Research Paper

Heavy metal biosorption by Extracellular Polymeric Substances (EPS) recovered from anammox granular sludge

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ABSTRACT

The recovery and conversion of Extracellular Polymeric Substances (EPS) from sewage sludge into bio-based commodities might improve the economics and environmental sustainability of wastewater treatment. This contribution explores the application of EPS from anammox granular waste sludge as biosorbent for the removal of heavy metals, specifically lead, copper, nickel, and zinc. Adsorption capacities equivalent or higher than well-established adsorbent media emerged from single-metal biosorption studies (up to 84.9, 52.8, 21.7 and 7.4 mg/gTS$_{EPS}$ for Pb^{2+}, Cu^{2+}, Ni^{2+} and Zn^{2+}, respectively). Combining spectroscopic techniques, a mechanistic hypothesis for metal biosorption, based on a combination of electrostatic interaction, ion exchange, complexation, and precipitation, was proposed. The adsorption mechanisms of extracted EPS and non-extracted EPS in the native biomass were indirectly compared by means of single-metal biosorption studies performed with pristine granules (adsorbing up to 103.7, 36.1, 48.2 and 49.8 mg/gTSgranules of Pb^{2+}, Cu^{2+}, Ni^{2+}, and Zn^{2+}, respectively). In comparison with pristine anammox granules, EPS showed lower adsorption capacities except for copper and different adsorption pathways as postulated based on the adsorption data interpretation via theoretical models. The multi-metal biosorption tests excluded significant competitions among different heavy metals for the EPS binding sites, thus opening further scenarios for the treatment of complex wastewaters.

1. Introduction

Autotrophic biological processes based on the metabolism of anaerobic ammonium oxidizing bacteria (anammox) allow removing ammonium from wastewater with significant savings in terms of energy spent for oxygen supply (−60%) and organic carbon supply (−100%); in addition, the production of excess sludge is reduced (−90%) compared to conventional nitrification/denitrification processes (Hu et al., 2013; Lotti et al., 2019a). For these reasons, anammox-based technologies are becoming the new standard for the treatment of nitrogen-rich wastewaters of municipal and industrial origins (Lackner et al., 2014). Anammox bacteria easily form granules (i.e., self-aggregated biofilms) without the presence of inert carriers. Thanks to these features, granular systems are characterized by higher biomass concentrations and volumetric conversion rates, if compared to flocculent or biofilm on carriers systems and as a matter of fact treat more than 50% of the total N-load

handled with anammox technologies worldwide (Lackner et al., 2014). Anammox-based process full-scale installations are mainly applied for the treatment of reject waters of anaerobic digestion systems. Considering the worldwide increasing trend of biogas production (Scarlat et al., 2018), and the effective savings offered by anammox-based technologies in the treatment of digestates (Lackner et al., 2014), the N-load treated in anammox plants, and therefore the anammox excess sludge production, is expected to increase in the future at a similar rate: strategies for the efficient anammox granular waste sludge management are therefore demanded (Feng et al., 2020). As in conventional biofilms, in anammox granules microorganisms are embedded in a matrix of hydrated extracellular polymeric substances (EPS) (Flemming and Wingender, 2010; Seviour et al., 2019, 2012). EPS, which account for up to 90% w/w of the organics in biofilm (Flemming et al., 2007), are a complex mixture of polysaccharides, proteins (structural proteins or exoenzymes), nucleic acids, (phospho)lipids, humic substances and intercellular polymers

(Flemming et al., 2016). Anammox EPS are mostly composed by proteinaceous material (>60%), with a low content (about 7%) of carbohydrates (Lotti et al., 2019a). Lotti et al. (2019b) observed the presence of amyloid-like fibrils in anammox EPS that possibly have a structural function in biofilms (Flemming et al., 2007; Taglialegna et al., 2017; Lin et al., 2018). The conversion of EPS recovered from waste sludge into bio-based commodities is getting increasing attention as appealing route to enhance sustainability and economics of wastewater treatment (Lin et al., 2015), promoting the development of circular economy chains. Waste sludge-derived EPS might be valorized for their metal sorption properties, as demonstrated in many studies (Guibaud et al., 2012; Li et al., 2017; Wei et al., 2016, 2019). The EPS matrix contains various functional groups, like carboxyl, phosphoric, amine and hydroxyl groups (Li et al., 2017) able to interact with cationic species in solution, thus representing potential metal-binding sites. Guibaud et al. (2012) observed that several functional groups (e.g., carboxyl, phosphoric, amine and hydroxyl sites) are involved in Cd^{2+} and Pb^{2+} binding by EPS from anaerobic granular sludge (AnGS). Li et al. (2017) noticed that hydroxyl and amino groups are the key functional groups involved in Ni^{2+} sorption by EPS from both AGS and AnGS. Based on the origin of the microbial aggregates, EPS can display different binding capacities towards metal cations: for instance, it is reported that EPS from AnGS provide a larger contribution to Ni^{2+} biosorption than EPS from aerobic granular sludge (AGS) (Li et al., 2017). However, despite these differences, the high protein content of EPS is generally positively correlated with the adsorption ability towards heavy metal cations: carbonyl and hydroxyl groups located on proteins are the main binding sites involved in the Pb^{2+}, Cd^{2+}, and Zn^{2+} adsorption by EPS from AGS (Liu et al., 2015). The abundant proteinaceous material of anammox EPS is therefore expected to promote the heavy metal biosorption phenomena: Li et al. (2020) observed that carboxyl groups of proteins of EPS from anammox granules have the fastest response in binding Cu^{2+} compared to polysaccharides and hydrocarbons.

The rapid industrial development and accelerating urbanization resulted in the production of various toxic substances, among which heavy metals have become an issue of great concern, due to their toxicity, non-biodegradability and potential to bioaccumulate in human body and food chain (He and Chen, 2014; Wang et al., 2018). Heavy metal contamination exists in aqueous waste streams of many industries, like metal plating facilities, mining operations, and tanneries (Bailey et al., 1999). Heavy metals in wastewaters of industrial origins (e.g., lead, copper, nickel, zinc, cadmium, chromium, etc.) could achieve high concentrations (Heidmann and Calmano, 2010; Sharma et al., 2020; Thomas et al., 2021). Conversely, the heavy metal concentrations found in civil wastewaters are generally lower (in the order of magnitude of µg/L); however, industrial wastewater discharges into municipal sewers might increase the heavy metal content of civil wastewaters.

Wastewater treatment plants (WWTPs) treating municipal sewage are not specifically designed to remove heavy metals. However, metals can be significantly displaced from the final effluents, as the result of the partitioning to the solid phase of the treatment systems: the sludge retains a great part of the metal content entering the WWTPs (Cantinho et al., 2016). The optimization of conventional treatment processes or the implementation of advanced treatment technologies (Hargreaves et al., 2018) may be required to be consistent with the current environmental regulation that imposes improved quality of both end-products of wastewater treatment (i.e., effluent and sludge). Land application of treated sewage sludge, worldwide considered as an economically and environmentally sustainable solution for sludge management (Cantinho et al., 2016), can be compromised by the high level of metals often retained in the excess sludge. If compared to many conventional treatment systems (e.g. chemical precipitation, ion exchange, electrochemical removal, membrane technology; Wang et al., 2018) affected by significant disadvantages (e.g., incomplete removal, high-energy requirements and production of toxic sludge), biosorption represents a highly cost-effective alternative for the treatment of heavy

metal-contaminated effluents, thanks to the low operational costs, high efficiency and comparatively less sludge production (Kratochvil and Volesky, 1998; Wang et al., 2018). Typical biosorbents can be recovered from non-living biomass (e.g., shrimp, krill, squid, crab shell, etc.), algal biomass, and microbial biomass (e.g., bacteria, fungi, and yeast) (Apiratikul and Pavasant, 2008). EPS extracted from sewage sludge could open up interesting perspectives in the field of the heavy metal biosorption. Excess sludge is the main waste product in WWTPS and its processing cost accounts for nearly half of the total operational capital (de Valk et al., 2019; Feng et al., 2020): the EPS extraction/recovery would reduce the mass of waste sludge to be treated/disposed concurrently with the production of a value-added biomaterial that could be valorized for its ability in binding heavy metals.

Many documents in literature investigate the anammox EPS-heavy metal interaction and binding mechanisms. Li et al. (2020) proposes a detailed description of the anammox EPS-Cu^{2+} interaction at molecular level, observing that functional groups in EPS exchange and coordinate with Cu^{2+} to form inner sphere complexes, by means of an endothermic and entropy-increasing process. Zhang et al. (2015) studied the behavior, distribution, and form dynamics of overloaded Cu^{2+} in anammox granular sludge reactors, disclosing that the Cu distribution migrated from the EPS-bound to the cell-associated Cu while the Cu forms shifted from the weakly bound to the strongly bound fractions over time. These pioneering studies highlighted the potentiality of using anammox EPS for metal remediation purposes, at least in the case of Cu. However, further research effort is demanded to evaluate the feasibility of exploiting EPS from waste anammox granular sludge as biosorbents for the treatment of heavy metal-contaminated effluents.

In this perspective, the present paper explores the recovery and valorization of EPS from anammox granular waste sludge as biosorbent for the removal of some heavy metals commonly found in wastewaters both of civil and industrial origins (lead, copper, nickel, zinc). Batch equilibrium biosorption tests were carried out by treating single- and multi-metal aqueous solutions with the recovered anammox EPS. The anammox EPS sorption properties were then compared to those of pristine granules to shed light on the adsorption mechanisms of extracted EPS and non-extracted EPS in the biomass. The biosorption mechanisms were investigated combining a series of spectroscopic techniques. The main goal of this work is therefore to provide proof-of-principle of the feasible conversion of EPS recovered from waste anammox granular sludge into value-added biomaterials suitable for the treatment of heavy metal-contaminated effluents. In addition, based on a comprehensive analysis of the anammox EPS adsorption properties, some guidelines for their potential industrial application are suggested.

2. Materials and methods

2.1. EPS extraction/recovery from anammox granular waste sludge

EPS were extracted from anammox granular waste sludge originating from a full-scale reactor in Rotterdam (Dokhaven-Sluisijesdijk WWTP, van der Star et al., 2007). The pH-based chemical extraction method developed by Lotti et al. (2019a) was applied with some modifications to obtain concentrated EPS dispersions directly suitable for single- and multi-metal equilibrium biosorption studies. Compared to the original protocol, the final acidic concentration step was not performed, and the EPS extracts were dialyzed (3.5 kDa molecular weight cut-off, MWCO) against ultrapure water (3 sequences of dialysis of about 8 h each) right after the alkaline extraction phase (4 h of granule incubation in 0.1 M NaOH aqueous solution, pH 12). The average EPS extraction yield was calculated based on the volatile solid content of the starting biomass ($mgTS_{EPS}/gVS_{granules}$) according to the method described in Supplementary material (Fig. S1).

2.2. Anammox EPS and granule characterization

The Total Solids (TS, g) were measured by placing the extracted EPS/granules in oven (105 °C) for about 24/48 h and then weighting the samples. The ash content (Ash, g) was determined by putting the dried EPS/granules in muffle-furnace (550 °C) for 2 h and then weighting the samples. The Volatile Solids (VS, g) were calculated as the weight difference between TS and Ash. The measurements were carried out in triplicate and the results (in terms of VS/TS ratio, g/g) are expressed as average values ± standard deviation. The granule particle size distribution was determined by an image analysis procedure adapted from Tijhuis et al. (1994) and using the software Image ProPlus®. Anammox EPS and granule samples were also analyzed in triplicate by a Varian 720-ES Inductively Coupled Plasma - Atomic Emission Spectrometer (ICP-AES) equipped with a pneumatic nebulizer and a cyclonic spray chamber to measure the metal concentrations (in terms of mg/KgTS).

2.3. Heavy metal aqueous solutions

For single-metal sorption tests, synthetic aqueous solutions, each containing one of the selected heavy metal ion M^{2+} (Pb^{2+}, Cu^{2+}, Ni^{2+} and Zn^{2+}), were prepared dissolving $Pb(NO_3)_2$, $CuCl_2 \cdot 2H_2O$, $NiCl_2 \cdot 6H_2O$ and $ZnCl_2$, respectively, in ultrapure water, with concentrations ranging between 10 and 1000 mgM^{2+}/L (pH=4.41, 4.59, 4.51 and 4.44 for Pb^{2+}, Cu^{2+}, Ni^{2+} and Zn^{2+} aqueous solutions, respectively, at 1000 mgM^{2+}/L). For multi-metal sorption studies, a solution containing all the selected heavy metal ions simultaneously was prepared dissolving $Pb(NO_3)_2$, $CuCl_2 \cdot 2H_2O$, $NiCl_2 \cdot 6H_2O$ and $ZnCl_2$ in ultrapure water (80 mgM^{2+}/L each, pH 5.25). $Pb(NO_3)_2$ (purity ≥ 99.0%), $CuCl_2 \cdot 2H_2O$ (purity ≥ 99.0%), $NiCl_2 \cdot 6H_2O$ (purity ≥ 99.9%) and $ZnCl_2$ (purity ≥ 98.0%) were purchased by Sigma-Aldrich. For the preparation of all samples, ultrapure water was used after filtration through a Milli-Q system (Millipore).

2.4. Heavy metal biosorption studies with anammox EPS

The biosorption properties of the extracted anammox EPS were assessed with equilibrium single-metal biosorption tests at laboratory scale, by treating synthetic aqueous solutions each containing one of the selected heavy metal ions M^{2+}. To evaluate the effect of the initial heavy metal concentration (C_0, mg/L) on biosorption, heavy metal solutions (4 mL) with concentrations ranging between 10 and 1000 mgM^{2+}/L were mixed anammox EPS dispersions (4 mL, 12.95 ± 1.65 gTS$_{EPS}$/L) in separate flasks and kept in contact under stirred conditions (120 rpm) at a temperature of 20 ± 0.5 °C for 6 h. Based on the information available in literature (Liu et al., 2015; Wei et al., 2016; Sajjad et al., 2017) and on preliminary kinetic studies (Fig. S2 in Supplementary material), a contact time of 6 h was considered as sufficient to attain sorption equilibrium. The pH of the mixed EPS-heavy metal aqueous systems was adjusted to 5.0 (for Pb^{2+} and Cu^{2+}) - 6.0 (for Zn^{2+} and Ni^{2+}) by adding 0.1 M HCl and NaOH aqueous solutions, in order to avoid precipitation of metal salts. The selected range C_0 reflected the metal content of various types of industrial effluents (e.g., galvanic wastewater, electroplating wastewater, metal plating wastewater); even if heavy metals are normally present in lower concentrations in municipal wastewaters, the level of contamination by heavy metals could increase in the case of discharges of industrial effluents in municipal sewers. Hence, the tested C_0 would allow to assess the capability of the extracted EPS in treating effluents of various origins with different levels of pollution by heavy metals.

Multi-metal biosorption studies were performed under conditions similar to those described for single-metal biosorption tests: an aqueous solution containing all the selected heavy metal ions simultaneously (4 mL, 80 mgM^{2+}/L each) was mixed with an anammox EPS dispersion (4 mL, 15.22 gTS$_{EPS}$/L), providing a contact time of 8 h under stirring (120 rpm) at a temperature of 20 ± 0.5 °C. Since the lower initial metal

concentrations, these tests were carried out at pH 6.0 (by adjusting the original pH of the EPS-metal aqueous system with 0.1 M HCl/NaOH), avoiding metal precipitation.

Both in single- and multi-metal biosorption tests, the measure of the residual metal concentrations at equilibrium (C_e, mg/L) was performed in triplicate by a Varian 720-ES Inductively Coupled Plasma - Atomic Emission Spectrometer (ICP-AES), after dialysis (3.5 kDa molecular weight cut-off) against ultrapure water. The removal efficiencies (%) and adsorption capacities (Q_e, mg/g) were calculated according to the following Eqs. (1) and (2), respectively:

$$Removal\ efficiency = \frac{C_0 - C_e}{C_0} \cdot 100\,(\%) \tag{1}$$

$$Q_e = \frac{C_0 - C_e}{m} \cdot V \left(\frac{mg}{g}\right) \tag{2}$$

where V (L) is the solution volume and m (g) is the sorbent mass. The experimental adsorption isotherm curves, describing the relationship between adsorption capacity (i.e., mass of solute adsorbed per unit mass of adsorbent, Q_e, mg/g) and residual metal concentration (C_e, mg/L) at equilibrium, were therefore determined. Langmuir and Freundlich adsorption isotherm models (Eqs. 3 and 4, respectively) were applied to interpret the observed profiles:

$$Q_e = \frac{b \cdot Q_m \cdot C_e}{1 + b \cdot C_e} \tag{3}$$

$$Q_e = K_f \cdot C_e^{1/n} \tag{4}$$

where Q_m (mg/g) is the theoretical maximum adsorption capacity, b (L/mg) is the Langmuir constant related to adsorption energy, K_f (L/g) is the energy binding constant reflecting the affinity of the sorbent for the sorbate and n is the Freundlich constant. The equilibrium parameter R_L, a dimensionless constant related to the separation factor (Balarak et al., 2017), was inferred from the Langmuir equation and determined as follows (Eq. 5):

$$R_L = \frac{1}{1 + b \cdot C_0^*} \; (-) \tag{5}$$

where C_0^* (mg/L) is the highest tested initial metal concentration. The value of R_L identifies the type of isotherm: irreversible ($R_L=0$), favorable ($0 < R_L < 1$), linear ($R_L=1$) or unfavorable ($R_L>1$).

2.5. Heavy metal biosorption studies with anammox granules

The biosorption properties of the extracted anammox EPS were compared to those of pristine granules to shed light on the adsorption mechanisms of extracted EPS and non-extracted EPS in the native biomass. Anammox granules were pre-treated to overcome their pH buffer capacity, which produced a progressive alkalizing effect in the aqueous medium, due to variable mineral and/or organic fractions present in the biomass: anammox granules were subjected to consecutive washing cycles with 0.45% (w/v) NaCl (aqueous solutions in demineralized water, pH 5) until reaching a stable pH 5 in the liquid bulk. The described pre-treatment method was time-consuming but also slightly invasive for the biomass: lower pH values might speed up the desorption processes, but also compromise the granular matrix and promote cell lysis phenomena. In addition, the use of buffer solution (e.g., containing phosphate) could promote competitions with the heavy metal cations to be adsorbed on the same binding sites. The pre-treated granules were drained by using a metal sieve (200 μm pore size): their VS/TS ratio (g/g) was determined and compared to that of the untreated biomass; the conversion factor between granule wet weight (WW, g) and dry weight (TS, g) was also evaluated (i.e., WW/TS, g/g).

To determine the experimental adsorption isotherm curves, a series of each metal ion solution with concentrations ranging between 5 and

500 mg/L were mixed with anammox granules (1.69 ± 0.20 gTS$_{granules}$/L) in separate flasks at pH 5.0 (for Pb^{2+} and Cu^{2+}) - 6.0 (for Zn^{2+} and Ni^{2+}) and maintained under stirring (120 rpm) for 6 h at a temperature of 20 ± 0.5 °C. Each sample was then filtered through 0.45 μm filter paper and analyzed by ICP-AES for the measurement of the residual heavy metal concentrations at equilibrium (C_e, mg/L). The removal efficiencies (%) and adsorption capacities (Q_e, mg/g) were calculated as previously described for the extracted EPS (Eqs. 1 and 2, respectively); similarly, the equilibrium sorption data were correlated by Langmuir and Freundlich adsorption isotherm models (Eqs. 3 and 4, respectively).

2.6. Biosorption mechanisms

Combining a series of spectroscopic techniques, a detailed description of the EPS/metal interaction at the molecular level was provided, proposing a mechanistic hypothesis for metal biosorption.

The occurrence of ion exchange mechanisms associated to the heavy metal adsorption was assessed measuring the concentration of alkali and alkaline earth metal ions (e.g., Ca^{2+}, K$^+$, Mg^{2+} and Na$^+$) released in solution simultaneously with the heavy metal uptake via ICP-AES.

Hydrodynamic diameter and Z-potential of EPS dispersions (0.01% w/v) in contact with increasing concentrations of the selected heavy metal ions (0.001–1 mM) were detected by Dynamic Light Scattering (DLS) at 90° and Phase Analysis Light Scattering, respectively (90Plus PALS, Brookhaven Instruments Corporation, USA). EPS samples treating highly concentrated heavy metal-contaminated aqueous solutions (C_0=500 mg/L) were also observed with a scanning electron microscope (SEM Zeiss Evo MA15; sample coating with gold) and analyzed with energy dispersive X-ray spectrometry (EDS Oxford Inca 250) to determine their elemental compositions.

To address the main EPS functional groups acting as metal-binding sites, Fourier Transform-Infrared (FT-IR) spectra of EPS samples before and after heavy metal biosorption were obtained in the transmission mode using a BioRad FTS-40 spectrometer (4 cm^{-1} resolution, 64 scans, spectral range 2000–400 cm^{-1}, DTGS detector).

3. Results and discussion

3.1. Anammox granule and EPS characterization

The particle size distribution of anammox granules is reported in Fig. 1; the characteristic diameters D10, D50 and D90 were 180.9, 317.0 and 871.2 μm, respectively. The average VS/TS ratio of anammox granules was 0.909 ± 0.002 g/g, while the granule wet weight on dry weight ratio was 11.54 ± 0.98 g/g. The EPS alkaline extraction yielded an average amount of EPS of 355.24 ± 4.48 mgTS$_{EPS}$/gVS$_{granules}$, in
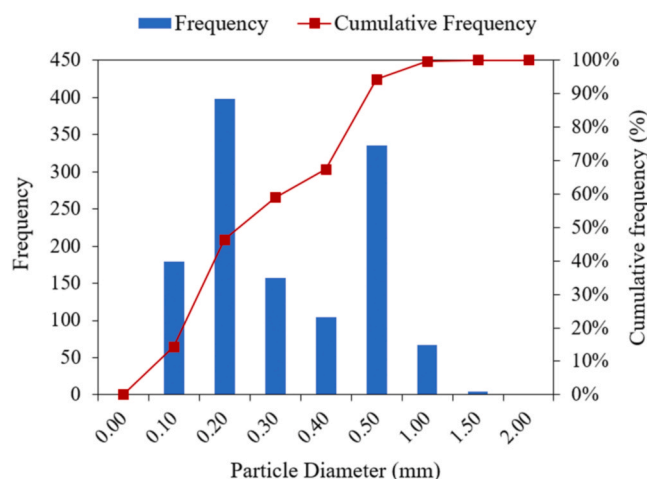

Fig. 1. Particle size distribution of anammox granules.

agreement with that reported by Lotti et al. (2019a). The VS/TS ratio of the extracted anammox EPS was 0.809 ± 0.020 g/g.

The tested granular sludge came from a full-scale reactor treating the reject water of a waste activated sludge anaerobic digester; since sewage sludge retains large part of the metal content entering the WWTP, the presence of alkali, alkaline earth and heavy metals was detected both in anammox granules and extracted EPS (Table S1 in Supplementary material). Some metals (e.g., Fe, Ni, Cu, Zn) are mostly present in the EPS (i. e., adsorbed by granules via extracellular uptake) and do not solubilize in the extraction process: considering an average EPS content in granules of about 36% (w/w), metal concentrations (in terms of mg/kgTS$_{EPS}$) in the extracted EPS can increase up to 2.8 times with respect to pristine granules. Other metals are mainly present in the cells (e.g., adsorbed by granules via intracellular accumulation and/or cell wall sorption) and/or as metal precipitates within the granular biomass: their concentration in the extracted EPS is therefore expected to be lower than in the original granules and eventually close to zero; this might be the case of Al, Co, Cr, Mo, V, Pb.

The applied granule pre-treatment allowed overcoming the biomass pH buffer capacity: a stable pH 5 was achieved in the aqueous medium via consecutive washing cycles of granules with 0.45% w/v NaCl at pH 5 (at least 3 changes of the liquid bulk), ensuring a total contact time (in 0.45% w/v NaCl, pH 5) in the range of 100–400 h dependent on the granule concentration (Fig. S3 in Supplementary material). The pretreatment permitted to maintain constant pH conditions during the metal sorption tests, thus avoiding uncontrolled formation of metal precipitates due to the increase of pH observed in preliminary sorption studies using untreated granules (data not shown). The alkalizing effect of the untreated granules under the conditions applied during the heavy metal biosorption experiments (pH 5–6) was likely due to the dissolution of carbonate-precipitates formed within the granular biomass during the operations in the parent full-scale reactor (operational pH>7) (Lin et al., 2013). A meaningful variation of VS/TS ratio was not detected after granule pre-treatment (VS/TS=0.888 ± 0.007 g/g for pre-treated granules).

3.2. Effect of initial metal concentration on heavy metal biosorption

The adsorption capacities (mg/g) and removal efficiencies (%) were determined as a function of the initial metal concentration (C_0, mg/L). Single-metal biosorption tests evidenced increasing heavy metal adsorption capacities at equilibrium (Q_e, mg/g) upon increasing C_0, both for extracted EPS and granules (Fig. 2a and b). C_0 provided an important driving force to overcome the mass transfer resistances of the metal between the aqueous and solid phase (Aksu and Akpinar, 2000), thus promoting adsorption phenomena. At the same time, with increasing C_0, the heavy metal ion competition for the available binding sites increased (Zheng et al., 2008), thus reducing the percentage removal efficiency (Fig. 2c and d). The highest EPS adsorption capacities obtained under the tested conditions were 84.9, 52.8, 21.7 and 7.4 mg/gTS$_{EPS}$ for Pb^{2+}, Cu^{2+}, Ni^{2+} and Zn^{2+} respectively (C_0=500 mg/L). Free Pb^{2+} was found in the treated solution above the detection limits of ICP-AES (in the order of μg/L) only for C_0> 40 mg/L, thus highlighting its large affinity towards EPS; a significant removal (about 93.9%) was detected also for the highest tested concentration (C_0=500 mg/L). Cu^{2+} and Ni^{2+} removals decreased from 90.8% and 60.9–62.2% and 28.3%, respectively, with increasing C_0 from 5 to 500 mg/L; lower removal efficiencies were obtained for Zn^{2+} (15.9% and 10.2% for C_0 equal to 5 and 500 mg/L, respectively). Concerning anammox granules, the highest observed metal uptakes at equilibrium were 103.7, 36.1, 48.2 and 49.8 mg/gTS$_{granules}$ for Pb^{2+}, Cu^{2+}, Ni^{2+} and Zn^{2+} respectively (C_0=500 mg/L). At the lowest C_0 of 5 mg/L, anammox granules were able to remove most of the metal content of the treated solutions (98.5%, 97.4%, 98.6% and 95.1% of Pb^{2+}, Cu^{2+}, Ni^{2+} and Zn^{2+}, respectively), while the removal efficiencies significantly decreased increasing C_0 up to 500 mg/L (33.5%, 12.6%, 15.5% and 14.2% for Pb^{2+}, Cu^{2+}, Ni^{2+} and
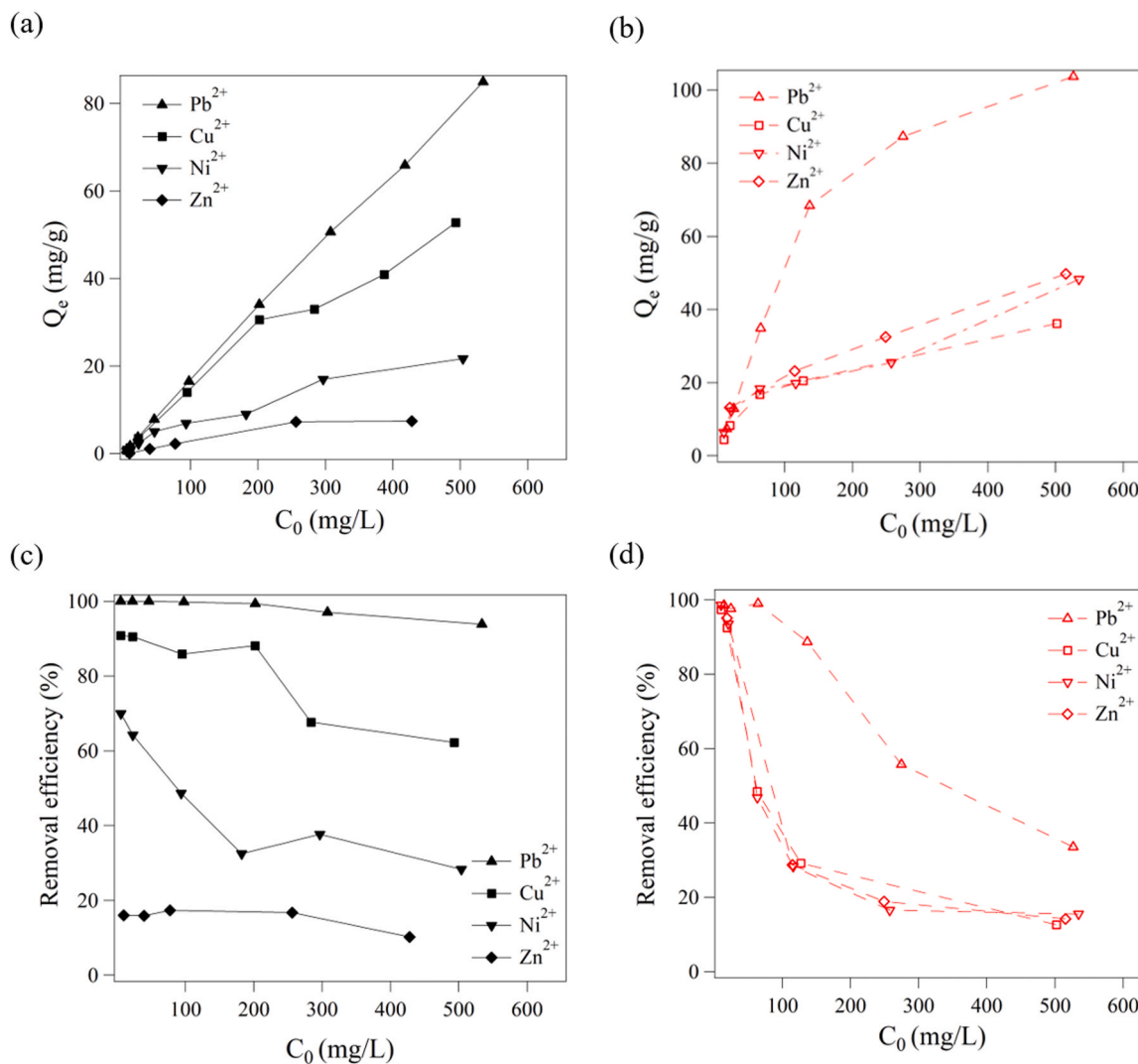
Fig. 2. Adsorption capacities (Q_e, mg/g) and removal efficiencies (%) observed for anammox EPS (a, c, respectively) and pristine granules (b, d, respectively) towards the selected heavy metal ions (Pb^{2+}, Cu^{2+}, Ni^{2+} and Zn^{2+}) at increasing initial heavy metal concentrations (C_0=5–500 mg/L). Adsorption capacities (Q_e, mg/g) are related to the total solid contents of anammox EPS and pristine granules (TS_{EPS} and $TS_{granules}$, respectively).

Zn^{2+}, respectively), consistently with the results obtained for the recovered EPS. The increase of the adsorption capacities of both extracted EPS and pristine anammox granules upon increasing C_0 can be exploited in potential applications for the pre-treatment of wastewaters containing high concentrations of M^{2+}.

3.3. Comparison between anammox granules and extracted EPS

Both anammox granules and extracted EPS exhibited metal-binding capacities equivalent or higher than other conventional and/or unconventional sorbent media, like activated carbons (Kongsuwan et al., 2009), clay minerals (Bertagnolli et al., 2011), magnetic sorbents (Lan et al., 2013) and various biomaterials such as fungal biomass (Bhainsa and D'Souza, 2008) and macroalgae (Pavasant et al., 2006). A more detailed literature survey of adsorbents and biosorbents for the heavy metal removal is reported in Table S2 in Supplementary material.

The extracted EPS showed lower efficiency in adsorbing Pb^{2+}, Ni^{2+} and Zn^{2+} compared to pristine granules. Conversely, the extracted EPS appeared more efficient in removing Cu^{2+} than native biomass.

The differences in terms of metal-binding ability observed for extracted EPS and pristine anammox granules might be ascribed to three main reasons:

- multiple mechanisms participating in the heavy metal biosorption by native granules, in addition to the heavy metal uptake by EPS;
- EPS chemical modifications induced by the extraction method applied;
- different polymer chain mobility and binding site availability of extracted EPS in aqueous dispersions and non-extracted EPS in pristine granules.

Describing granules as dense microbial aggregates, comprising metabolically active microbial cells embedded in an EPS matrix (Liu and Tay, 2004), it is expected that the heavy metal uptake by EPS present within the biomass plays a crucial role in the biosorption phenomena. Indeed, EPS are rich in ionizable functional groups (e.g., carboxyl and hydroxyl groups) able to interact with cationic species in solution, thus representing potential metal-binding sites. Hypothesising that all the active metal-binding sites of anammox granules are localized on the EPS matrix, greater metal sorption capacities would be expected for the extracted EPS compared to pristine granules (up to 2.8 times higher in terms of mgM^{2+}/gTS, considering an average EPS content of about 36% w/w in the native biomass). However, other mechanisms might participate in the heavy metal sorpion promoted by anammox granules: cell wall adsorption and intracellular accumulation are usually common ways for biosorption (Vijayaraghavan and Yun, 2008). This could

especially explain the lower zinc uptake disclosed for the extracted EPS compared to pristine biomass: zinc is an essential co-factor for a large number of proteins, contributing to many structural and/or catalytic functions and hence bacteria are able to rapidly accumulate it on their cell walls as observed by Limcharoensuk et al. (2015). Furthermore, anammox granules presented adsorption capacities similar to other granular biomasses, like AGS and AnGS, as evidenced in Table S3 in Supplementary material. The microbial communities populating the above cited sludges are different, and therefore their EPS are expected to have different biochemical compositions. Therefore, regardless of these intrinsic differences among EPS at molecular level, the comparable adsorption capacities would suggest that there are also other driving factors that determine the mechanisms of M^{2+} biosorption, probably related to the 3D porous structure of granules, and relative EPS chain arrangement and biding site availability. In other words, given the similar biosorption figures for different granular sludges, biosorption does not seem to be only dependent on the specific nature of the sludge/EPS considered, but also connected to structural aspects of the granules (e.g., high porous granular structure, polymer chain arrangement, binding site availability).

Besides the potential contribution in metal adsorption exerted by other components than EPS, it should be highlighted that the availability of binding sites of EPS themselves could be altered by the chemical extraction method applied. Some reports have shown that EPS extracted with chemical methods display meaningful differences in their ability to bind heavy metal ions, likely due to contamination of the recovered EPS with the chemicals used during the extraction (d'Abzac et al., 2010). The chemical extraction protocol here applied is based on the use of 0.1 M NaOH as alkaline agent to solubilize the EPS matrix. Although the extraction protocol includes dialysis (3.5 kDa MWCO), the EPS extracts showed a relatively high content of Na^+ ions (11.6 mg/gTS$_{EPS}$, Table S1 in Supplementary material), which could compete with the heavy metal ions to be adsorbed on the available binding sites. The extraction under alkaline conditions might also affect the conformation of EPS components through the loss of protein tertiary and/or quaternary structure. This can promote specific interactions among proteins, resulting in more significant entanglement and progressive folding (i.e., association) of the peptidic chains, with subsequent reduction of the specific surface area and binding site availability.

Moreover, structural aspects of the sorbent medium can affect the performance in removing metal cations. While the highly porous structure of granules, with abundant and accessible sorption sites, promote the sequestration of contaminants upon diffusion into their deeper layers and channels (Liu et al., 2003), the use of the extracted EPS in the form of dispersions could result in weaker solute-sorbent interactions (or not strong enough to overcome the forces extended by the solvent). The relatively lower metal-binding capacities of the EPS dispersions compared to pristine granules in single-metal biosorption tests (especially in the case of Ni^{2+} and Zn^{2+} solutions) can be also attributed to the different impact that the EPS/metal complex formation has on the structural arrangement of EPS in suspension and on the EPS present within the native granules. Due to the higher mobility of the EPS molecules dispersed in water, a potential explanation of the lower metal-binding capacity of the EPS dispersions could be that, when a M^{2+} is bound to EPS dispersed in water, the 3D conformation of the EPS molecules (free to move into the liquid aqueous phase) is strongly altered due to the arrangement of the EPS molecules around the M^{2+}. An experimental evidence of that could be the increase of the hydrodynamic diameter of EPS dispersed in water, observed upon loading M^{2+} (Fig. S4 in Supplementary material). Instead, this effect is strongly inhibited for the EPS present within the granules due to their limited mobility. On the basis of this hypothesis, for dispersed EPS, with respect to EPS in granules, a higher number of EPS/M^{2+} bonds is expected in the EPS/M^{2+} complexes thus resulting in a lower availability of binding sites for the formation of further bonds with other M^{2+} ions.

3.4. Langmuir and Freundlich adsorption isotherm models

The interpretation of the equilibrium biosorption data via Langmuir and Freundlich adsorption isotherm models confirmed some differences in the sorption pathways followed by anammox EPS and granules. Fig. 3 shows the experimental sorption isotherm curves and the related fits via Langmuir and Freundlich models. Based on the model statistitical indicators (Table S4 in Supplementary material), using anammox granules as biosorbent medium, a Zn^{2+} mono-layer biosorption was predicted due to the better statistical fitting via Langmuir model; Pb^{2+}, Cu^{2+} and Ni^{2+} biosorption data were better simulated by Freundlich model, thus suggesting a multi-layer biosorption on a heterogeneous surface. Using the EPS extracts as biosorbent medium, a Pb^{2+} multi-layer biosorption was predicted because of the better statistical fitting via Freundlich model; Langmuir model was more representative of Cu^{2+} and Zn^{2+} equilibrium biosorption data, thus implying an uniform and mono-layer adsorption on the EPS surface: the EPS-Cu^{2+}/Zn^{2+} interactions could be not strong enough to overcome the forces extended by the solvent except in the first layer, being both solute and sorbent in a sol-like state. The slight differences in R^2 values observed for Ni^{2+} adsorption by anammox EPS, reflecting the simultaneously applicability of both theoretical models, suggested as mono- or multi-layer attachment, possibly with chemical interactions between EPS and metal ions, and also between the sorbates molecules, could explain the biosorption phenomena (Wang et al., 2018). Further information can be deducted from the model parameters (Table S4 in Supplementary material). The $0 < 1/n < 1$ values in Freundlich model (0.422–0.680 and 0.227–0.336 for extracted EPS and anammox granules, respectively) implied a favorable metal biosorption by anammox EPS and granules (Wang et al., 2018), consistent with the $0 < R_L < 1$ values (0.005–0.369 and 0.004–0.280 for extracted EPS and anammox granules, respectively) in Langmuir model. Similarly to the Langmuir constant b, the values of the energy binding constant K_f in Freundlich model reflected the metal binding affinity of the sorbent in the following orders: $Pb^{2+}>Cu^{2+}>Ni^{2+}>Zn^{2+}$ for extracted EPS and $Pb^{2+}>Cu^{2+}{\sim}Ni^{2+}{\sim}Zn^{2+}$ for anammox granules.

3.5. Multi-metal biosorption studies

Similar metal-binding capacities were observed for the extracted EPS in multi- and single-metal biosorption experiments (Table 1): significant competitions among different heavy metal ions for the available EPS binding sites were ruled out. Moreover, the total heavy metal uptake in the case of multi-metal systems (14.81 mg/gTS$_{EPS}$) was higher than the amount of each metal ion adsorbed in single-metal sorption tests, indicating the capability of EPS in adsorbing multi-metal ions. Similar evidences were found by Sun et al. (2009) for Zn^{2+} and Co^{2+} binary sorption on EPS from aerobic granules, thus supporting the consistency of these considerations, regardless of the quality of EPS and original microbial aggregate. Many conventional techniques for the heavy metal removal (e.g., ion exchange resins, nanofiltration membranes) are often designed to target one contaminant at a time, thus making their use impractical for environmentally polluted waters and/or wastewaters where several contaminants occur simultaneously (Bolisetty et al., 2019). Conversely, the sorption capability of extracted anammox EPS in multi-metal aqueous systems might be exploited in technologies for the treatment of effluents contaminated by several heavy metals.

3.6. Biosorption mechanisms

The process scale-up should be supported by a deeper molecular-level understanding of the biosorption processes, made challenging by the complexity of the mechanisms involved. This is hampered by the fact that the fine physic-chemical characterization of EPS recovered from biofilm-based engineered systems is still far from complete (Seviour et al., 2019). The investigation should start with the surface properties of the sorbent, representing a key element in all adsorption phenomena.
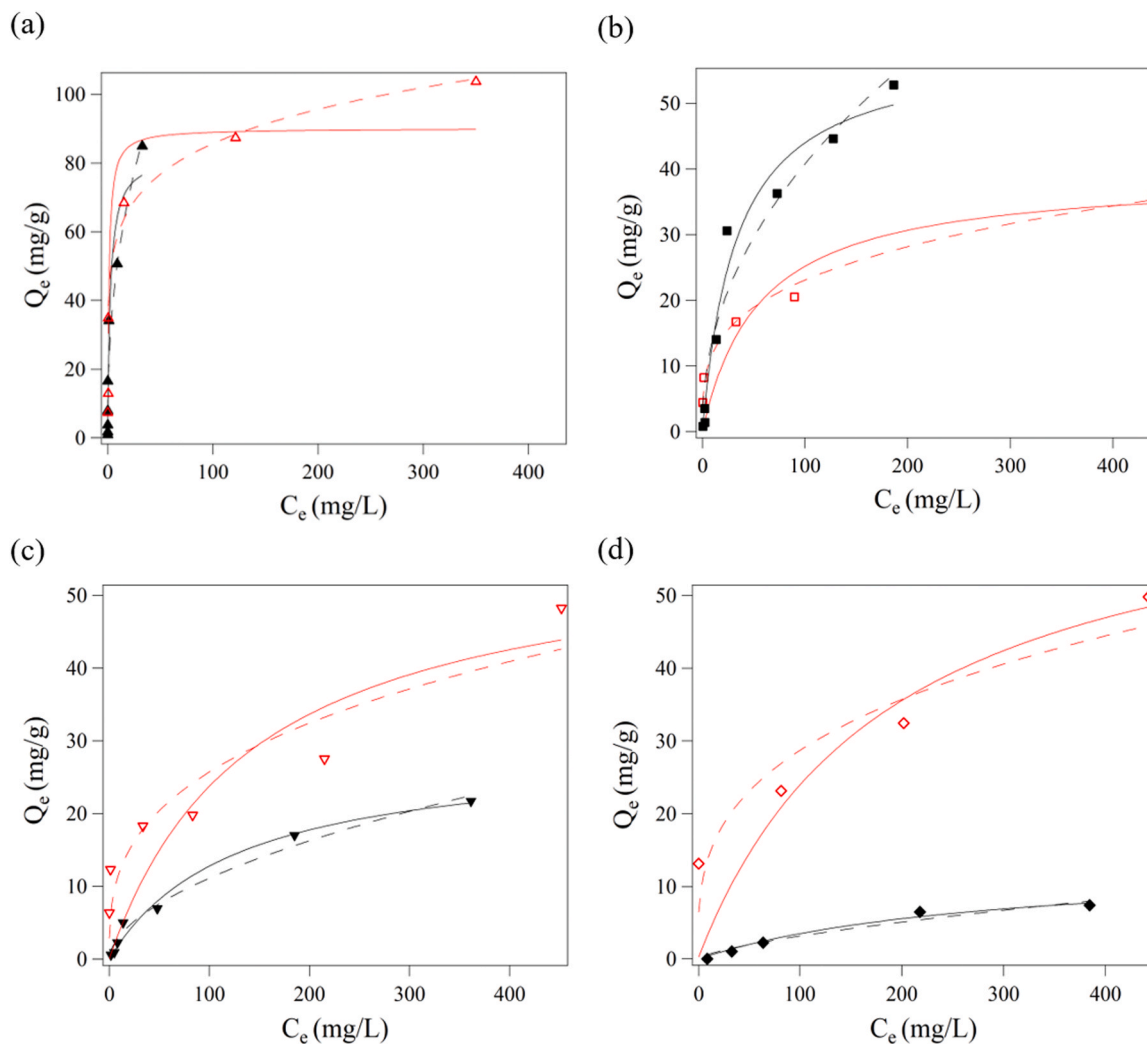
Fig. 3. Experimental adsorption isotherm curves and related fits via Langmuir and Freundlich models (in continuous and dotted lines, respectively) for anammox EPS (full markers, black lines) and pristine granules (empty markers, red lines) towards Pb^{2+} (a), Cu^{2+} (b), Ni^{2+} (c) and Zn^{2+} (d). Adsorption capacities (Q_e, mg/g) are related to the total solid contents of anammox EPS and pristine granules (TS_{EPS} and $TS_{granules}$, respectively).

Table 1

Adsorption capacity of anammox EPS in single- and multi-metal biosorption tests ($C_0 = 40$ mgM^{2+}/L).

	Adsorption capacity (mg/gTS$_{EPS}$)	
	Multi-metal sorption	*Single-metal sorption*
Pb^{2+}	5.56	7.83
Cu^{2+}	4.45	4.35
Ni^{2+}	2.36	3.06
Zn^{2+}	2.43	1.07

The removal efficiencies of contaminants are strongly dependent on both their species and sorbent surface charge through mechanisms of electrostatic attraction. Modifications of the surface properties of the sorbent could be promoted by the pH of the medium, that not only affects the speciation of the solutes, but can also change the surface charge of the sorbent via protonation or deprotonation (Wang et al., 2018). The effect of pH on the Z-potential of aqueous solutions containing EPS extracted from anammox granular sludge was recently reported in the literature (Lotti et al., 2019a). In brief, at pH $\leq$ 4, EPS surface is largely protonated and Z-potential is close to neutrality |$Z_{pot} \leq 10$ mV|: in these conditions H$^+$ ions compete with the heavy metal ions for the adsorption sites. For higher values of pH, deprotonation occurs, and the EPS surface

becomes negatively charged, thus increasing the ability to interact with positively charged metals. In the tested sorption conditions (pH 5–6), anammox EPS were negatively charged ($Z_{pot} \leq -20$ mV, Lotti et al., 2019a) and could attract metal cations via electrostatic interactions as first step of biosorption, thus favouring further polymer-metal interactions (Wang et al., 2018). A detailed description on how biosorption proceeds is difficult: physic-chemical interactions between heavy metal cations and EPS (anionic) functional groups, based on ion exchange, surface complexation, redox reaction and micro-deposition, might be involved, individually or in combination (Wang et al., 2018). FT-IR spectroscopy suggested the involvement of multiple functional groups and components in the heavy metal biosorption provided by anammox EPS. The FT-IR spectra of the EPS samples presented some changes in terms of peak shifts after the interaction with the heavy metal ions (Fig. 4). The highest shifts were observed for the peaks at 1547 cm^{-1} and 1047 cm^{-1} wavenumbers, related to the C-N stretching and N-H bending of proteins (Amide II), and C-H in plane bending of polysaccharides (Lotti et al., 2019a; Niu et al., 2016). The metal-binding capability of anammox EPS might be therefore ascribed to protein- and polysaccharide-like substances. In agreement with the literature, where the ion exchange is reported as a common mechanism for the metal biosorption by EPS (Li and Yu, 2014) and/or biomass (Sajjad et al., 2017), the heavy metal uptake by anammox EPS and granules was
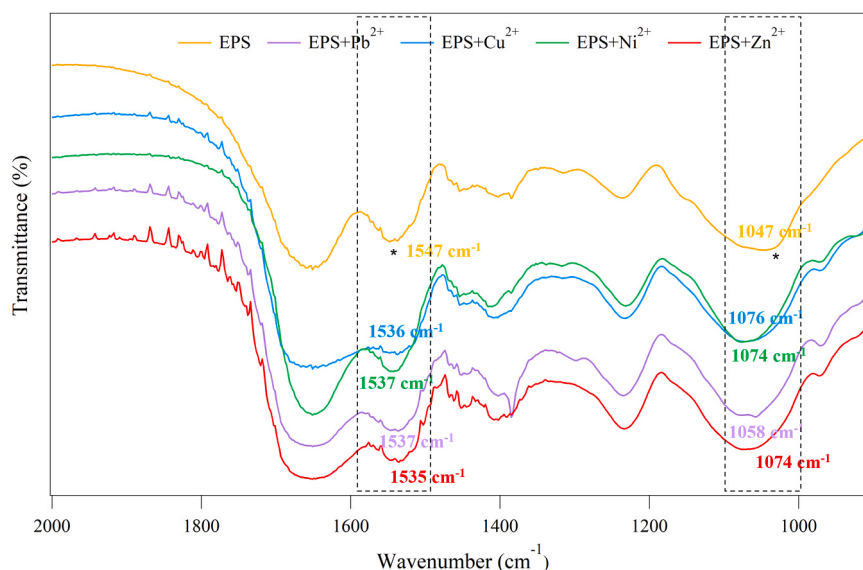
Fig. 4. FT-IR spectra of EPS samples before and after the heavy metal biosorption. The spectra were obtained in the transmission mode using a BioRad FTS-40 spectrometer (4 cm^{-1} resolution, 64 scans, spectral range 2000–400 cm^{-1}, DTGS detector). The peaks with the highest shifts after the heavy metal uptake are evidenced.

associated to releases of alkali and alkaline earth metal ions (e.g., Ca^{2+}, Na^+, Mg^{2+} and K^+) in the bulk medium, resulting from a relatively lower affinity and competitiveness of the exchangeable cations to bind with the sorbent (Wang et al., 2010). Fig. 5 compares the amounts of Pb^{2+}, Cu^{2+}, Ni^{2+} and Zn^{2+} adsorbed by anammox EPS and granules with those of Ca^{2+}, K^+, Mg^{2+} and Na^+ found in the aqueous medium after the heavy metal sorption tests (C_0=500 mg/L). It was noticed that the heavy metal adsorption by anammox EPS was mainly associated to Ca^{2+} and Na^+ releases in solution, which accounted for 93.5%, 93.4%, 88.3% and 95.6% of the total amount of metal ions discarded by EPS following the Pb^{2+}, Cu^{2+}, Ni^{2+} and Zn^{2+} adsorption, respectively, whit a lower release of K^+ and Mg^{2+} also detected. Ion-exchange mechanisms were also observed for the pristine granules: Ca^+ represented less than 1% of the total release following adsorption, while Na^+ ions were the most abundant (93.1%, 94.2%, 75.2% and 92.4% of the total metal ion release for Pb^{2+}, Cu^{2+}, Ni^{2+} and Zn^{2+} biosorption, respectively), with a lower amount of K^+ (5.7%, 5.8%, 24.1% and 6.7% of the total metal ion release for Pb^{2+}, Cu^{2+}, Ni^{2+} and Zn^{2+} biosorption, respectively) detected in the aqueous medium after biosorption. A non-stoichiometric exchange (i.e., release/adsorption ratios < 1 meq/meq) was observed both for anammox EPS and pristine granules, suggesting that this process was not the only mechanism at play in biosorption. In addition to ion exchange, complexation and surface precipitation are considered as two of the major phenomena responsible for the heavy metal-EPS/biomass interaction (Li and Yu, 2014). Depending on metal speciation in response to pH, directly affecting the metal solubility, many heavy metals can be also easily precipitated into EPS/biomass or onto the surface of some mineral fraction contained in EPS/biomass (Guibaud et al., 2009), thus greatly complicating the understanding of sorption processes. In particular, for C_0= 500 mg/L, ion exchange accounted for 46.4%, 22.4%, 26.1% and 32.6% of Pb^{2+}, Cu^{2+}, Ni^{2+} and Zn^{2+} biosorption, respectively, by anammox EPS and 88.6%, 99.1% and 76.1% of the Cu^{2+}, Ni^{2+} and Zn^{2+} uptake, respectively, by anammox granules (data related to Pb^{2+} sorption are not clear, due to the release/sorption ratios > 1 meq/meq).

It was observed that the ion exchange contribution to the heavy metal biosorption by anammox EPS increased by 45%, 110%, 157% and 88% in the case of Pb^{2+}, Cu^{2+}, Ni^{2+} and Zn^{2+} contaminated aqueous systems, respectively, upon decreasing C_0 from 500 to 200 mg/L. This evidence suggested a complex interplay of other adsorption mechanisms more active for the highest initial metal concentrations. Indeed, in

single-metal biosorption tests treating C_0= 500 mgM^{2+}/L, the Ni-EPS and Zn-EPS aqueous systems appeared slightly unstable (likely due to the potential formation of micro- and/or nano-precipitates, not visually detectable), while the formation and subsequent precipitation of Pb-EPS and Cu-EPS composite aggregates was observed (aggregate size in the order of magnitude of millimetres, i.e., clearly visible). The Pb/Cu-EPS composite precipitates were observed with a scanning electron microscope (SEM) for the surface morphology investigation: the surface roughness indicated the presence of polymeric components, while regular crystalline structures expected for metal precipitates were not appreciated (Fig. 6). Considering the molecular composition of anammox EPS reported in literature (i.e., $C_1H_{1.629}O_{0.503}N_{0.209}P_{0.009}$, 24.97 gVS_{EPS}/C-mol$_{EPS}$, Lotti et al., 2019a), the metal/EPS weight and atomic ratios of the composite aggregates were calculated based on the elemental analysis obtained via energy X-ray dispersive spectroscopy (EDS) (Table 2). Details on the EDS analysis are reported in Supplementary material (Fig. S5 and S6). The EPS presence in the aggregates was confirmed by the O/C atomic ratios in agreement with those reported for pristine anammox EPS (Lotti et al., 2019a). As emerged from EDS analysis, carbon, oxygen, and heavy metals (i.e., Pb and Cu, respectively) were prevalent (in terms of atomic% and weight%) in the aggregate composition, with weight and atomic ratios consistent with the polymer-heavy metal co-precipitation. The EPS-heavy metal co-precipitation phenomena observed in biosorption tests treating highly concentrated heavy metal-contaminated aqueous solutions was probably not due to pH and/or ionic strength of the aqueous systems: the experiments were designed selecting operative conditions that avoided the precipitation of metal salts. The effect of ionic strength was not investigated in this study, but the applied experimental conditions (Table S5 in Supplementary material) would have allowed to overcome the competition with electrolyte cations, thus promoting the metal adsorption processes. More likely, increasing C_0, and therefore the amount of heavy metal ions adsorbed on the EPS binding sites, multiple-bridging complexation phenomena might be promoted. In line with this hypothesis, divalent cations M^{2+} could act as bridges for different EPS units. This would result in a strong increase of EPS particle size, and in a reduction of the repulsive force among EPS units, thus leading to aggregation and finally to precipitation, as also suggested by preliminary Zeta-potential and Dynamic Light Scattering (DLS) measurements (Fig. S7 in Supplementary material). This evidence agreed with what reported by Li et al. (2020), who observed a decreasing

(a)

(b)
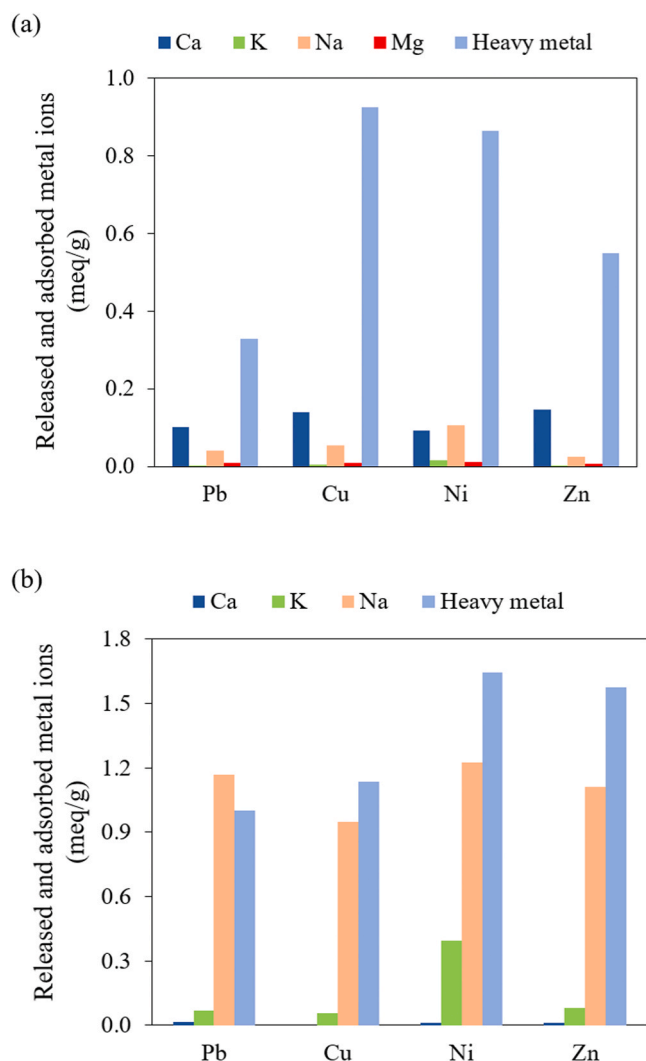
Fig. 5. Metal cations released and adsorbed (in meq/gTS) by anammox EPS (**a**) and pristine granules (**b**) during heavy metal biosorption tests at $C_0 = 500$ mg/L (i.e., 2.58, 7.76, 8.59 and 6.55 mM for Pb^{2+}, Cu^{2+}, Ni^{2+} and Zn^{2+}, respectively).

repulsive force between the EPS colloidal particles (i.e., a reduction of the absolute value of the Zeta-potential) upon increasing the Cu^{2+} concentration in contact with anammox EPS. Regardless of the mechanistic hypothesis, these findings would suggest that anammox EPS might be applied as flocculant/complexing agent to pre-treat very concentrated heavy metal-contaminated wastewaters: EPS would concentrate heavy metals in the form of composite aggregates (i.e., metal concentration in the polymer-metal aggregates higher than those adsorbed by EPS in aqueous dispersions), easily separable from the clarified effluent via sedimentation or filtration, thus greatly simplifying the post-treatment separation phase.

In summary, Fig. 7 proposes a schematic representation of the heavy metal biosorption mechanisms provided by extracted EPS and pristine anammox granules.

3.7. Outlook and perspectives

Anammox granular waste sludge might be valorized as high-performance and cost-effective biosorbent medium for the treatment of heavy metal-contaminated effluents. If compared to conventional suspended microbial flocs, granular biomasses feature the advantages of compact structure and superior settleability, which can facilitate the

post-separation (Adav et al., 2009) in water and/or wastewater treatment systems. After the heavy metal biosorption (i.e., transfer of the heavy metals from liquid to solid phase), granules could be easily separated from treated water by gravity and concentrated up to 100 Kg/m^3; eventually after further concentration (e.g., centrifuge, belt presses), the granular sludge could be then subjected to incineration, thus concentrating the metals in the ashes, or anyway disposed similarly to pristine biomass.

Compared to the direct application of pristine granules, the recovery and conversion of EPS into value-added products pave the way to interesting scenarios in the treatment of heavy metal-contaminated effluents. Even considering the low growth yield of anammox bacteria (0.11 gVS/gNH$_4$-N, Lotti et al., 2014), significative heavy metal removal potentials could be speculated for the extracted EPS, especially in treating highly concentrated heavy metal-contaminated effluents (up to 3.57, 2.22, 0.91 and 0.31 g of Pb^{2+}, Cu^{2+}, Ni^{2+} and Zn^{2+}, respectively, per kilogram of NH_4-N removed from wastewater). The metal-binding capability of anammox EPS could be therefore exploited for designing technologies able to remove a large content of heavy metals from concentrated wastewaters. However, the quality of the native anammox granular sludge, strictly dependent on the type of wastewater treated, should be pivotal in the evaluation of the most efficient and cost-effective management strategy. For example, sludges containing high levels of heavy metals could not be suitable in agriculture (usually considered one of the most desirable waste sludge valorization strategy): in this case, the application of pristine biomass and/or extracted EPS for treatments of heavy metal biosorption could be an interesting alternative.

A potential process scale-up concerning the anammox EPS recovery and application as high-performance and cost-effective metal biosorbent in WWTPs and/or in other industrial sectors requires further investigations (e.g., optimization of the extraction process, development of composite media, implementation of technological aspects, evaluation of the economic feasibility, etc.). Anammox EPS could be applied as functionalizing agent for designing EPS-based composite media (e.g., EPS-activated carbon composite adsorbents, composite hydrogels, hybrid membranes). Based on the results described above, an interesting route to engineer the metal-binding ability of anammox EPS might be the application of EPS-based flocculant/complexing agent to pre-treat very concentrated heavy metal-contaminated effluents (e.g., galvanic and mining wastewaters). Conceiving the treatment unit in this way, technological limitations due to the use of EPS in the form of aqueous dispersions would be avoided, in addition to the advantage in using a bio-based commodity recovered from a waste product in substitution to chemical agents usually applied in these types of process. Moreover, the amount of heavy metal-contaminated sludge to be disposed after this kind of pre-treatment could be significantly lower compared to the application of pristine biomass, as demonstrated by the adsorption potentialities: 171 mgPb/gTS$_{EPS}$ and 68 mgCu/gTS$_{EPS}$ for EPS acting as flocculant agent (Table 2) versus 103.7 mgPb/gTS$_{granules}$ and 36.1 mgCu/gTS$_{granules}$ for native granules (Fig. 2). In the case of effluents contaminated by a single heavy metal (condition that can be relevant for many kinds of industrial wastewaters, Bolisetty and Mezzenga, 2016), the use of anammox EPS as flocculant/biosorbent media might also provide heavy metal recovery strategies. For example, the amyloid fibrils present in anammox EPS (Lotti et al., 2019b) could be able to reduce metal ions into metal nanoparticles via a bio-mineralization processes as reported for synthetically formed amyloid fibrils by Bolisetty and Mezzenga (2016), thus enabling the recovery of expensive heavy metal ions (Bolisetty et al., 2011; Bolisetty and Mezzenga, 2016).

Another aspect to be considered in developing this type of EPS-based processes is the digestibility and dewaterability of the remaining (i.e., non-extracted) waste sludge, that could be enhanced by the alkaline treatment involved in the EPS extraction process, as suggested by Lotti et al. (2019a): in this way, the efficiency of the excess sludge treatment via aerobic/anaerobic digestion would be improved, and its mass to be
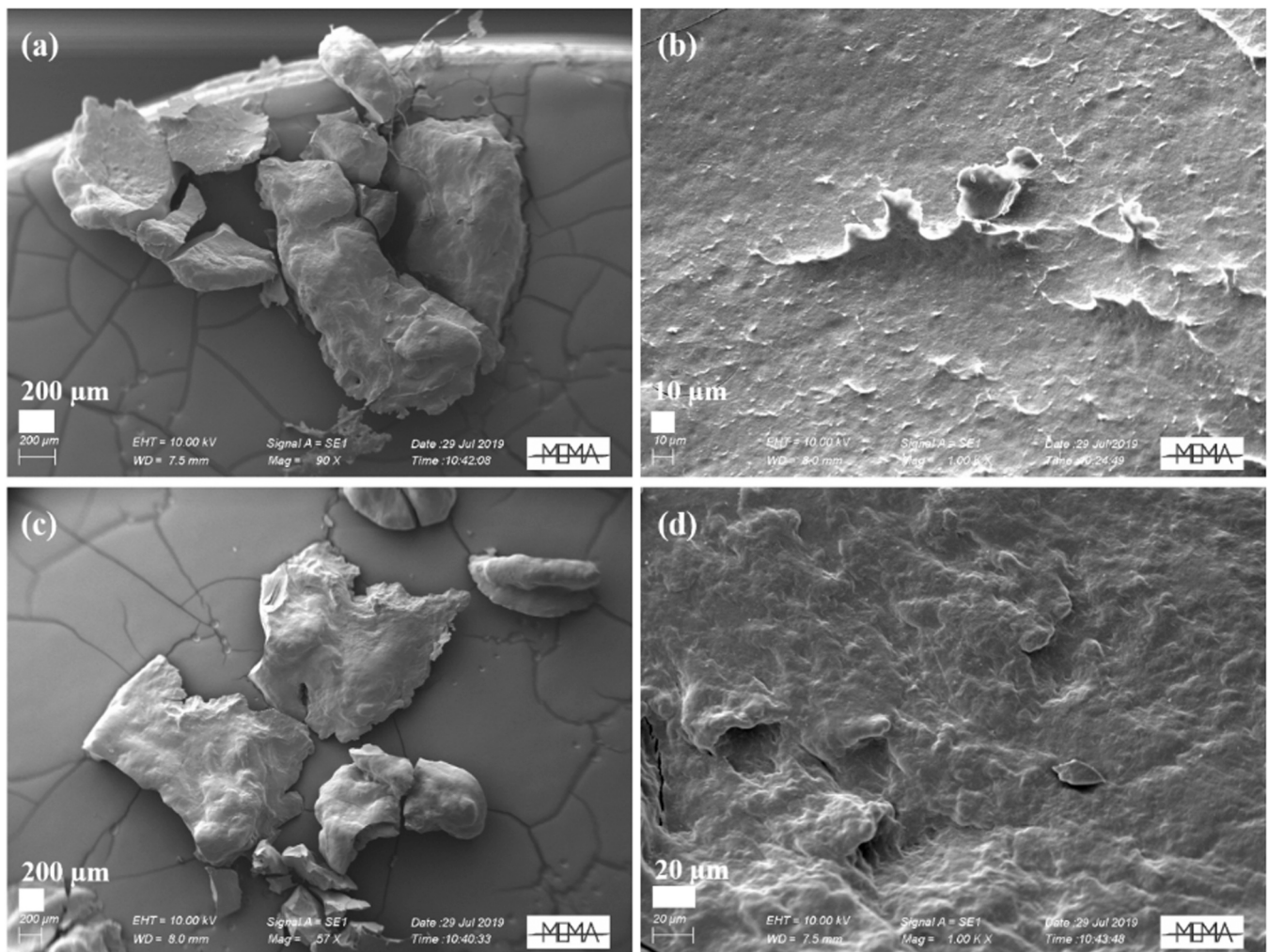
Fig. 6. EPS-Pb composite aggregates (a, b) and EPS-Cu composite aggregates (c, d) precipitated in single-metal biosorption tests treating C_0= 500 mgM^{2+}/L observed through a scanning electron microscope (SEM Zeiss Evo MA15) at different magnifications.

Table 2

Elemental analysis of EPS-Pb/Cu composite aggregates (precipitated in single-metal biosorption tests at C_0 =500 mg/L) detected by energy dispersive X-ray spectrometry (EDS). Average values $\pm$ standard deviations (n. 4 and 3 points of analysis for composite EPS-Pb and EPS-Cu aggregates, respectively).

Composite EPS-Pb aggregates			Composite EPS-Cu aggregates		
C	40.51 ± 5.63	Weight%	C	53.55 ± 2.47	Weight%
	66.12 ± 7.38	Atomic%		63.90 ± 1.44	Atomic%
O	34.59 ± 4.79	Weight%	O	38.17 ± 0.04	Weight%
	30.80 ± 5.36	Atomic%		34.22 ± 0.85	Atomic%
Pb	9.85 ± 7.84	Weight%	Cu	8.28 ± 2.42	Weight%
	1.54 ± 0.63	Atomic%		1.88 ± 0.59	Atomic%
O:C	0.495 ± 0.104	mol/C-mol$_{EPS}$	O:C	0.536 ± 0.025	mol/C-mol$_{EPS}$
Pb:C	0.023 ± 0.007	mol/C-mol$_{EPS}$	Cu:C	0.029 ± 0.007	mol/C-mol$_{EPS}$
Pb:EPS	0.171 ± 0.056	g/gTS$_{EPS}$	Cu:EPS	0.068 ± 0.023	g/gTS$_{EPS}$

treated reduced, thus contributing to the economic and environmental sustainability of the overall treatment chain.

4. Conclusions

This work proposed a detailed investigation of the heavy metal biosorption processes provided by EPS recovered from waste anammox granular sludge. Thanks to their high metal-binding capacities both in single- and multi-metal contaminated aqueous systems, anammox EPS could be successfully applied for the treatment of heavy metal-contaminated wastewaters. With the aim of shedding light on the adsorption mechanisms of extracted EPS and non-extracted EPS in the native biomass, extracted EPS and pristine anammox granules were compared. Different adsorption capabilities were observed for extracted EPS (84.9, 52.8, 21.7 and 7.4 mg/gTS$_{EPS}$) and pristine anammox granules (103.7, 36.1, 48.2 and 49.8 mg/gTS$_{granules}$) towards Pb^{2+}, Cu^{2+}, Ni^{2+} and Zn^{2+}, respectively (C_0= 500 mgM^{2+}/L) and different biosorption pathways were postulated. Besides the potential metal uptake exerted by other components than EPS in pristine granules (e.g., cell surface adsorption, intracellular accumulation, etc.), the metal-binding capability of EPS could be altered by the chemical modifications induced by the extraction protocol applied and by the spatial rearrangement allowed by the much higher mobility of EPS in aqueous dispersion compared to EPS in native granules. A molecular-level analysis, supported by a complete set of spectroscopic techniques, disclosed a multifaceted adsorption mechanism, involving a combination of electrostatic interaction, ion-exchange, complexation, and precipitation.

Even if further research effort is required to engineer the processes here proposed, the obtained results pave the way to interesting routes to promote circularity in the wastewater treatment field as well as to move towards environmentally and economically sustainable treatment
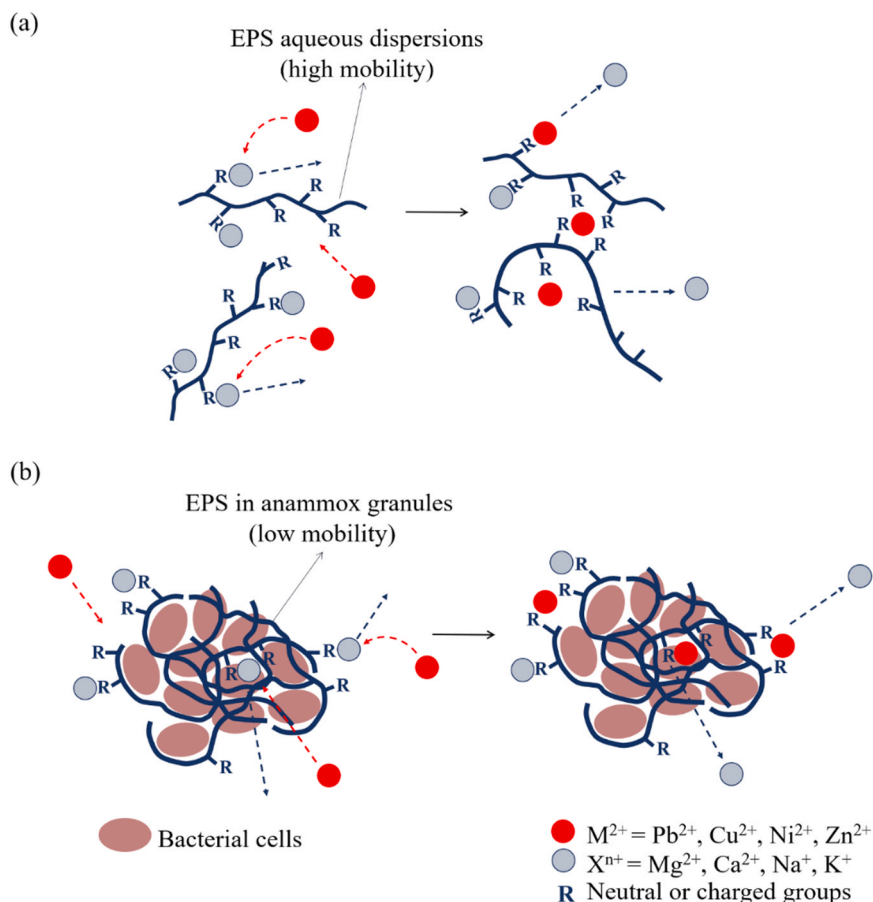
Fig. 7. Schematic representation of the adsorption mechanisms provided by extracted EPS (a) and pristine anammox granules (b). Heavy metal cations M^{2+} (Cu^{2+}; Ni^{2+}; Zn^{2+}; Pb^{2+}) form complexes with the functional groups R of EPS (i.e., neutral or charged acidic or alkaline groups) causing the partial substitution of the cations naturally bond to the EPS (i.e., Ca^{2+}; Mg^{2+}; Na^+; K^+) through ionic exchange processes.

systems for heavy metal-contaminated effluents.

CRediT authorship contribution statement

B.P. conceived the original idea, conceived and planned the experiments, carried out the experiments, contributed to the interpretation of the results, took the lead in writing the manuscript. E.C. contributed to conceive the original idea, conceived and planned the experiments, carried out part of the experiments, contributed to the interpretation of the results. M.S. carried out part of the experiments, contributed to the interpretation of the results. D.B. contributed to conceive the original idea, conceived and planned the experiments, contributed to the interpretation of the results. C.L. conceived the original idea, conceived and planned the experiments, contributed to the interpretation of the results, supervised the project. T.L. conceived the original idea, conceived and planned the experiments, contributed to the interpretation of the results, supervised the project. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Declaration of Competing Interest

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

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2021.126661.

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