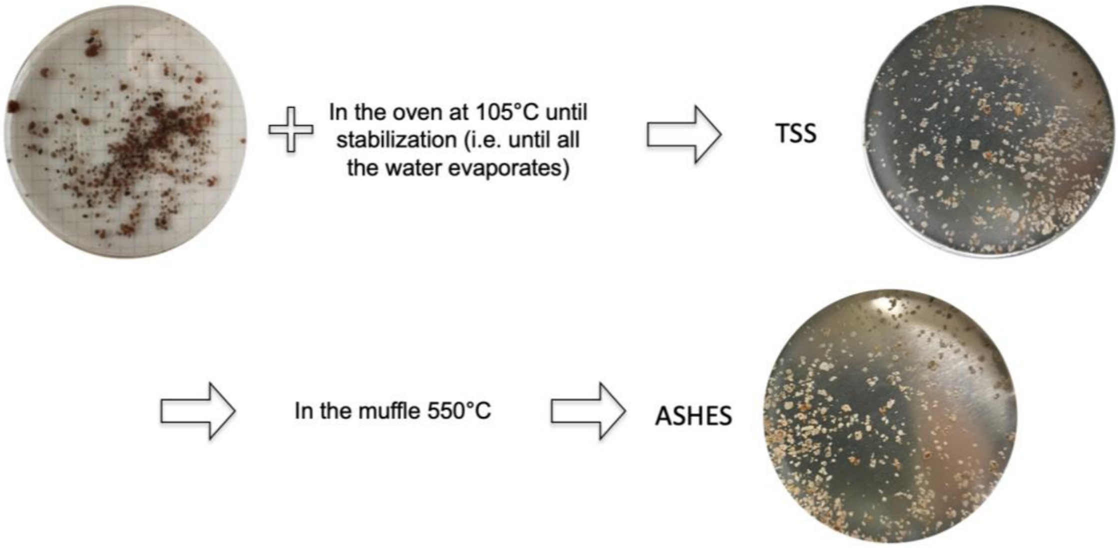


Figure 11 Granules composition analysis experimental scheme

After collecting the EPS, further analyses aimed at comparing the composition and yield of the samples were conducted. Firstly, TSS, VSS, and ashes amounts in EPS were calculated to derive the EPS_{yield} = g VSS in EPS/g VSS in GS.

3.4 Particle size distribution (PSD) analysis with ImageJ

The software ImageJ was adopted to compare different granules sizes from a quantitative point of view. ImageJ is an image-processing tool able to count the number and the dimension of the particles in a picture. To obtain a meaningful PSD analysis, ten photos were taken for each sample, each capturing a different portion of the sample $\simeq$ 250 granules per picture. This tool receives a picture as input and calculates the number of particles present in it and the area covered by each one of them. The first step of the analysis, is setting a scale and a unit of measure; then, the software renders the image binary, i.e. black and white, see Figure 12; and, finally, it calculates the size and number of grains.

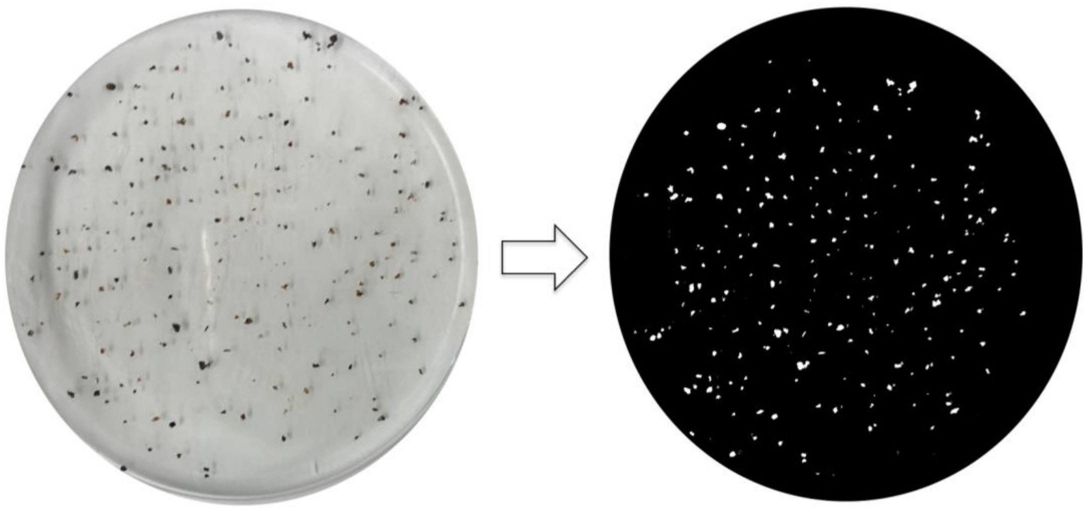


Figure 12 Image J binary transformation

3.5 Protocol of EPS extraction from granular sludge with heating-Na_2CO_3 based method

EPS were extracted from granular sludge using heating at 80oC with addition of Na_2CO_3, according to previous studies (Feng et al., 2019). Firstly, 0.4 g (dry weight) of granular sludge was transferred to a 100 ml blue-cap bottle with 40 ml with 0.5% Na_2CO_3. Then the bottle containing the mixture was put into the water bath preheated at 80°C for 30 mins. Afterwards, the tube containing the mixture was transferred to a 50 ml falcon tube for centrifuge at 4000 rpm and 4°C for 20 minutes. Then the supernatant was collected and filtered using a 0.45 µm membrane directly in a new 50 ml centrifugation tube. The supernatant was then thickened for 1 day at 60°C to a final volume of 5 ml to increase the polymer concentration in the supernatant. The sample was stored at -20°C and transferred into a freeze drier at -50°C for 2 days to remove water. The procedure was depicted in Figure 13.

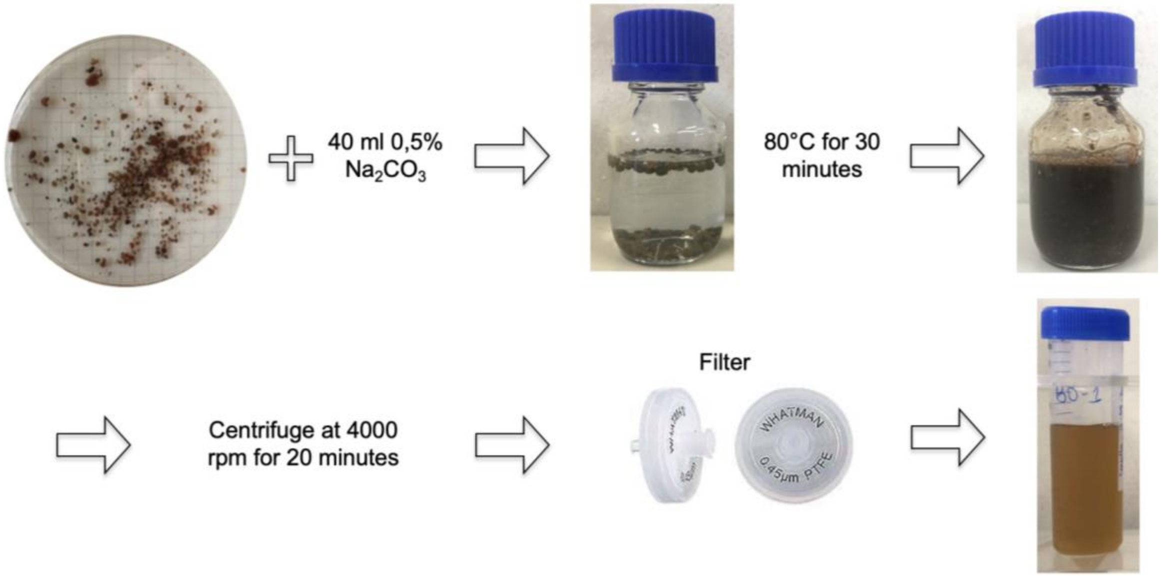


Figure 13 Experimental scheme of EPS extraction using the method of heating with Na_2CO_3 method (Feng et al., 2019)

3.6 Carbohydrates analysis using the phenol-sulfuric acid method

Measurement of carbohydrate contents in a variety of samples is a fundamental analytical operation to understand the composition of various EPS samples. The phenol-sulfuric acid method is widely used cause it is an accessible, reliable and sensitive method for polysaccharides measurement (DuBois et al., 1956). Due to its simplicity and speed, it is suitable for the analysis of large numbers of samples.

Assay

1. Prepare a 1ml sample.
2. Add 1ml of 5% phenol-solution and mix thoroughly.
3. Leave for 10 minutes at room temperature.
4. Add rapidly (in stream) 5mL of 97% sulfuric acid.
5. Leave for 5 minutes at room temperature for cooling.
6. Mix (shake) the test tube again.
7. Leave for another 25 minutes at room temperature.
8. Measure the absorbance at 490 nm against a blank (Milli Q water).

The first step was the creation of the calibration curve (Figure 14) using standard samples with known concentrations (Table 7). The calibration curve $y = 111.01x -$

7.0679 allows the derivation of the concentration of polysaccharides in other samples by comparison. The statistical value of R2 is equal to 0.9952. In a best-fit line, a high (close to the maximum 1) R2 value indicates a strong correlation between the independent variable (x), the measured absorbance, and the dependent variable (y), the PS concentration. The R2 value is very high, so the dependence is reliable.

Table 7 Calibration curve for carbohydrates analysis

Number	Standard (µg/ml)	Solution A (ml) in a 100 ml total volume	Absorbance at 490 nm
1	200	20	1.834
2	100	10	0.946
3	50	5	0.579
4	20	2	0.204
5	10	1	0.134
6	5	0.5	0.071

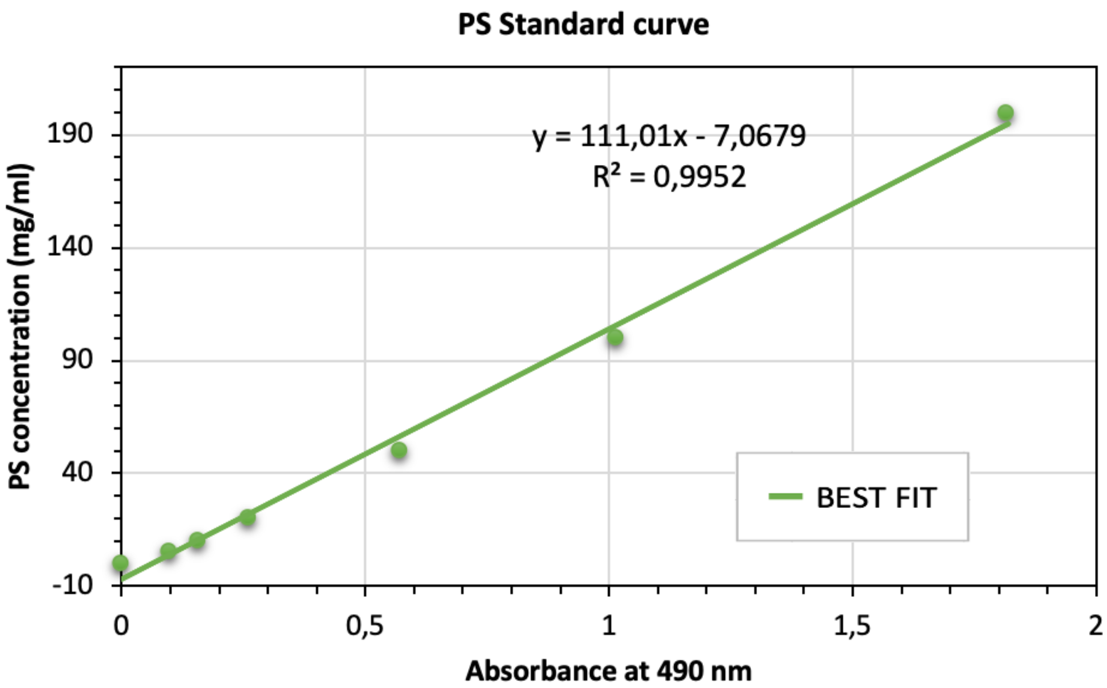


Figure 14 Carbohydrates analysis calibration curve

3.7 Bicinchoninic Acid Protein Assay (BCA)

The protein determination was based on the BCA assay, since it is better than other techniques as it is user-friendly, provides stable colour complex, it has a lower susceptibility to detergents, and it is widely applicable (Smith et al., 1985).

Assay

1. Prepare the BCA working reagent;
2. Prepare standards of different concentrations;
3. Add 2 ml of the BCA working reagent to 0.1 ml of each BSA standard, blank and unknown sample. Vortex gently for thorough mixing. The total liquid volume is 2.1ml;
4. 37°C for 30 minutes;
5. If required, allow the tubes to cool down at room temperature;
6. Transfer the reaction solutions into a cuvette;
7. Measure the absorbance of the solution at 562nm.

The working reagent adopted is a mixture of bicinchoninic acid and copper sulphate pentahydrate with a ratio 50:1. As for the polysaccharides measurement, firstly the calibration curve (Figure 15) was prepared using Protein Standard (Bovine Serum Albumin - BSA) as stock a solution (Table 8). The calibration equation is y= 929.15x-17.046, and the high R_2 index of 0.9988 indicates a strong correlation and excellent reliability. It is quite significant to notice the colour trend: it goes light green to purple with increasing protein concentration (Table 8).

Table 8 Standard concentration, measure absorbance and standard appearance

NUMBER	1	2	3	4	5
Sample (ml)	0.1	0.1	0.1	0.1	0.1
[BSA] Protein Standard (µg/ml)	0	200	600	800	1000
BCA working reagent (ml)	2	2	2	2	2
Absorbance at 562nm	0.106	0.358	0.780	0.980	1.187
Image					

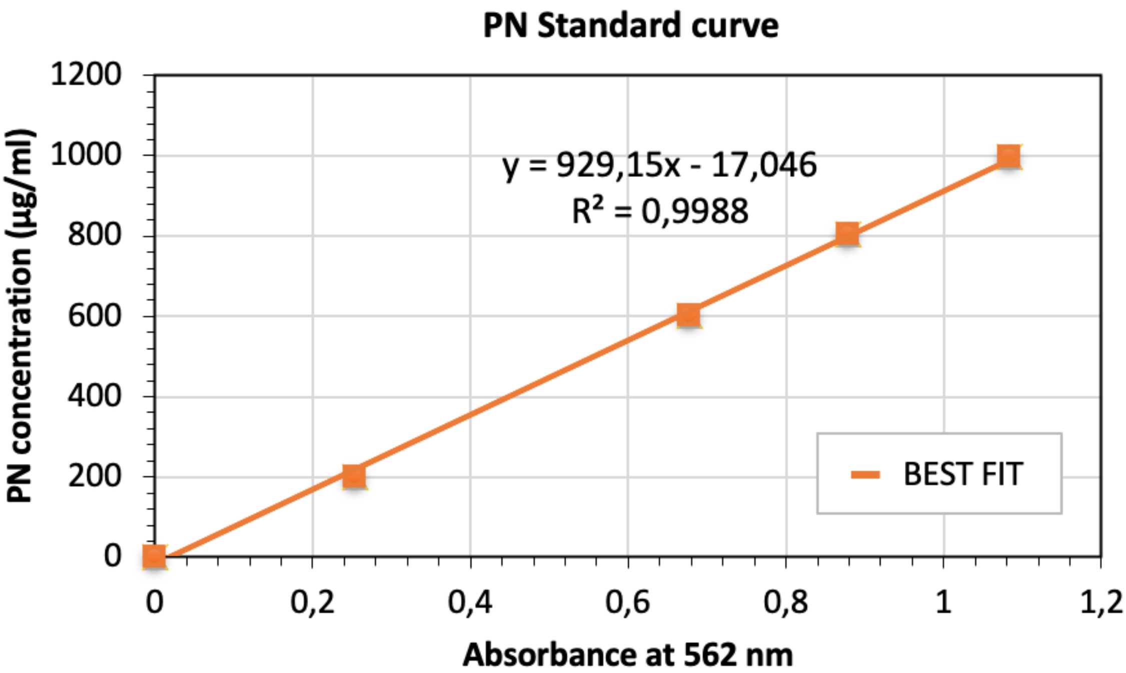


Figure 15 Proteins analysis calibration curve

3.8 UV-vis spectra collection

UltraViolet-Visible Spectroscopy was performed to determine the functional groups in EPS. Spectroscopy is a way to quantitatively study on the matter, resulting from analysing the spectra arising from the electromagnetic radiation interaction with matter. The interaction depends upon both radiation properties and the material structure. When electromagnetic radiation passes through a solution, it can be absorbed, reflected or transmitted depending on the wavelength and the body type. When the transmitted part of the light passes through a monochromator - prism or grating that select a specific wavelength of light - the absorption spectra are obtained. With absorption, electrons pass from the ground state to an excited state. Thanks to spectrophotometers absorbance of light can be measured and, based on the spectra, the elements and composition of the material can be obtained *(Orbitbiotech, 2018)*. Figure 16 schematically represents the operation of a spectrophotometer.

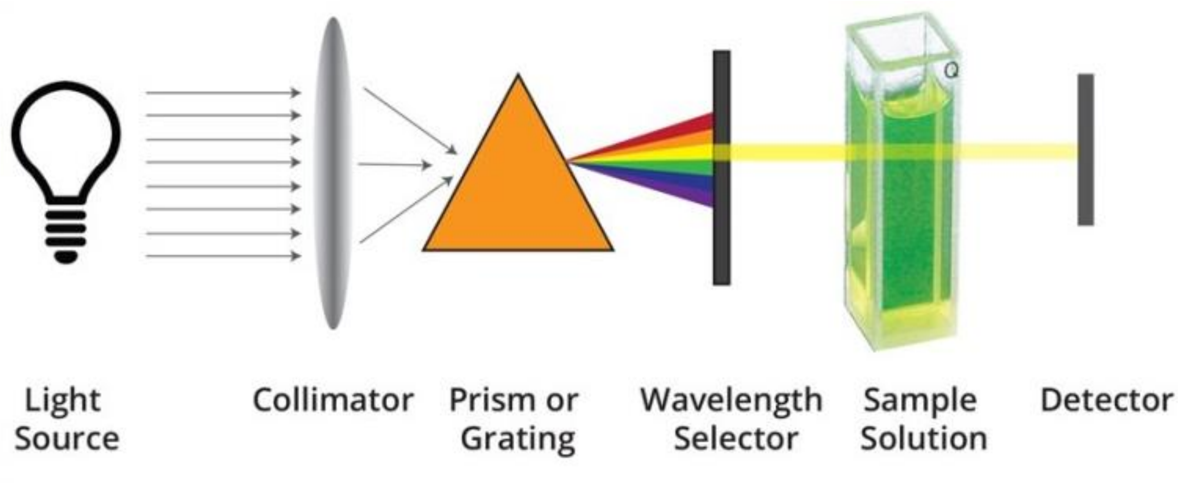


Figure 16 Schematic spectrometry functioning representation from Orbitbiotech (Orbitbiotech, 2018)

UltraViolet-Visible Spectroscopy (UV-vis) covers the range of the electromagnetic spectrum of UV between 100-400 nm and the visible region 400-800 nm. The UV light can be further divided into two regions far UV (100-200 nm) and near UV (200-400 nm) (Orbitbiotech, 2018).

3.9 Polymer size fraction analysis

The EPS supernatant, 2 ml for each sample, was centrifuged to concentrate the EPS supernatant, using different sizes centrifugal filter units: 3 and 30 kDa MWCO (Molecular weight cut-off).

Assay

1. Insert 2 ml of Milli-Q water in the centrifugal filter (3 and 30 kDa);
2. Centrifuge for 40 minutes at 4000 rpm;
3. Remove the water;
4. Insert 2 ml of EPS solution in the filter and mark the volume;
5. Centrifuge for 40 minutes (20 minutes in case of 30 kDa) at 4000 rpm;
6. Add around 2 ml of Milli-Q water and mix;
7. Centrifuge for 40 minutes (20 minutes in case of 30 kDa) at 4000 rpm;
8. Add water until the mark signed in point 4.
9. Try to dissolve the pellet.

Chapter 4 – Results and Discussion

4.1 General granular sludge characteristics

As shown in Figure 17, a morphological analysis can be done visually by confronting particles shape, colour and size. Generally, the granules have a spherical shape except for B5. For B5, the structure was decomposed, and its colour differs from other anammox samples, maybe due to a malfunction of the reactor or additional external factors. It can be seen that the colour of anammox GS (B0-B4) are reddish, which can be attributed to the presence of heme *c* group of the protein cytochrome *c* (Feng et al., 2019). In contrast, anaerobic GS are dark grey.

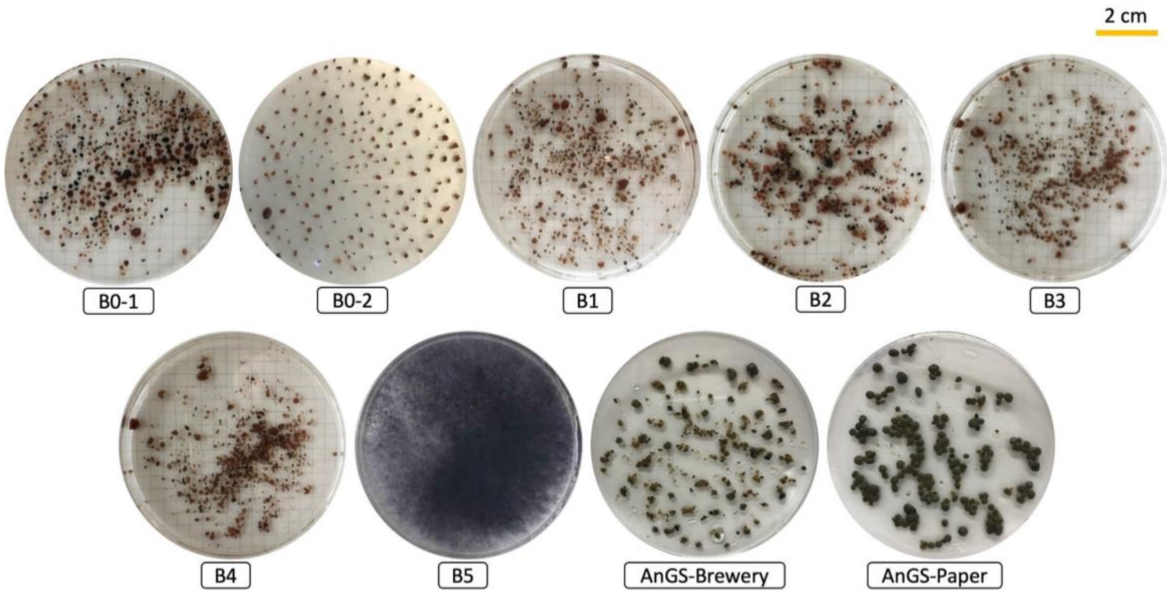


Figure 17 Granules pictures

PSD of granular sludge samples is a critical step to characterise the granules. The analysis was conducted on all samples except B5, due to its small dimensions. Particles distribution (Figure 18) is dominated by small granules (diameters below 0.3 cm), and the maximum diameter detected is close to 1.1 cm. In every graph, the bar charts follow the same trend, and the peak is registered slightly after the diameter of 0.1 cm. The anammox samples PSD curves basically overlap (Figure 19a), the ones with a higher fraction of granules close to 0.2 cm are B1 and B2. Monitoring anammox granules might lead to the individuation of a gradual increase of the particles size with time. Indeed the formation of the granular shape is a long process that might last hundreds of days starting with small aggregates to the final granules (Fan et al., 2020). As it will be illustrated in Chapter 4, the anammox reactor under study is not yet stable and worked with different biomass, so it is not possible to notice granules progressive formation.

AnGS-Paper granules are the more massive and compact; accordingly, the curve peak of AnGS-Paper is shifted to the right with respect to AnGS-Brewery sample, and it is close to 0.3 cm (Figure 19b). Indeed, the two AnGS samples have very different distributions; if the shape of AnGS-Brewery is similar to the anammox one, the curve of AnGS-Paper is unique (Figure 19c).

Table 9 shows the particle diameter of different granules. For anammox granules, the mean diameter ranges from 0.2058 (B1) to 0.2438 cm (B2), excluding B5 value since it is an outlier while the mode diameter is within 0.0618 (B3) and 0.125 (B2) cm. Both from morphological observation and mean diameters information B2, B3, B4 are characterised by higher average mean areas. Moreover, B5 granules are visibly tiny: the average diameter is smaller than other samples of about 0.2 cm. AnGS have a more compact structure with respect to anammox granules (Figure 17), AnGS-Paper is the samples with the biggest granules, 0.2614 and 0.1882 cm, respectively, for mean and mode diameter.

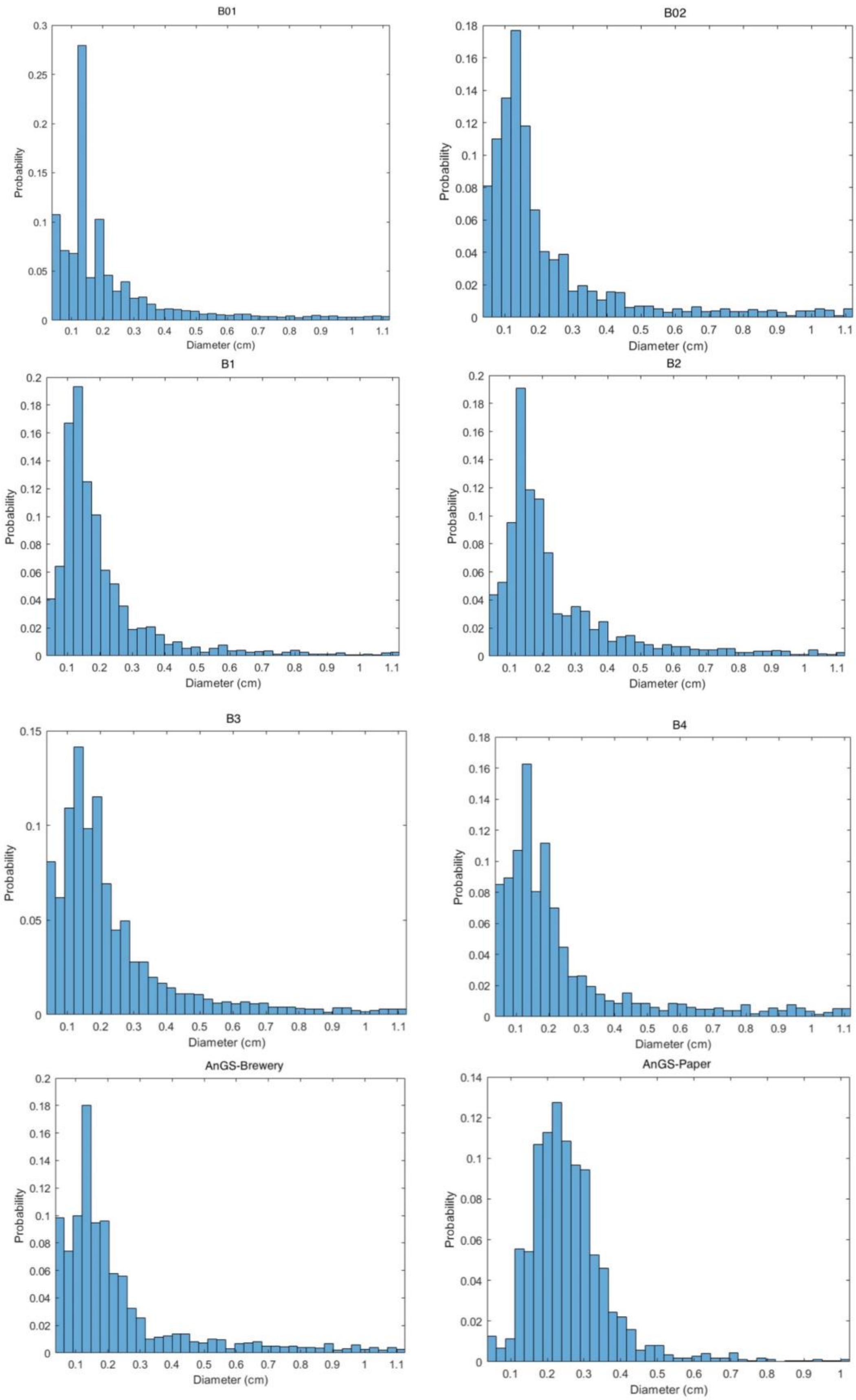


Figure 18 Plot of particle size distribution analysis

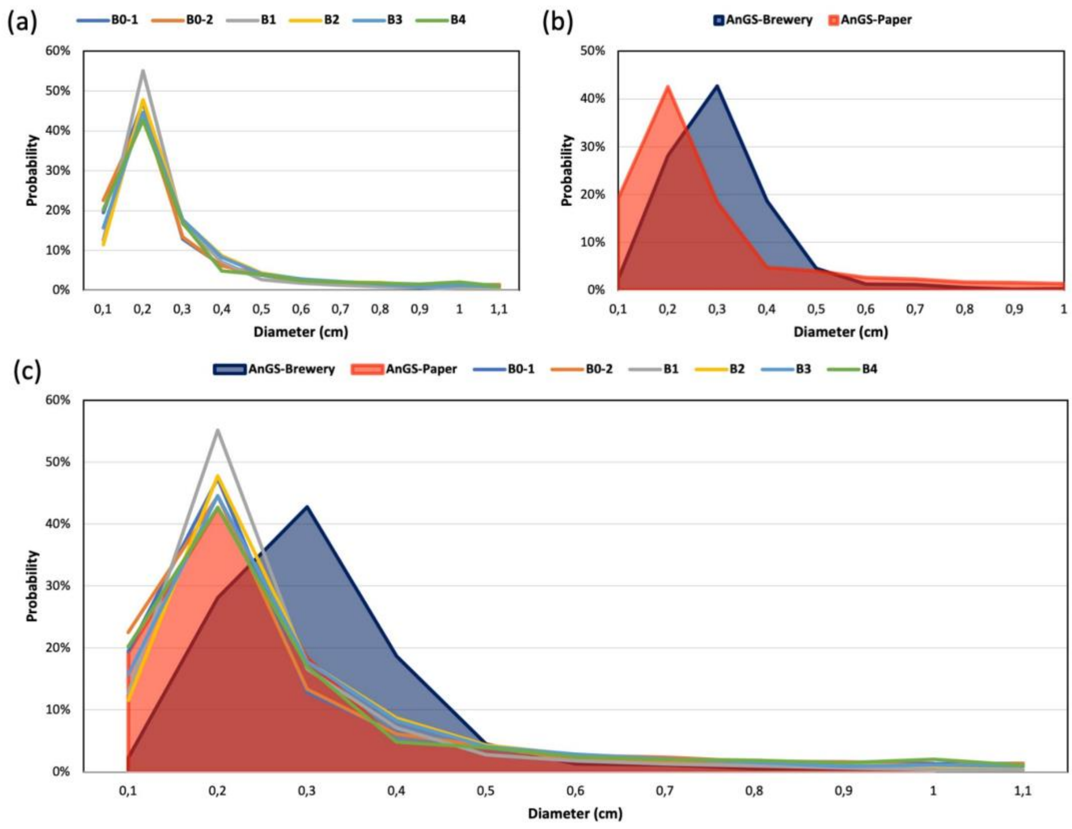


Figure 19 (a) comparison of particle size distribution analysis of anammox granules (b) comparison of particle size distribution analysis of anaerobic granules (c) comparison of particles size distribution analysis of anammox and anaerobic granules.

Table 9 Granules size information

Type	Name	Mean Diameter* [cm]	Mode Diameter** [cm]
Anammox GS	B0-1	0.2298	0.1293
	B0-2	0.2233	0.1362
	B1	0.2058	0.1335
	B2	0.2438	0.1524
	B3	0.2370	0.0618
	B4	0.2403	0.0798
	B5	0.0822	0.0357
Anaerobic GS	AnGS-Brewery	0.2367	0.0505
	AnGS-Paper	0.2614	0.1882

*Mean diameter: the average diameter by summing all the diameter value and diving the sum by the number of values.

**Mode diameter: the value of the diameter that appears more frequently in each sample.

Granules composition analysis was performed, the experiments mainly encompass ashes fraction calculation together with TSS and VSS content measurement. As shown in Figure 20, the VSS take the fractions within 83% and 93% of TSS in all samples. B4 has the lowest VSS content of all the anammox granules, 83.67%, while B2 has the highest, 94.02%. B0-1 and B0-2 have medium VSS values: 0.1100 and 0.1131 g/g SS, respectively; B1, B2, B3 and B5 by a medium-high VSS fraction. Distinct VSS contents characterise AnGS, AnGS-Brewery has a value above the anammox average, 92.71%, while AnGS-Paper has one below the average, 83.31%, despite the compactness of its granules.

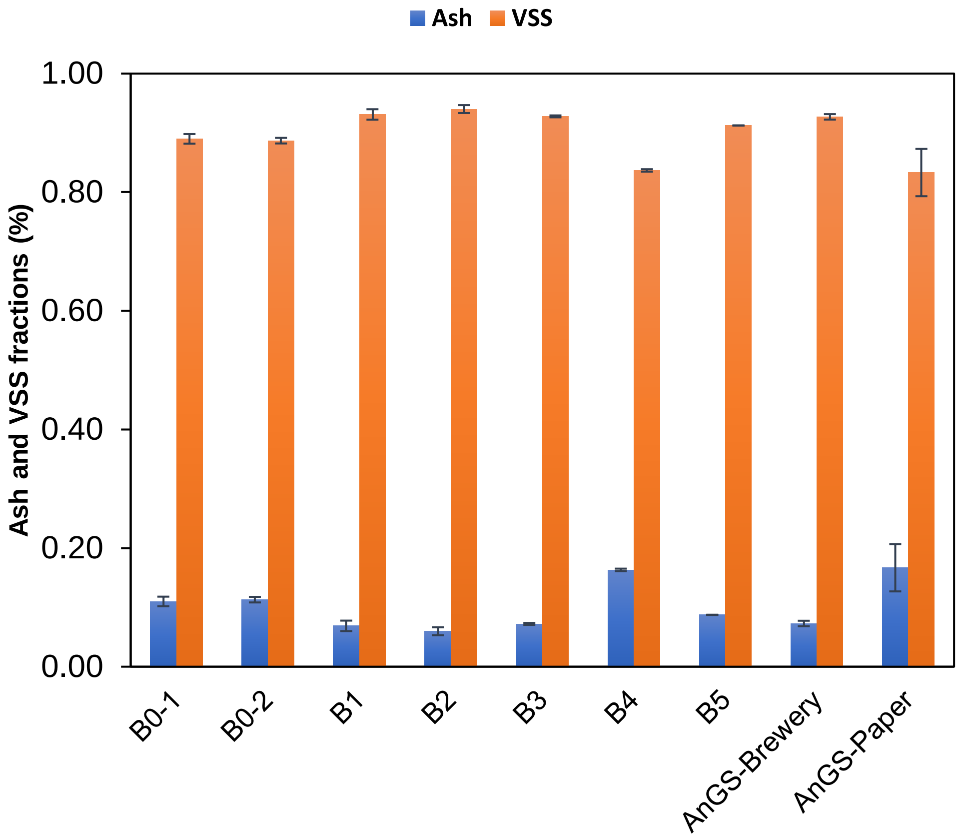
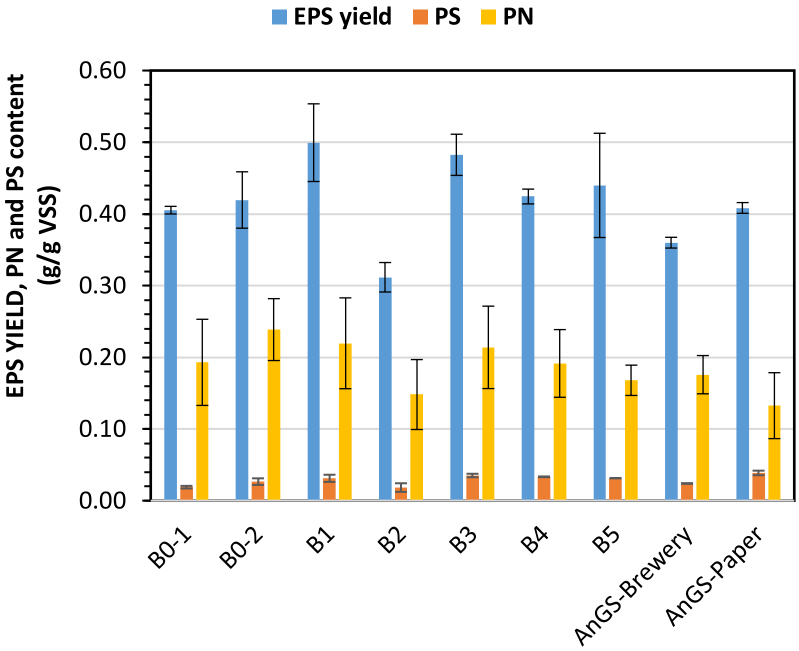


Figure 20 Ashes and volatile suspended solids content in the samples. Each test was performed in triplicates

4.2 EPS yield and EPS composition

The EPS yields are shown in Figure 21a for anammox granular sludge, EPS yields of B1 and B3 are highest, respectively 0.4994 and 0.4835 g/g VSS; while B2 has the lower production, 0.3116 g/g VSS. Although B5 granules diverged from the other samples in appearance, colour and size, no significant effect was found on the EPS content of 0.4398 g/g VSS. AnGS have a slightly lower yield, in particular, AnGS-Brewery has a lower EPS production of 0.3599 g/g VSS. In agreement with previous studies (Lotti et al., 2019, Feng et al., 2018), proteins and polysaccharides take the major fractions of EPS. In this study they represent around 50% of the total (Figure 21b). The highest fraction characterises B0-2, 63.24% while AnGS-Paper by the lowest, 41.93%. More precisely, among different samples, PS values are in a small range, from 0.0465 to 0.785 g/g VSS. As to the proteins contents, the fractions account for 32% and 57% of EPS. The proportions in B5 and AnGS-paper are lower, around 38% and 32%, respectively. Comparted with other anammox GS samples, protein contents of B5-derived EPS is quite far from other values, in line with the strange appearance of granules in Figure 17. Another thing that needs to be pointed out is that PN and PS contents in anaerobic-Paper are low, which can be attributed to the influent constitutes containing cellulose (Haq et al., 2020).

(a)



(b)

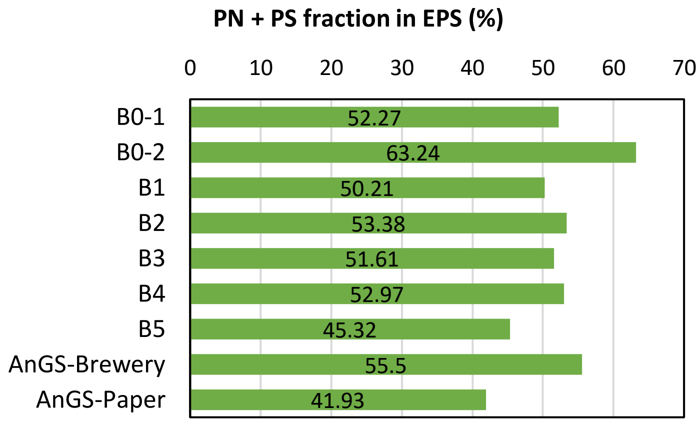


Figure 21 EPS yield, polysaccharides (PS) and proteins (PN) contents; (b) Fractions of PN+PS in EPS

4.3 Polymer size fraction analysis

Biopolymers distribution in extractable EPS was calculated using filters with different molecular weight cut-off: 3 and 30 kDa. Therefore, polysaccharides and protein fractions were derived (Figure 22). Three portions can be distinguished: small molecular weight

(MW) compounds (< 3 kDa), medium MW compounds (3-30 kDa) and large MW compounds (> 30 kDa) (Feng et al., 2019). For proteins, B01 has the largest amount of medium MW compounds; B3 has the highest large compounds fraction (≃ 60%); B2 and B5 are the samples with the higher small MW compounds presence. AnGS values remain within the average. On the other hand, the results of polysaccharides analyses reveal in every sample a meager fraction of medium MW compounds. Brewery industry has the highest fraction of small MW carbohydrates concentration, perhaps to the high quality of the granules that were already outstanding for the compactness and solidity of the structure.

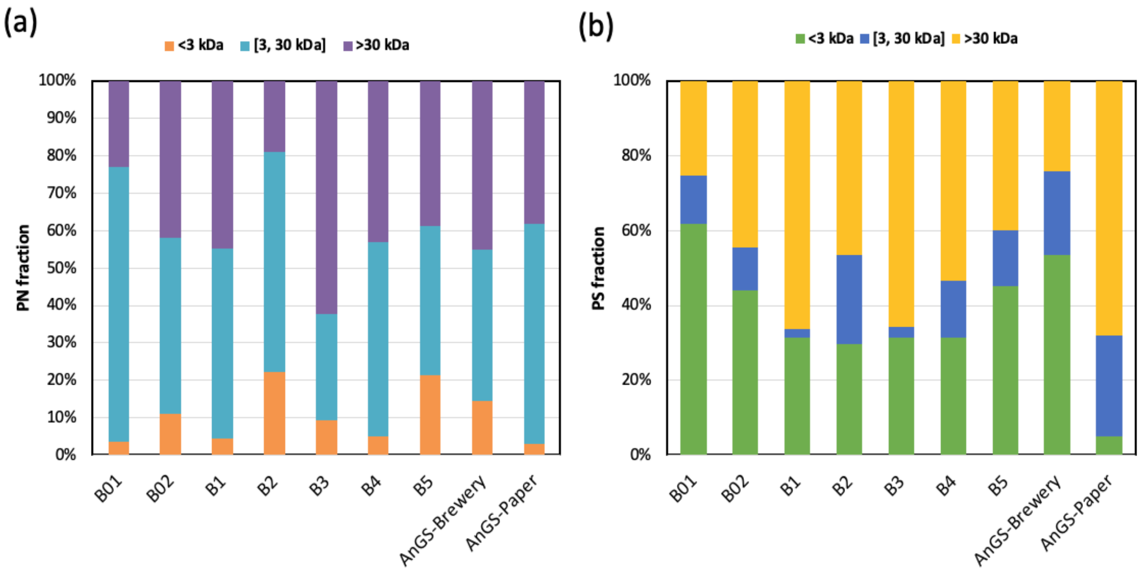


Figure 22 (a) Proteins distribution in different size ranges(b) Polysaccharides biopolymer distribution in different size ranges adapted from Feng et al. (2019)

4.4 UV-vis analysis

The spectrum was collected at 200-800 nm. The absorption spectroscopy is a useful tool to evaluate functional groups in EPS. Indeed, various functional groups are present in EPS since it is rich in aromatic structures and unsaturated fatty chains (Sheng et al., 2010). To smooth peaks the concentration of the EPS solution was adjusted with a dilution factor of 10.

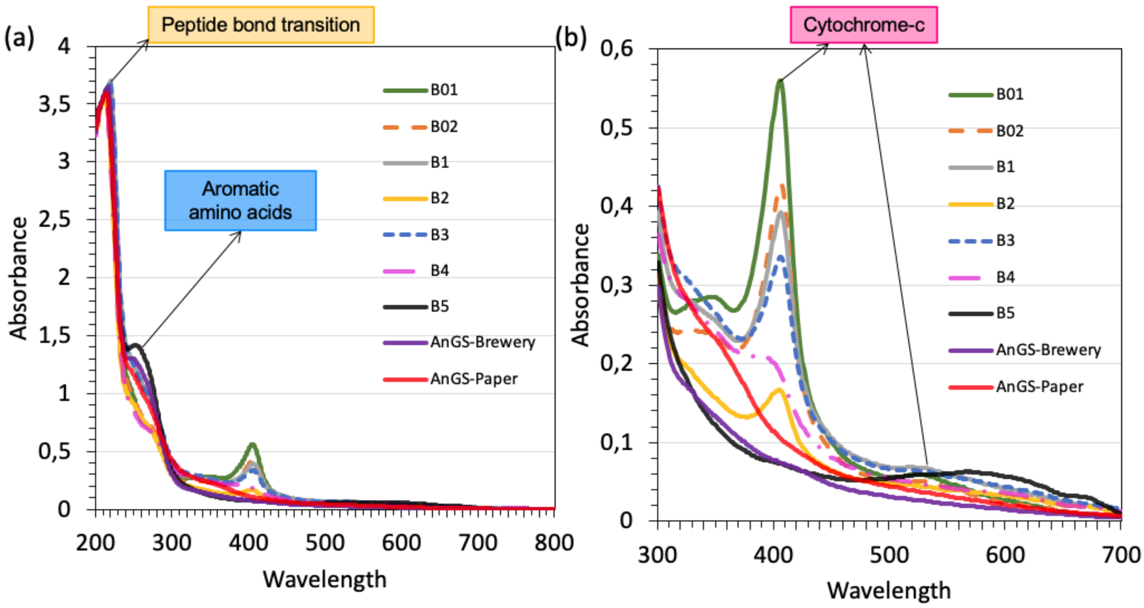


Figure 23 (a) UV-Vis analysis at pH ≃ 10 in the wavelength between 200 and 700 nm; (b) UV-Vis analysis at pH ≃ 10 in the wavelength between 300 and 700 nm

In Figure 23a, representing the UV-Vis analysis, four peaks along the curves are distinguished each related to a different functional group. In the range between 210-220 nm, the peak could be attributed to the dominance of peptide bond transitions in proteins (Lotti et al., 2019). At this stage, the recorded absorbance among the samples overlaps, highlighting a similar peptide concentration. While, the second peak around 270 nm stresses the abundance of aromatic and poly-aromatic amino acids (Lotti et al., 2019). Moreover, it is possible to start noticing a difference in absorbance between the samples: B5 has the highest value reaching an absorbance of 1.4, while the sample with the lowest value is B4 with 0.7. Anaerobic granules samples show a significant peak, above the anammox average, at around 270 nm wavelength. The peaks at 410 nm and 520-550 nm, represented on a smaller scale in Figure 23b, can be assigned to cytochrome-c (Lotti et al., 2019; B. Bin Wang et al., 2018). The highest cytochrome-c peak was detected in B01 as forecasted from the colour of the granules visualised in Figure 17: since it was composed of the darkest granules. As previsioned too, the lowest cytochrome-c value was found to be in B4 and B2, both characterised by a more reddish colour. B5 is the only anammox sample that did not show any peak close to 410 nm, as suggested by its dark colour. Also, AnGS granules have no peak at this wavelength, showing an absence of cytochrome-c excess.

PH neutralization was performed. Indeed, pH variations control EPS functional groups that affect their structure and alter their surface and chemical properties (L.-L. Wang et al., 2011). Figure 24b shows how the absorbance curves changed in response to the lowering of pH.

At a lower pH, properties such as hydrophobicity and hydrogen bonds help to create a more compact structure of functional groups. To better understand the effect of pH neutralisation, we can compare the curve for each sample before and after adjusting the pH as is represented in Figure 25. Each graph reveals a downward shift of the absorbance curve after pH neutralisation, that signifies a lower relative abundance of functional groups. Moreover, in each sample the most significant absorbance drop is close to 270 nm, and it could be attributed to the destruction of amide groups resulting in lowering the pH (L.-L. Wang et al., 2011).

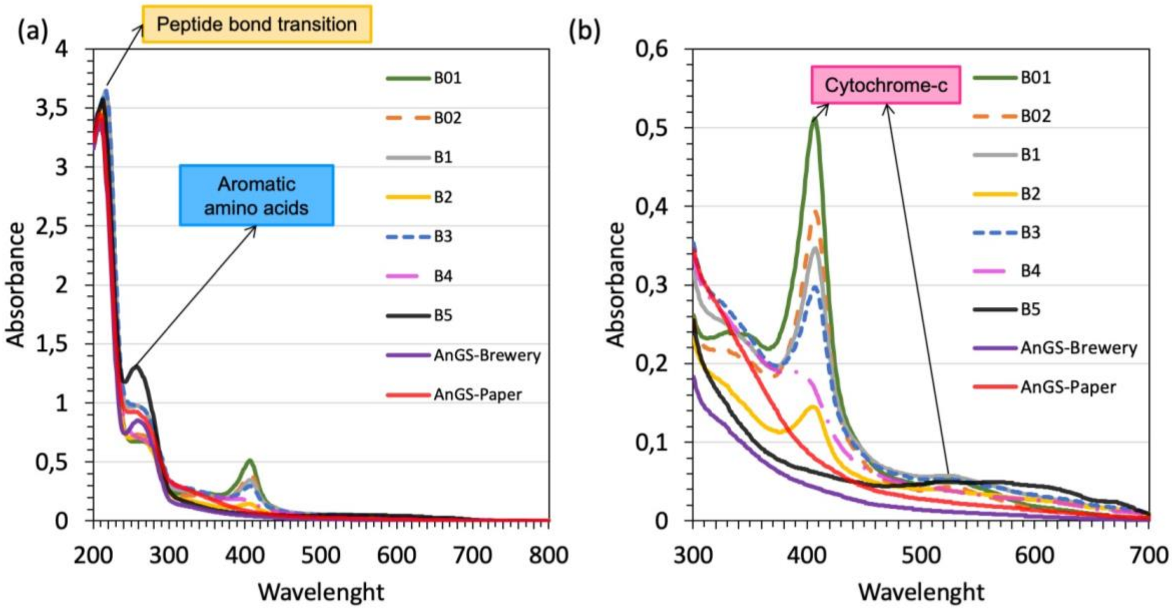


Figure 24 (a) UV-Vis analysis at pH ≃ 7 in the wavelength between 200 nm and 700 nm; (b) UV-Vis analysis at pH ≃ 7 in the wavelength between 300 nm and 700 nm

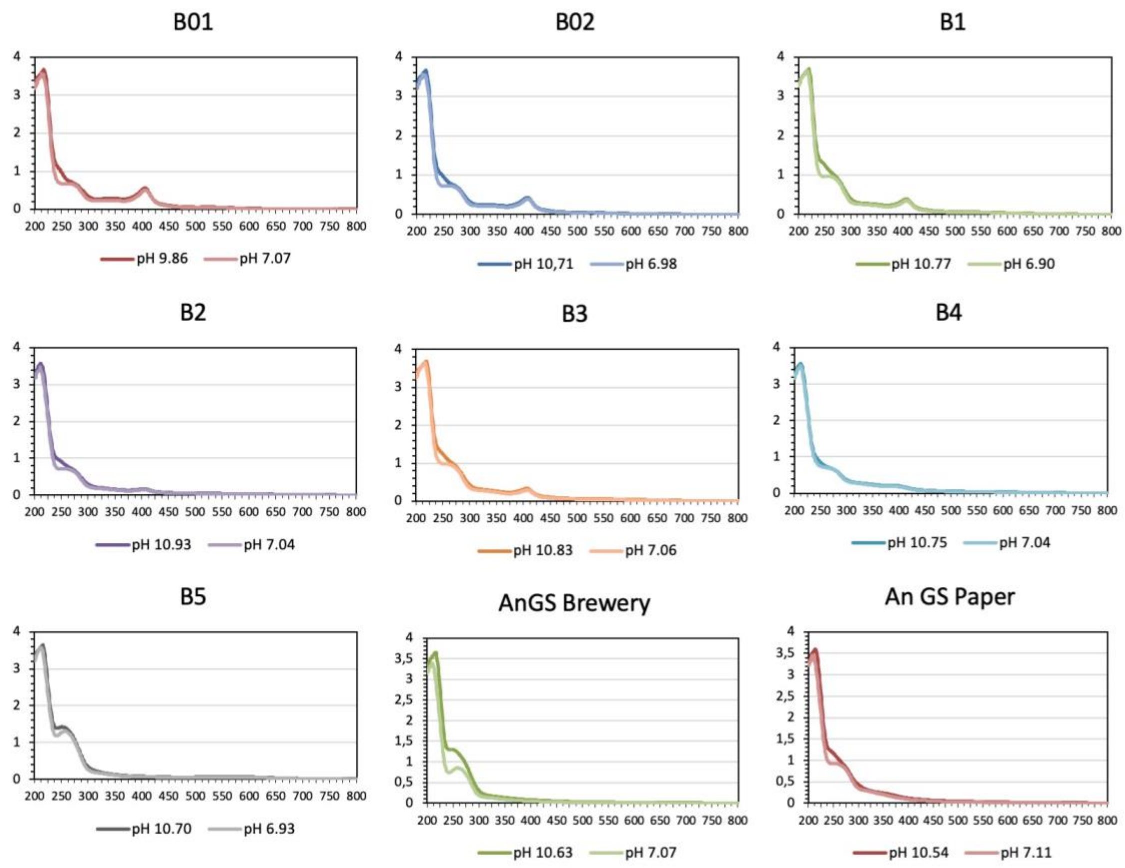


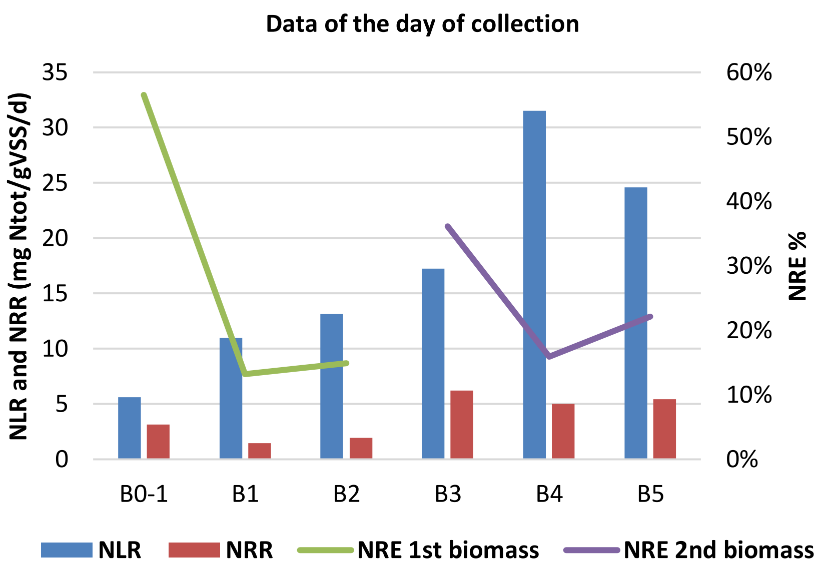
Figure 25 B01, B02, B1, B2, B3, B4, B5, AnGS-Brewery and AnGS-Paper Uv-Vis analysis at different pH

4.5 Anammox reactor performance linked with bacterial activity

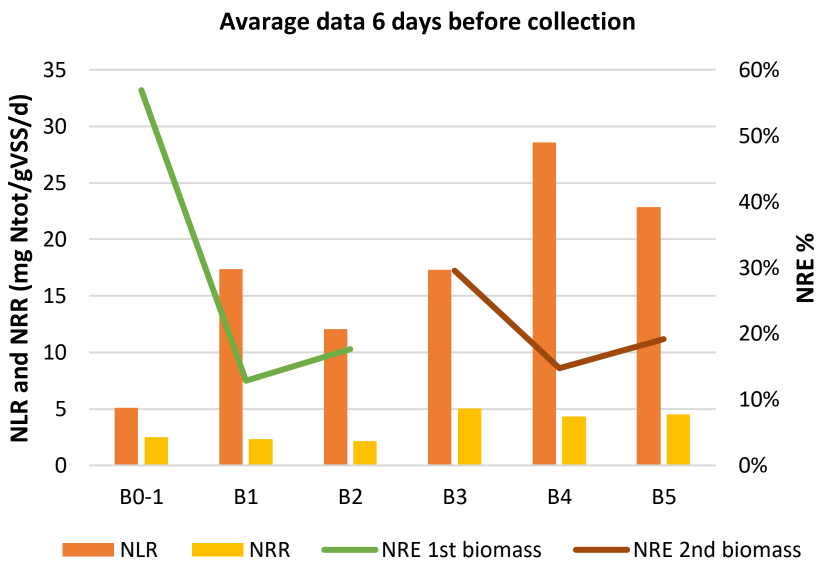
It is meaningful to compare reactor performance results with EPS physicochemical properties. In this study, the nitrogen loading rate (NLR), the nitrogen removal rate (NRR) and the nitrogen removal efficiency (NRE) were analysed to characterise the reactor performances. NRE is equal to the ratio of NLR and NRR. New biomass was added twice to the reactor: at the beginning of July (B0-1); in October (B3) due to a toxicity occurrence that caused the drop of NRE.

Figure 26 illustrates data at different sampling dates and average values of the periods before collection. All graphs display a similar trend: NLR has grown steadily from July to November. A turnaround was reported in December with an unexpected decrease of the NLR, as it was previously noticed during the period in with B5 was collected the reactor was probably not working at full capacity. For what regards NRR, we cannot detect a particular trend. Finally, after adding new biomass, we have a peak of NRE, and then a decreasing trend. However, data are not enough to detect a clear tendency of NRE. Moreover, its behaviour is not reliable due to the reactor instability; it has not reached regime working conditions.

(a)



(b)



(c)

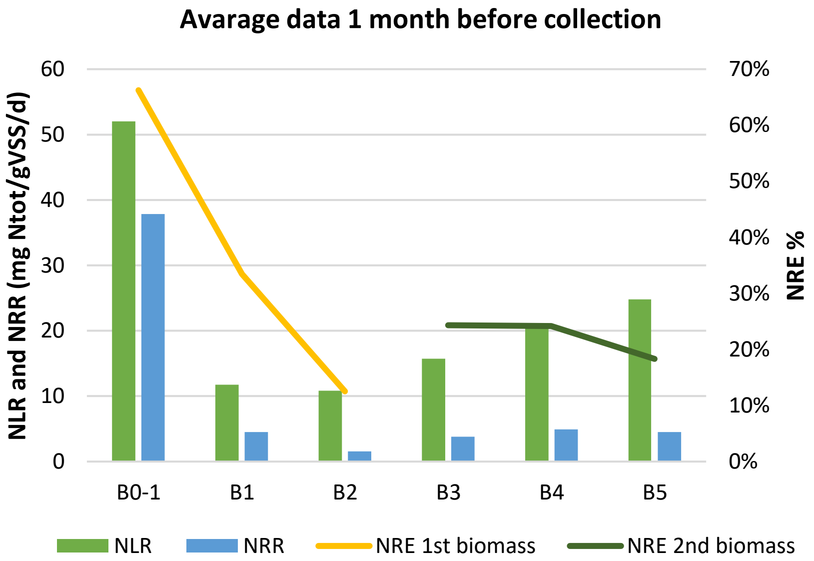


Figure 26 Nitrogen loading rate (NLR), nitrogen removal rate(NRR) and nitrogen removal efficiency (NRE) of SCR reactor considering: (a) values on the day of collection; (c) average values 6 days before collection; (d) average values 1 month before collection

Reactor inlet pH ranges from a minimum of 7.1 to a maximum value of 9.4; while outlet pH is within 6.5 and 10.2 (Figure 27). Figure 28 compares the EPS yield and concentration trends with pH values on the date of collection of the sample. In B5 (09/12/19), there is a peak, 8.3, while the lowest value, 6.9, corresponds to B2 (16/09/19).

The graph does not show any correlation between the considered EPS parameters and pH. Indeed, influent and effluent pH value do not significantly influence anammox bacteria activity (Yang et al., 2018). On the other hand, the variation in EPS is characterised by different proteins and polysaccharides concentrations which is an index of unstable reactor performances. Since many factors influence the status of the reactor, it is not possible to find a unique reason for the instability of the parameters.

Another parameter that distinguishes reactor performances is the total suspended solids (TSS) in the effluent (mg/L) (Figure 29). There is probably a relationship between the compactness of the and the effluent TSS (Thanh, Visvanathan, & Aim, 2009). Indeed the lowest value, 76 mg/L, was registered when B5 was collected. On the contrary, the highest values correspond to the introduction of new biomass: B0-1 with a peak of 648 ml/L and B3 with 350 mg/L.

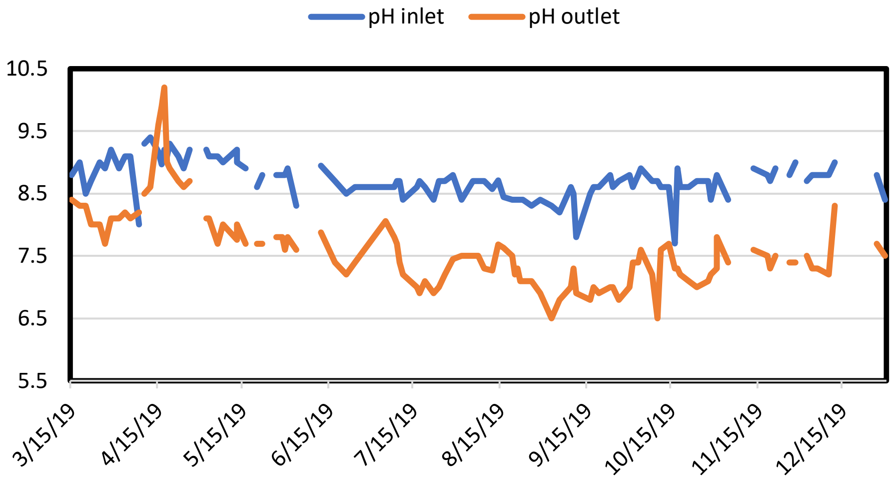


Figure 27 inlet and outlet pH trend of SCR reactor

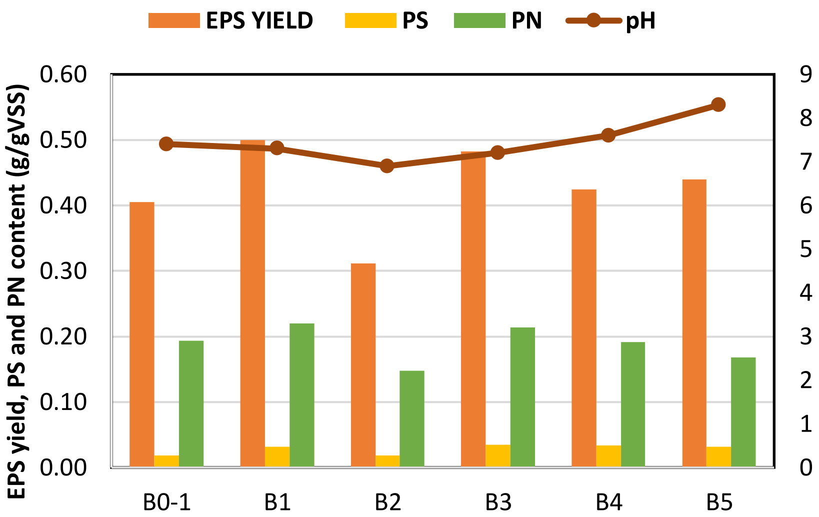


Figure 28 EPS yield, proteins and polysaccharides of anammox samples and effluent pH value of the SCR reactor

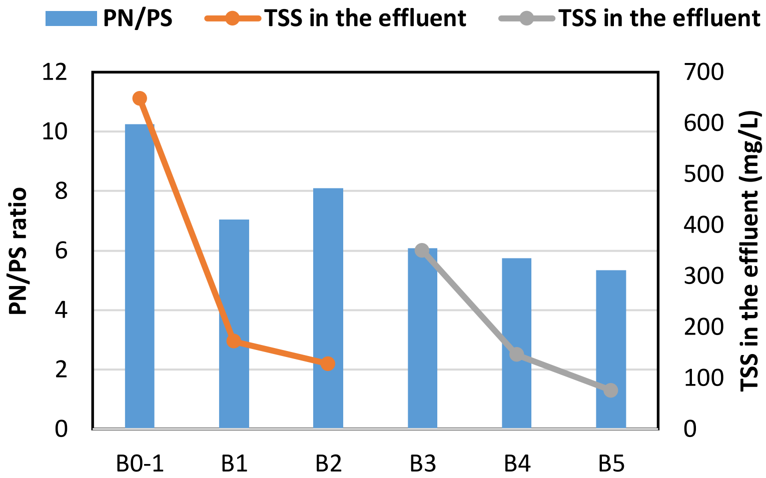
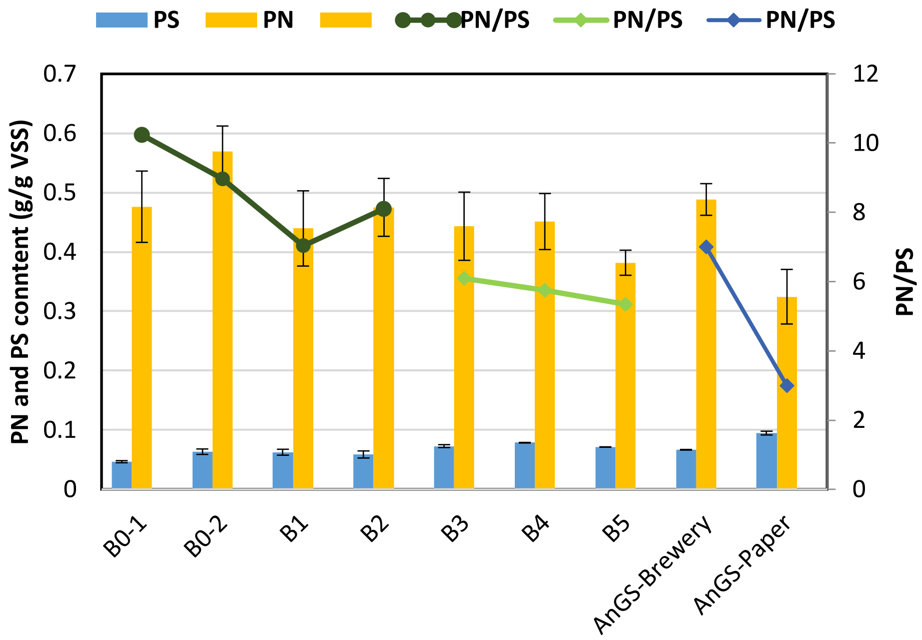


Figure 29 Proteins and polysaccharides ratio (PN/PS) in EPS and TSS in the effluent

PN/PS ratio is a key index that relates to EPS quality the reactor performances. PN/PS ratio is a performance indicator: a high value corresponds to a good reactor functioning and more compact granules (Chen et al., 2009). Moreover, there is a linear correlation between relative hydrophobicity of EPS and the PN/PS ratios (Z. Wang et al., 2015). Therefore, it is crucial to analyse their behaviour. First of all, it can be seen that PN/PS ratios of granule-based EPS are at a high level. Secondly, a negative trend is observable (Figure 30a). Introducing new biomass in the reactor, in correspondence of B0-1 and B3, generated an increase of PN/PS. Then, it gradually decreases in the following months, probably due to reactor deteriorating performances and instability. On the other hand, it is interesting to notice the low PN/PS ratio in AnGS-Paper despite the compact structure of the granules.

In future research, PN/PS might be linked with NRE (Figure 30b). So far, a reliable correlation cannot be detected both due to the problems of October that did not allow for continuity in the study on PN/PS and lack of data. Extend the work for a more extended period might lead to recognise an interesting correlation.

(a)



(b)

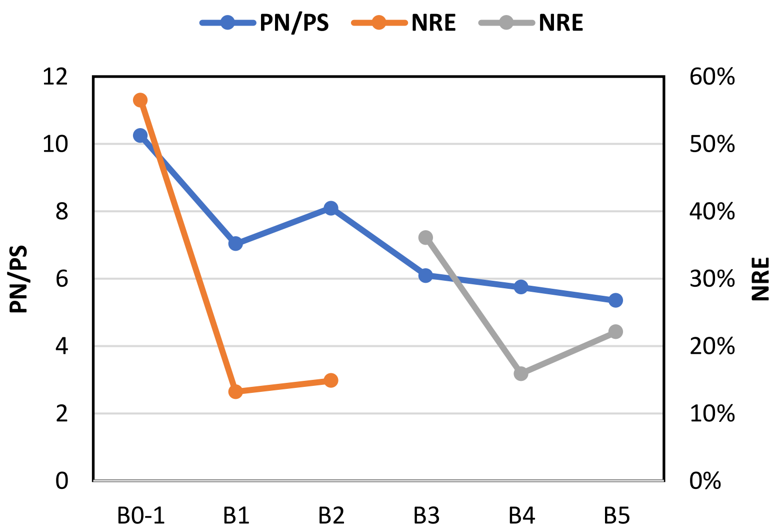


Figure 30 (a) Proteins (PN) and polysaccharides (PS) contents in EPS, and PN/PS ratio (b) PN/PS and nitrogen removal efficiency (NRE)

Chapter 5 – Conclusions

The dissertation aimed at achieving three objectives: compare the EPS of different anammox granules samples, distinguish anammox and anaerobic granules performances and find a correlation among reactor performances and samples quality. From the discussed results, we can draw the following conclusions:

- Anammox samples have different characteristics, but they have similar features except for B5. This sample is an outlier, its granules are black and tiny, and cytochrome c is not present.
- Overall anammox and anaerobic granules have similar characteristics. However, some differences need to be underlined. First of all, anaerobic granules are darker compared to the reddish anammox granules. Also, anaerobic granules have a more compact structure, especially AnGS-Paper. The protein cytochrome c is not present in anaerobic granules, it is a specific feature of anammox granules, and it gives them the characteristic reddish colour.
- In line with previous studies (Lotti et al. 2019; Feng et al. 2019), proteins and polysaccharides compose the most significant fraction of EPS. In all the tested samples, they constitute about 50% of the total.
- The anammox reactor is unstable. Indeed, the performance parameters change very frequently. However, a trend can be recognised in the PN/PS parameter. It has a descending behaviour, which means that the reactor characteristics are probably deteriorating.

This work could lead to developing many future kinds of research. First of all, monitoring the PN/PS ratio might reveal a correlation with the nitrogen removal efficiency and the quality of the granules. Moreover, the potential EPS application can be further tested and investigated.

Acronyms list

EPS – extracellular polymerics substances

PS – polysaccharides

PN – proteins

HS – humic substances

TB-EPS – tightly bound EPS

LB-EPS – loosely bound

WWTP – wastewater treatment plant

AnGS – anaerobic granular sludge

UASB – up-flow anaerobic blanket

USFF – up-flow stationary fixed film

ASB – the anaerobic sequencing batch reactor

SBR – sequencing batch reactor

MBBR – moving bed biofilm reactor

EGSB – expanded granular sludge bed

SGBR – static granular bed reactor

ETDA – ethylenediaminetetraacetic acid

CER – cation exchanges resins

COD – chemical oxygen demand

nZVI – nanoscale zero-valent

ALE – alginate like substances

TSS – total suspended solids

VSS – volatile suspended solids

Uv-Vis – UltraViolet-Visible Spectroscopy

NLR – the nitrogen loading rate

NRR – nitrogen removal rate

NRE – nitrogen removal efficiency

MWCO – Molecular weight cut-off

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Publications

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