# Application of anammox within an integrated approach to sustainable food waste management and valorization

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**Abstract.** In this study, the anammox process was applied for the first time to the treatment of ammonium-rich liquid residues produced by the two-stage anaerobic digestion of food waste (2sAD-FW); such residues may represent a significant environmental issue if not properly managed. A granular anammox reactor was fed with a progressively increasing share of partially nitritated 2sAD-FW wastewater. An alternative operating strategy based on partial by-pass of the partial nitritation unit was tested, in order to regulate the influent $NO_2/NH_4$ molar ratio without chemical addition. High nitrogen removal efficiency (89±1%) and negligible nitrite discharge rates were achieved, together with high nitrogen removal rate / nitrogen loading rate (NRR/NLR, 97±1%) and stable specific anammox activity (0.42±0.03 $gN_2$-N/gVSS·d). The observed $NH_{4\text{-removed}}/NO_{2\text{-removed}}/NO_{3\text{-produced}}$ molar ratio was in agreement with anammox stoichiometry, as confirmed by the low contribution (< 5%) of denitrification to nitrogen removal. Moreover, the possibility of using digital color characterization of granular biomass as a novel, simple tool for the monitoring of anammox biomass enrichment and process performance was investigated under dynamic conditions, using real wastewater: changes in granule color correlated well with the increasing share of 2sAD-FW wastewater in the influent ($R^2$=83%), as well as with the decrease of anammox biomass abundance in the reactor ($R^2$=68%). The results suggest that anammox may be successfully integrated into a 2sAD-FW system, thus enhancing its environmental sustainability.

**Keywords**. Ammonium; anaerobic digestion; anammox; CIE-Lab; food waste; sequencing batch reactor

*Abbreviations:* 2sAD, two-stage anaerobic digestion; 2sAD-FW, two-stage anaerobic digestion of food waste; Anammox, anaerobic ammonia oxidation; AOB, ammonium oxidizing bacteria; BLR, biomass loading rate; CIE, Commission Internationale de l'Eclairage; COD, chemical oxygen

demand; $C_{org}$, organic carbon; DOC, dissolved organic carbon; EU, European Union; FA, free ammonia; FID, flame ionization detector; FISH, fluorescence in situ hybridization; FNA, free nitrous acid; FW, food waste; HRT, hydraulic retention time; IA, image analysis; Mt, $10^6$ metric tons; NitDR, nitrite discharge rate; NLR, nitrogen loading rate; NRE, nitrogen removal efficiency; NRR, nitrogen removal rate; OFMSW, organic fraction of municipal solid waste; PN, partial nitritation; SAA, specific anammox activity; SBR, sequencing batch reactor; TKN, total Kjeldahl nitrogen; TNb, total nitrogen bound; TP, total phosphorus; TSS, total suspended solids; VFA, volatile fatty acids; VSS, volatile suspended solids.

## Introduction

Every year, approximately 1,200 Mt of the food produced worldwide are lost or wasted through the food supply chain, which causes significant social, environmental, and economic issues [1,2]. According to European waste statistics, approximately 245 Mt of municipal solid waste were generated in the EU-28 in 2016, out of which food waste (FW) accounted for 35% by weight [3]. Rather than being considered an environmental issue, FW may be seen as a potential source of material and energy which should be recovered within an eco-sustainable approach. The two-stage anaerobic digestion process (2sAD) aiming at the recovery of hydrogen (dark fermentation) and methane has proved to be a promising option, since it enhances overall energy recovery compared to conventional one-stage processes [4,5]. Moreover, the possibility of recovering energy as hydrogen and methane, rather than only methane, is of interest due to the positive environmental features of hydrogen as an energy carrier, especially if generated from renewable non-fossil sources [3].

However, maximization of energy recovery from FW by a 2sAD process must be accompanied by minimization of its potential environmental impacts, in order to promote an environmentally sound approach. Since FW consists mainly of carbohydrates, fats and proteins [6], the anaerobic digestion of such a substrate results in the transfer of nutrients from the solid to the liquid phase, whose direct discharge into municipal sewers is not a viable option as it would unbalance the chemical oxygen demand / total Kjeldahl nitrogen / total phosphorus (COD/TKN/TP) ratio of municipal wastewater [7]. The use of the digested effluents in agriculture as organic fertilizer may also be limited by factors such as transport requirements, water content, or the presence of unwanted substances and pathogenic microorganisms [8].

The relatively low organic carbon to nitrogen ratio makes such effluents potentially suitable for treatment by completely autotrophic nitrogen removal processes, which combine partial nitritation (PN) and anaerobic ammonia oxidation (anammox). In the PN reactor, roughly 50-55% of influent ammonium should be converted into nitrite by ammonium oxidizing bacteria (AOB), so that the residual ammonium can be converted into dinitrogen gas in the anammox reactor by Planctomycetes (a distinct phylum in the Bacteria domain), using nitrite as electron acceptor [9]. PN/anammox represents a cost- and technically effective alternative to conventional biological treatment based on full nitrification and denitrification, as well as to chemical-physical processes, requiring less energy and fewer chemicals (no exogenous organic substrates such as methanol or glucose are required) [10,11]. Moreover, the PN/anammox footprint is low in terms of greenhouse gas emissions [12,13], which makes it an eco-sustainable option worthy of being investigated; compared with conventional wastewater treatment based on nitrification and denitrification, $CO_2$ emissions may be reduced by up to 80%, and $N_2O$ is absent in anammox physiology (conversely, it is an intermediate in denitrification) [13].

Despite such promising features, limited work has been focused on the application of PN/anammox to the treatment of the liquid effluents originated by the one-stage anaerobic digestion of the municipal solid waste organic fraction (OFMSW) [7,14]: in one report, the possibility was successfully investigated of upgrading the Florence wastewater treatment plant by integrating the anaerobic co-digestion (waste activated sludge + OFMSW) with the completely autotrophic removal of nitrogen from the supernatant, using a nitritation membrane bioreactor and an anammox sequencing batch reactor [7]. The latter showed variable $NH_4^+$-N and $NO_2^-$-N removal rates, ranging from 1.3 to 47.1 mgN/L·d and from 0.5 to 47.6 mgN/L·d, respectively. Others [14] performed batch experiments to investigate the response of anammox biomass exposed to the liquid fraction of digested and co-digested OFMSW, using conductivity as an aggregate parameter to evaluate inhibition capacity: although the undiluted liquid fraction of digested OFMSW was found to have the strongest inhibitory capacity (anammox activity reduction of 73-89%), likely due to the high overall conductivity, anammox biomass was found to have a potential adaptation capability.

As for the anaerobic digestion of food waste in a two-stage system (2sAD-FW), so far only preliminary studies have been reported concerning the application of PN/anammox to the treatment of simulated (synthetic) 2sAD-FW wastewater [15,16]. In particular, anammox biomass was able to withstand the high nitrogen loading rates applied to the PN reactor (up to 1.5 gN/L·d), showing high nitrogen removal efficiencies (NRE > 90%) and negligible nitrite discharge rates [16]. However, specific knowledge about anammox behavior with real 2sAD-FW wastewater is not available, so that an important gap has to be filled in view of possible process scale-up.

In this study, the liquid effluent produced by 2sAD-FW and treated in a PN unit was fed into a granular anammox sequencing batch reactor, in order to evaluate its applicability and achieve process optimization using real wastewater. An alternative process layout based on partial by-pass of the PN unit was tested in order to regulate the influent $NO_2/NH_4$ molar ratio without chemical (e.g., ammonium chloride) additions, which would represent a significant cost at pilot/full scale. A

novel approach to anammox sludge characterization was also implemented, based on the determination of biomass color as a potentially quick, simple and cost-effective indirect measure of process performance and biomass enrichment.

## Materials and Methods

### *Reactor set-up and operation*

The granular anammox reactor was the second unit of a two-step laboratory-scale treatment system based on partial nitritation and anammox. A brief description of the PN unit and a schematic representation of the whole PN-anammox system are provided in supplementary material (SM1). The anammox unit consisted of a 3 L sequencing batch reactor (SBR) with a working volume of 2.1 L, operated at controlled temperature (35±0.5 °C) and pH (7.0±0.1). In order to reduce start-up time, granular anammox biomass originating from a previous experimental campaign concerning refinery wastewater treatment [10] was used as inoculum. The reactor was operated in fed-batch mode with a 6h cycle configuration (267 min mixed feeding, 83 min reaction, 5 min settling and 5 min effluent withdrawal). Mechanical mixing was provided by a marine impeller (80±5 rpm). At the beginning of each working cycle, inert $N_2$ gas was flushed for 5 min, in order to assure anoxic conditions inside the SBR. The influent flow rate was set at 2.0 mL/min; the volumetric exchange ratio was kept equal to 0.25, corresponding to a hydraulic retention time (HRT) of 1 d. The vessel was completely covered with tin foil, in order to avoid any penetration of light which would hinder anammox activity [17]. The organization of the experimental activity is summarized in **Table 1**.

### *Influent wastewater*

The granular anammox reactor was fed with a synthetic medium simulating PN-treated 2sAD-FW wastewater [16], in order to avoid any influence of the previous experimental campaign [10], as well as to achieve stable process performance under strictly controlled conditions, which constituted the starting point for the present study. The synthetic medium consisted of $NH_4HCO_3$ 3,848mg/L, $NaNO_2$ 4,030 mg/L, $MgSO_4 \cdot 7\ H_2O$ 200 mg/L, $KH_2PO_4$ 6.25 mg/L, $CaCl_2$ 300 mg/L, $FeSO_4 \cdot 7\ H_2O$ 12.5 mg/L and trace element solution [18] (1.25 mL/L) in distilled water. The synthetic medium was progressively replaced by the PN-treated 2sAD-FW wastewater according to a conservative exponential rule [10] (see supplementary material, SM2). The main characteristics of both untreated and PN-treated 2sAD-FW wastewater are summarized in **Table 2**.

Correction of influent $NO_2/NH_4$ molar ratio was initially applied by appropriate $NH_4Cl$ dosage, and total influent nitrogen was kept at 1,500 mg/L (Phases 1 and 2), corresponding to a nitrogen loading rate (NLR) of 1.5 gN/L·d. Thereafter, part of the PN influent was sent directly to the anammox unit and manual correction by $NH_4Cl$ addition was avoided (Phase 3).

*Analytical Methods*

Ammonium (as $NH_4^+$-N), nitrite (as $NO_2^-$-N) and nitrate (as $NO_3^-$-N) concentrations were determined at least twice weekly on influent and effluent samples, as well as on samples drawn from the anammox reactor during specific anammox activity (SAA) assessments. Ammonium concentration was determined according to Standard Methods [19], while nitrite and nitrate concentrations were determined by ion-chromatography using a DIONEX ICS-90 (Dionex-Thermofisher Scientific Inc., USA) equipped with an AS14A Ion-PAC 5 μm column. All samples were filtered (0.45 μm) before analysis. Total nitrogen bound concentration (TNb, the sum of organic and inorganic nitrogen forms) was measured to characterize the untreated and PN-treated 2sAD-FW wastewater used for feed preparation, using a Hach DR6800 spectrophotometer (LCK338 cuvette test kit, Hach Lange GmbH, Germany). Samples were not filtered. Free Ammonia (FA) and Free Nitrous Acid (FNA) concentrations were estimated according to [20]. The nitrogen removal efficiency (NRE, the actual removal of influent nitrogen, which takes into account the production of nitrate), the nitrogen removal rate (NRR, the amount of $NH_4$-N and $NO_2$-N removed per liter of reactor per day), and the nitrite discharge rate (NitDR, the amount of $NO_2$-N discharged per liter of reactor per day) were calculated as described in [10].

Alkalinity of untreated and PN-treated 2sAD-FW wastewater (unfiltered samples) was measured periodically (i.e., every time a new feed was prepared) by potentiometric titration to preselected end-point pH, using an AT-510 automatic titrator (KEM electronics, Japan). The content of organic matter in influent and effluent filtered samples (0.45 μm) was measured at least twice weekly as dissolved organic carbon (DOC) by means of a TOC-V analyzer (Shimadzu Corp., Japan). Volatile fatty acids (VFA) concentration was determined in the untreated and PN-treated 2sAD-FW wastewater every time a new feed was prepared, as well as in anammox reactor effluent (at least twice per week), using a 6890-N gas chromatograph (Agilent, USA) equipped with a headspace auto-sampler (Agilent, mod. 7694), a DB-FFAP column (30 m × 0.25 mm × 0.25 μm) and a flame ionization detector (FID). All samples were acidified with 2M $H_2SO_4$ and filtered (0.45 μm) before analysis. Total suspended solids (TSS) and volatile suspended solids (VSS) concentrations in the anammox reactor and in the effluent were determined according to Standard Methods [19] at least weekly.

*Characterization of anammox granular sludge*

Specific anammox activity (SAA) assessments were based on a chemical tracking method [21], and carried out periodically according to a published protocol [10]. Granule density was determined according to the dextran blue method [22] on samples drawn weekly.

Granular aggregates were characterized periodically in terms of size (particle size distribution) and aspect (roundness, aspect ratio) through image analysis (IA) technique, according to a published protocol [10] using Image-Pro Plus v.6 (Media Cybernetics, USA). More than 200 granules were considered for each sample.

Digital color analysis of anammox granules was performed using a CM 3610 spectrophotometer (Konica Minolta, Japan). Color was represented using Konica Minolta SpectraMagic NX software as three numeric coordinates, labeled L*, a* and b* color components, ranging from 0 to 100 (L*) and from -128 to +127 (a* and b*), according to CIE Lab color space [23]. L* is a measure of lightness; a* and b* are related to Hering's color opponent process theory, i.e. a* represents the green-red opposition (negative and positive values, respectively), while b* the blue-yellow opposition (negative and positive values, respectively). The (a*, b*) Cartesian coordinate duplet was converted to corresponding polar coordinates, labelled as C* (chroma, which can also be intended as saturation), and h (hue, or hue angle, expressed as degrees), according to the following equations:

$$C^* = \sqrt{(a^*)^2 + (b^*)^2} \qquad \text{Eq. 1}$$

$$h = arctan\left(\frac{b^*}{a^*}\right) \qquad \text{Eq. 2}$$

Color analysis was performed on samples collected on days 50, 73, 89, and 105, corresponding to a share of PN-treated 2sAD-FW wastewater of 30%, 50%, 70% and 100%, respectively. To prepare granular biomass a mixed liquor sample (10 mL) was collected from the reactor; granules were sieve-drained and then carefully disrupted using a glass mortar and pestle, in order to obtain a homogeneous suspension expressing not only the color of the surface of the granules, but also of their internal part. The suspension was then filtered through a glass fiber membrane with a porosity of 1.2 µm, and the resulting filter cake was analyzed to determine color composition.

The absolute C* value and the a*/b* ratio (the latter related to hue changes, according to Eq.2 and [24]) were chosen as color main indicators. Since PN-treated 2sAD-FW wastewater has its own color and represents a potential darkening/coloring agent itself, its contribution was also assessed and used as blank: in order to rule out the coloring/darkening effect of PN-treated 2sAD-FW wastewater, color difference between samples and blanks was calculated according to CIE $dE_{00}$ standard [25] for each "sample/blank" pair, and CIE $dE_{00}$ color difference was chosen as the third color main indicator. For each processed sample, color indicators were plotted versus (i) the corresponding share of PN-treated 2sAD-FW wastewater on total influent, (ii) the anammox biomass abundance and (iii) the SAA, and linear fitting was applied in order to highlight potential correlations.

*Microbiological characterization*

Microbiological characterization was performed during Phase 1 by fluorescence in situ hybridization (FISH) on representative granules samples, according to [26]. Hybridizations with group specific probes for anammox bacteria (AMX820, specific for "*Candidatus* Brocadia anammoxidans" and "*Candidatus* Kuenenia stuttgartiensis") were carried out simultaneously with probes EUB338, EUB338-II and EUB338-III combined in a mixture (EUB338mix) for the detection of most bacteria, and with DAPI staining for quantifying the total number of cells. Anammox bacteria

abundance was expressed as the percentage of AMX820-positive cells out of EUB338mix-positive cells. All probes were purchased from Eurofins Genomics Gmbh (Germany), and synthesized with 5'-FITC (green) and 5'-Cy3 (red) labels. Details on oligonucleotide probes are available at ProbeBase [27]. Slides were examined with an epifluorescence microscope (Olympus BX51) at different magnifications (100, 400 and 1000x); images were captured with an Olympus XM10 camera using Cell-F software (Olympus Corporation, Japan). DAIME software [28] was used for FISH quantification of hybridized cells.

## Results and discussion

*Overall anammox performance*

During Phase 1, the increasing share of PN-treated 2sAD-FW wastewater did not affect process performance in terms of nitrogen removal (**Figure 1**): NRE and the NRR/NLR ratio averaged 90±1% and 98±1%, respectively, and effluent nitrite concentration was always negligible. The observed "$NH_{4\text{-removed}}/NO_{2\text{-removed}}/NO_{3\text{-produced}}$" molar ratio was in good agreement with the stoichiometric range reported in literature for anammox metabolism [29,30] (Figure 1c), indicating no significant competition for nitrite between anammox and heterotrophic denitrifying bacteria. Indeed, most of the readily degradable organic substrates contained in the untreated 2sAD-FW wastewater (e.g., VFAs) were completely removed by the previous aerobic step (PN reactor), and were unavailable for denitrification; despite the progressively increasing availability of organic matter, the resulting influent organic carbon to nitrogen ratio ($C_{org}/N$) at the end of Phase 1 was around 0.1 mol/mol, much lower than the threshold level proposed by others [31], above which heterotrophic bacteria outcompete anammox biomass ($C_{org}/N=1$). The amount of nitrogen potentially removed by heterotrophic denitrification accounted for only 0.9±0.4% of total observed nitrogen removal (based on the observed organic matter depletion).

As the share of PN-treated 2sAD-FW wastewater in the influent reached 100% (Phase 2), process performance remained stable in terms of NRE (89±2%) and the observed NRR/NLR ratio was still high (97±2%), as shown in Figure 1b. Again, the amount of nitrogen potentially removed by heterotrophic denitrification was low (0.8±0.4%), and the observed $NH_{4\text{-removed}}/NO_{2\text{-removed}}/NO_{3\text{-produced}}$ molar ratio remained in good agreement with the stoichiometric range. More specifically, the $NO_{2\text{-removed}}/NH_{4\text{-removed}}$ molar ratio substantially followed the fluctuations of the influent $NO_2$-N/$NH_4$-N molar ratio, and remained within the stoichiometric range reported in literature (with the exception of a few points), as well as the observed $NO_{3\text{-produced}}/NH_{4\text{-removed}}$ molar ratio (Figure 1c).

In order to regulate the influent $NO_2$-N/$NH_4$-N molar ratio without external $NO_2$ or $NH_4$ addition, in the perspective of process application at pilot- or full-scale, an operating strategy based on partial by-pass of the PN unit was adopted (Phase 3). Feeding the anammox reactor with a mixture of PN-treated (85%) and untreated (15%) 2sAD-FW wastewater resulted in temporary inhibition, with a corresponding worsening of nitrogen removal: the NRE decreased from day 122 and reached its

lowest on day 135 (72%). Nitrite accumulated up to 113 mg$NO_2$-N/L (day 139). However, complete process recovery was achieved within 20 days: from day 145, the observed NRE and NRR/NLR were 89±1% and 97±1%, respectively, and NitDR was negligible.

As expected, the availability of readily degradable organic matter enhanced the occurrence of denitrification: DOC removal efficiency increased up to 67% and denitrification contribution to nitrogen removal rose, peaking at approximately 5% of total removed nitrogen. Despite such increase, the dilution of untreated 2sAD-FW wastewater minimized the competition for nitrites between anammox and heterotrophic bacteria, since the resulting influent $C_{org}$/N ratio (<0.3) remained below critical levels and the available readily degradable VFAs were low (< 10 mg/L).

Although different authors [30,39] have proposed mixing of PN-treated and untreated wastewater as the simplest way to correct the influent $NO_2$-N/$NH_4$-N ratio, such a solution was applied only once in an anammox reactor fed with a mixture of PN-treated and raw livestock manure digester liquor (relative share not reported) [32]: nitrite accumulation was observed after the first week of operation, ascribed to an increase in influent nitrite concentration due to unstable operation of the PN reactor. Severe inhibition of anammox activity occurred and process recovery could be achieved by adjusting process pH from 7.9 to 7.5. Neither positive nor negative effects could be unequivocally ascribed to the presence of untreated wastewater in the influent.

With regard to specific anammox activity (**Figure 2**), an increasing trend was observed in the first part of Phase 1, with a maximum value of 0.71 g$N_2$-N/gVSS·d (day 60, 36% of PN-treated 2sAD-FW wastewater). However, SAA decreased progressively as the share of PN-treated 2sAD-FW wastewater was further increased (it was 0.44 g$N_2$-N/gVSS·d at the beginning of Phase 2, when 100% PN-treated 2sAD-FW wastewater was fed to the reactor). The results suggest that PN-treated 2sAD-FW wastewater may contain compounds which stimulate anammox activity, at least if the share of PN-treated 2sAD-FW wastewater in the influent is low enough. A similar trend was observed by others [10] in a granular anammox reactor fed with progressively increasing share of PN-treated refinery (IGCC) wastewater; higher SAAs (maximum value: 0.27 g$N_2$-N/gVSS·d) were achieved when PN-treated wastewater was fed to unacclimated anammox biomass, compared to those measured using a synthetic medium [32].

During Phase 2, no significant biomass inhibition due to prolonged exposure to PN-treated wastewater was observed. The lowest SAA was at the beginning of Phase 3 (0.39 g$N_2$-N/gVSS·d), when untreated 2sAD-FW wastewater was added to the influent. Such a decrease in SAA was consistent with the overall worsening of process performance observed on days 122-140. Given the complex composition of such effluents [14,33], it was not possible to identify any specific compound or parameter at the origin of the temporary decrease in SAA and NRE. A hypothesis may involve interference of organic matter to anammox metabolism, whose mechanisms are still unclear and depend on the organic compounds and their concentration [34].

Although overall process performance recovered during the second half of Phase 3, SAA did not show a particular increase and stabilized at 0.42±0.03 g$N_2$-N/gVSS·d. The reduced SAA may be

considered as a measurable effect of competition between anammox and heterotrophic bacteria, which was less evident when NRE, NRR and NitDR were considered.

As far as we are aware, to date no other studies have focused on anammox application to 2sAD-FW wastewater, so that an extensive direct comparison of SAA cannot be drawn. The SAAs observed here were comparable to, or higher than, those previously reported concerning the application of anammox to similar substrates (i.e., the digestate of OFMSW), although reported data are limited [7,35]. Moreover, SAAs were also comparable with those reported in previous studies concerning anammox systems treating conventional substrates (reject water) [36,37].

*Granular anammox physical and morphological properties*

With regard to the average size of anammox granules, no significant differences were detected throughout the experimental period (mean diameters measured during Phase 1, 2 and 3 were 0.66±0.01, 0.64±0.02 and 0.66±0.02 mm, respectively). On closer inspection (**Figure 3**), the evolution of particle size distribution showed changes in the frequencies of smaller size (<1 mm) as the share of PN-treated 2sAD-FW wastewater in the influent increased: compared to the beginning of Phase 1, at the end of Phase 2 (day 123) a decrease in 0.3-0.7 mm classes abundance from 53 to 40% was observed, together with a corresponding increase from 2.3 to 12.9% in 0.1 mm and from 16 to 20% in 0.9-1.1 mm class abundances, suggesting the formation of new granules and the simultaneous enlargement or disruption of part of existing ones. By the end of Phase 3 (day 161), the increase up to 20% in 0.3 mm and up to 12.4% in 1.1-1.3 mm class abundances combined with the corresponding decrease from 24.4 to 15.8% in 0.7-0.9 mm class seemed to confirm this behavior. Consistent with average granule size, the other morphological parameters investigated, namely aspect ratio and shape regularity (roundness), did not change significantly during the entire operation. Only a slight decrease in shape regularity was observed, as indicated by the decreasing rate of granules with a roundness value above 0.8, at 62%, 51% and 39% for days 1, 123 and 161, respectively.

Neither the increasing share of PN-treated 2sAD-FW wastewater (Phase 1) nor the partial by-pass of the PN unit (Phase 3) affected anammox granule density, which was stable during the whole experimental campaign (69.5±6.1 gTSS/$L_{gran}$), and comparable to other anammox granular sludge systems previously reported in literature [10,38,39].

Stressful operating conditions, such as prolonged shear stress, exposure to toxic substances, etc., were found to strongly affect physical and morphological properties of both anaerobic and aerobic granular aggregates [38,40]. The stable behavior observed in this study can be likely ascribed to the conservative feeding strategy applied during Phase 1, which minimized any potentially stress condition for granular anammox biomass and facilitated acclimation to the real wastewater, as previously reported [10].

Biomass concentration inside the anammox reactor did not change significantly during the entire SBR operation (VSS 5.0±0.75 g/L; VSS/TSS 90±2%), although an increasing trend was observed

during Phase 1. Effluent TSS concentration reached its maximum values at the end of Phase 1 and during Phase 2 (143±6 mg/L), while the average observed during Phase 3 was 103±20 mg/L. Only a few granules were lost with the effluent at the end of each cycle, and TSS mostly consisted of floc-shaped biomass or small particles probably produced by granule breakage due to abrasion/collision.

*Microbiological characterization*

During Phase 1, biomass samples were collected under dynamic conditions at progressively decreasing time intervals (due to the exponential rule adopted for replacement of the synthetic medium), and microbiological characterization by FISH was used to draw an indicative trend of anammox biomass abundance with increasing share of PN-treated 2sAD-FW wastewater in the influent (**Figure 4**).

Initial anammox biomass abundance represented 62% of total bacteria, in agreement with previously reported studies [41]; a linear correlation was observed between the share of PN-treated 2sAD-FW wastewater and abundance, the latter decreasing as the synthetic medium was progressively replaced by real wastewater. Such behavior can be reasonably ascribed to the progressive enrichment of other microbial populations due to the increasing availability of substrates other than ammonium nitrogen, as confirmed by the increase in EUB338mix/DAPI ratio from 74% (Day 1) to 87% (day 105, end of Phase 1).

*Digital color characterization of granules*

For color evolution of granular biomass, a representation on CIE Lab color space is provided in **Figure 5**: as the share of PN-treated 2sAD-FW wastewater increased up to 100% (Phase 1), the progressive darkening of biomass samples was observed, as indicated by the decrease in L* (i.e., lightness) values. Moreover, the progressive decrease in a* and b* values indicated a shift from red towards green opposites, and from yellow towards blue opposites, respectively, which resulted in the progressive loss of the initial vivid orange-reddish color typical of anammox enriched biomass. The analysis of blank (influent) samples showed a similar decreasing trend in L* values, negligible variations of a* and, as expected, a slight increase of b* values toward the same yellowish hue of the PN-treated wastewater: these results demonstrated that the influent contained progressively increasing amounts of darkening (e.g., suspended solids) and coloring (e.g., dyed compounds) agents which may affect biomass color characterization, if not properly taken into consideration.

As shown in **Figure 6**a-b, both C* and a*/b* trends showed a linear correlation with the increasing share of PN-treated 2sAD-FW wastewater on total influent ($R^2$=83 and 79%, respectively), thus confirming that the progressive replacement of the synthetic medium with real wastewater altered the initial biomass color. As CIE $dE_{00}$ color difference was considered as color indicator,

linear correlation was still maintained (Figure 6c, $R^2$=68%), thus showing that changes in biomass color were related not only to a direct coloring effect of the influent, but also to changes in other biomass properties, which were in turn influenced by the increasing share of PN-treated 2sAD-FW wastewater.

On this premise, the possible correlation between changes in granular biomass color and anammox biomass abundance was investigated, in order to evaluate if digital color analysis may be used to track biomass evolution in anammox based systems. As previously discussed, the progressive replacement of the synthetic medium with PN-treated 2sAD-FW wastewater was accompanied by a corresponding slight decrease in anammox biomass abundance. The decrease was correlated linearly with changes in C* and a*/b* values ($R^2$=68 and 63%, respectively), as shown in Figure 6d-e. When the direct influent contribution to biomass color was subtracted, a linear correlation still remained (Figure 6f, $R^2$=50%), thus indicating that relatively small changes in anammox biomass abundance can be detected effectively through digital color analysis of the granules, even in a system fed with real wastewater under dynamic conditions.

Conversely, none of the chosen color indicators showed a significant linear correlation with SAA ($R^2$=12, 8 and 2% for C*, a*/b* and $dE_{00}$, respectively). This result was predictable, given the unusual SAA trend observed during Phase 1 which increased as the share of PN-treated 2sAD-FW wastewater increased up to 36%, and subsequently decreased (Figure 2). Indeed, SAA was affected by the decreasing trend of anammox biomass abundance, which correlated well with changes in biomass color as well as with the ascending share of PN-treated 2sAD-FW wastewater, but was also directly affected by the changing composition of influent wastewater, as previously discussed. Although the correlation of each of these influencing factors with SAA should have been investigated with specific assessments, it was beyond the aims of this study.

Digital color characterization of granular anammox through color space representation was apparently tested only once previously [24]. In that study, three reactors were fed with synthetic influent at different biomass loading rate (BLR, the ratio between NLR and biomass concentration). Granular biomass drawn from the reactors showed three different surface colors, namely black, brown and red; only the a*/b* ratio was chosen as main color indicator, showing a linear correlation with SAA and BLR, while no correlation with anaerobic oxidizing bacteria abundance or cytochrome-c concentration was observed. A direct comparison between the results achieved in [24] and those reported here is not possible, due to significantly different operating conditions, namely steady state vs. dynamic conditions, and synthetic medium vs. a progressively increasing share of real wastewater in the influent. However, both studies highlight the potential of digital color analysis as a simple method for monitoring of granular anammox based systems.

## Conclusion

The anammox process was proved to be a feasible and valuable option for the treatment of ammonium-rich liquid digestate produced by the 2sAD-FW process, in a two-step PN/anammox

system. In view of process scale-up, the dosage of ammonium chloride was avoided by adopting an alternative treatment scheme layout based on partial by-pass of the PN unit to regulate the $NO_2/NH_4$ molar ratio in the influent to the anammox reactor (Phase 3); no significant effects of competition between anammox and heterotrophic bacteria were observed in terms of overall process performance, although a decrease in SAA occurred. The application of PN/anammox would strongly contribute to reducing the overall environmental impact of the 2sAD-FW process.

Color characterization was applied for the first time on granular anammox fed with real wastewater: a good correlation between biomass color change and the increasing share of PN-treated 2sAD-FW wastewater on total influent was observed. Moreover, relatively small changes in anammox biomass abundance under dynamic conditions were detected by FISH and confirmed by digital color analysis, which may be used as a quick, simple and cost-effective indirect measurement of anammox biomass enrichment in pilot- or full-scale plant application.

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**Figure and Table Legends**

Figure 1. Anammox reactor performance throughout the whole experimental period. (a) Composition of NLR; (b) NRE, NLR, NRR and NitDR; (c) measured influent and effluent stoichiometric ratios. Yellow horizontal belts indicate a range of stoichiometric values, considering those proposed in literature.

Figure 2. (a) Specific Anammox Activity (SAA) vs time profiles measured during the whole experimental campaign; (b) average SAA, $NO_{2\text{-removed}}/NH_{4\text{-removed}}$, and $NO_{3\text{-produced}}/NH_{4\text{-removed}}$ molar ratios measured in each experimental Phase.

Figure 3. Anammox granule size distribution during the experimental period.

Figure 4. Evolution of anammox biomass abundance with increasing share of PN-treated 2sAD-FW wastewater in the influent (Phase 1).

Figure 5. Representation of samples (blue circles) and blanks (white circles) color and lightness in the CIE Lab color space.

Figure 6. Scatter plots correlating color parameters with increasing share of PN-treated 2sAD-FW wastewater on total influent (a-c) and anammox biomass abundance (d-f).

Table 1: Organization of the experimental activity.

Table 2: Average composition of the untreated and PN-treated 2sAD-FW wastewater.