



From waste activated sludge to polyhydroxyalkanoate: Insights from a membrane-based enrichment process

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ABSTRACT

Polyhydroxyalkanoate (PHA) production is a promising technology fostering the spread of the circular bio-economy approach. However, the environmental implication of the process is usually neglected. This paper shows the results of a membrane-based PHA production pilot plant fed with no-pretreated waste activated sludge (WAS). The system was monitored for effluent water quality, nitrous oxide (N₂O), and PHA production by dynamic accumulation over a long-term period to assess the consistency of the results over several fluctuations. The experimental study was characterized by three C/N ratios of 9, 4.5, and 4 g COD/g N. The system achieved a stable and high removal efficiency for carbon and nitrogen (96.3 ± 2.6 % and 89.9 ± 6.7 %, respectively), despite the only legislation limit respected being the biological oxygen demand concentration discharge limits imposed by 2020/741/EU. Low N₂O gaseous and liquid concentrations were achieved over the 200-day experimental period, never exceeding 0.52 mg N₂O-N/L. Despite the high concentration, the N₂O emission factor accounted for only 0.21 ± 0.14 % of the influent nitrogen. Finally, the system produced an average of 36.3 ± 1.8 g PHA/g VSS with a storage yield of up to 0.42 g COD_{PHA}/g COD_{VFA}. The system revealed a high stability over a long-term experimental period, achieving a considerable amount of PHA while maintaining a low N₂O emission. Promising effluent water quality was achieved, highlighting the potential of applying the water reuse practices.

1. Introduction

Polyhydroxyalkanoates (PHA) production is an emerging methodology being studied, showing potential to foster the transition towards a circular bio-economy model. Waste-recovered biopolymers were established as an attractive choice for the operators mainly because of the high substrate availability and the rising market demand [1,2]. Among the several alternatives, PHA has gained much attention in the last 30 years because of its properties similar to conventional plastics and the production process carried out by the mixed microbial cultures (MMC) in sewage sludge [3,4]. Part of the MMC produces PHA as an energy storage product by adopting volatile fatty acids (VFA) as a carbon source. Among the various strategies studied to enrich PHA producers in MMC, aerobic dynamic feeding (ADF) stands out as one of the simplest and most effective methods [5]. Despite the full aerobic conditions and a balanced feast-to-famine ratio (around 0.2) favored ADF application, the technology still hasn't reached full-scale application, especially regarding the one based on waste-activated sludge (WAS) [6]. Despite

the high potential, due to low cost and high availability, WAS is not considered the best feedstock to produce PHA mainly because of the low VFA production rate, which leads to low PHA accumulation [7,8]. Few pilot-scale studies evaluate the feasibility of producing PHA from WAS, mainly adopting pre-treated WAS [9–11] or co-fermentation with organic fraction municipal waste [12,13]. The pilot studies managed to produce a minimum of 20 % w/w of PHA up to a maximum of 60 % w/w, thus indicating the possibility of producing a considerable amount of PHA from WAS. In the above-referred papers, thermal pre-treatment and/or temperature control (≥ 37 °C) are applied for the WAS fermentation. Despite being key steps to achieve high PHA production rates, these techniques will lead to a considerably high economic and environmental impact for pilot reactors. As reported by the recently approved Directive of the European Parliament and the Council concerning urban wastewater treatment, several environmental challenges can no longer be neglected, especially regarding the wastewater treatment plants (WWTPs) operation [14].

Water scarcity is one of the most significant issues of the

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contemporary era, mainly caused by population growth and climate change [15,16]. As one of the many solutions proposed, treated wastewater reuse gained a lot of attention due to the high availability of the source [17]. Water reuse has to be applied by always considering environmental protection, meaning that several limits have to be used to ensure the water quality before it is released back into the environment or reused [18]. Due to this reason, more severe limits are applied to nitrogen (N) and phosphorus (P) concentrations in the effluent water [14]. While asking for a more stringent concentration of contaminants, the EU directive also mandates the monitoring and reducing greenhouse gas (GHG) emissions from WWTPs. Many of the facilities currently operating will have to face, within the next 10 years, a technological revamping as water reuse will become, almost worldwide, a fundamental requirement with low nutrient concentrations in the effluent [19,20]. Moreover, the process has to be sustainable by limiting GHG emissions and many emerging contaminants, such as microplastics [21]. Regarding water reuse, membrane bioreactors (MBRs) have been established as a robust technology for producing high-quality effluent suitable for reuse [22–24]. MBRs combine biological treatment processes with membrane filtration, offering compact and efficient contaminants removal from wastewater. In the current era, the PHA production process cannot further neglect the environmental impact of the solutions adopted. A first attempt to evaluate the GHG emission was recently carried out on an aerobic/anoxic enrichment process [25]. The system performance was studied in view of external C/N ratio variations, mainly focusing on the environmental impact of the nitrous oxide (N₂O) emission by separate reactors. The effluent water quality was only partially addressed in view of studying the complete nitrogen removal process without tertiary treatment units, which lacks a comprehensive view, including the removal of contaminants. Based on the circular bio-economy approach, if the PHA production has to be implemented within the Water Resource Recovery Facility (WRRF), factors such as contaminants removal and GHG emission should not be abandoned. Especially regarding VFA production, the main stream and side stream are strongly interconnected and require a plant-wide approach [26–29]. Changes in the operating conditions of one treatment unit within the overall treatment train can significantly affect both downstream and upstream performance.

To the best of the authors' knowledge, no studies have yet explored or established constitutive relationships among treatment lines and units. This study explores the interconnection of the main stream and side stream (PHA line) operating parameters in view of deriving a preliminary empirical constitutive relationship to predict fermented WAS features. The long-term consistency of the WAS-based PHA production process at a pilot scale was assessed. An external MBR was applied to evaluate the efficiency of producing high-quality effluent. Indeed, the system was comprehensively monitored to remove contaminants, GHG emissions and PHA production in view of future applications of this technology. WAS fermentation was carried out to produce VFA, used as a carbon source in an ADF enrichment process. The enrichment reactor was coupled with an MBR to produce a high-quality effluent potentially eligible for reuse. The system performance in contaminant removal was compared to the limits imposed by legislation. In particular, effluent COD concentration was compared to the BOD concentration discharge limits imposed by 2020/741/EU (10 mg/L,) while N and P concentrations were compared to the limits for water reuse imposed by the Italian national decree 39/2023, 15 mg/L for N and 2 mg/L for P for WWTP serving less than 100,000p.e.. Direct N₂O emissions were measured during the experimental period to assess the GHG emissions. PHA accumulation was carried out in a pilot-scale reactor to determine the PHA production yield.

2. Materials and methods

2.1. Pilot plant layout

The experimental activities were carried out at the WRRF of Palermo University [30]. The PHA production line is fed by the WAS produced by the mainstream wastewater treatment pilot plant, as described in Fig. 1. Overall, the PHA line is composed of three units: i) a fermenter equipped with an MBR to produce and recover VFA; ii) a selection sequencing batch reactor (S-SBR) equipped with an MBR aimed to enrich the PHA producers within the mixed microbial cultures by applying ADF; iii) an accumulation SBR (A-SBR) aimed to accumulate PHA [31]. The experiments were carried out over an experimental period of 206 days, where three periods were identified based on the different fermented WAS liquid C/N composition: period I (days 1–70) 9 g COD/g N, period II (days 71–130) 4.5 g COD/g N, period III (days 131–206) 4 g COD/g N. These periods were identified according to the varying features of the WAS fermentation liquid described below. The C/N variation studied in this study is not related to external chemical dosage. The different features are related to the seasonality of the influent wastewater inside the Palermo University campus, which is related to preparing the meals at the canteen and the dormitory crowding.

2.2. VFA production

WAS acidogenic fermentation was carried out in the fermenter reactor (200 L working volume) for 5 days, with mixed liquor total and volatile suspended solids (MLSS and MLVSS, respectively) concentration of 3.03 ± 0.41 g/L and 1.75 ± 0.51 g/L, respectively. The temperature was monitored but not controlled, accounting for 21 ± 4 °C. No pre-treatments were performed on the WAS before the fermentation. At the end of the fermentation, the mixture was filtered by the MBR unit (working volume 48 L) equipped with a hollow fiber membrane.

The fermenter was fed only with the WAS collected from the main wastewater treatment line of the WRRF. During the experimental activities related to this study, the main wastewater treatment line adopted two different configurations aimed at COD and N removal. During the first 97 days of operation of the fermenter, the main wastewater treatment line adopted an Integrated Fixed Film Activated Sludge (IFAS) – MBR configuration with intermittent aeration (IA), defined as IFAS-MBR-IA [32]. From day 98 until the end of the experimental activities, the plant kept the IFAS- IA configuration with two parallel lines, an MBR and a settler-UF line [33]. The return sludge collected from the MBR and the settler-UF line returned to the same IFAS-IA biological reactor. This configuration was defined as IFAS-MBR-UF-IA. The features of the suspended sludge adopted for the fermentation are reported in Table 1.

2.3. Biomass enrichment and PHA accumulation

Fermented WAS liquid was used to feed the S-SBR and A-SBR. The S-SBR (working volume of 50 L) was run following the ADF enrichment. The cycle lasted 12 h and adopted a volume exchange ratio of 25 %. The cycle was composed of feeding, aerobic reaction and effluent discharge through MBR filtration. An HRT of 2 days was adopted through the experimental activities while the SRT was maintained by withdrawing sludge to feed the A-SBR. A feast-to-famine ratio of 0.22 was kept in the reactor. Dissolved oxygen (DO) and temperature (T) were continuously monitored using a probe (WTW FDO 925) installed inside the reactor. The operational conditions for the S-SBR are reported in Table 2.

The PHA accumulation was run in fed-batch mode with a 50 L reactor (working volume of 10 L). Briefly, the mixed liquor withdrawn from the S-SBR was left to settle to remove the supernatant. The biomass was then left aerated overnight before starting the accumulation. The accumulation was carried out employing homemade software installed in a Raspberry Pi4 Model B. The software uses the DO as the only parameter to run the accumulation, as previously described in the

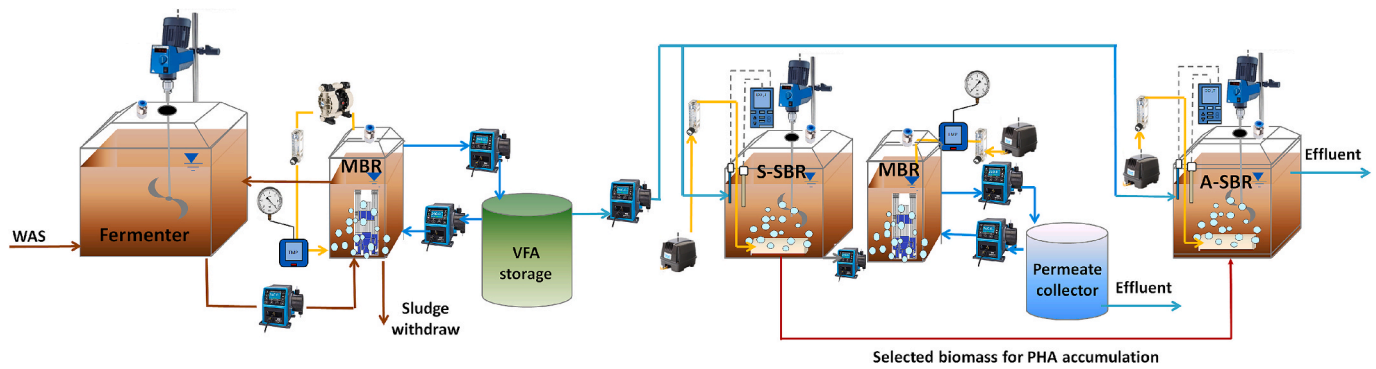


Fig. 1. Layout of the adopted configuration.

Table 1

Features of the waste activated sludge adopted for the fermentation process. F/M and SRT refer to the conditions applied in the main wastewater treatment line from which the sludge was withdrawn.

| Days | EPS | | SMP | | TCOD | SCOD | NH ₄ ⁺ -N | PO ₄ ³⁻ -P | F/M | SRT |
|------|----------|---------------|----------|---------------|------|-------|---------------------------------|----------------------------------|--------------------|------|
| | Protein | Carbohydrates | Protein | Carbohydrates | | | | | | |
| | mg/g VSS | mg/g VSS | mg/g VSS | mg/g VSS | mg/L | mg/L | mg N/L | mg P/L | kg BOD / kg SS day | days |
| 7 | 150.9 | 19.6 | 14.9 | 2.4 | 4338 | 134.8 | 18.1 | 3.8 | 0.2 | 6.5 |
| 14 | 106.4 | 10.4 | 20.3 | – | 4169 | 166.1 | 21.4 | 2.7 | 0.2 | 7.1 |
| 21 | 441.9 | 37.3 | 15.3 | – | 3821 | 189.4 | 25.6 | 4.3 | 0.2 | 8.0 |
| 27 | 315.7 | 29.4 | 1.1 | 0.6 | 5629 | 128.0 | 20.2 | 3.8 | 0.2 | 6.7 |
| 34 | 454.3 | 46.6 | 26.3 | 3.9 | 4524 | 110.2 | 15.6 | 4.6 | 0.2 | 6.3 |
| 43 | 586.8 | 34.2 | 34.0 | 3.5 | 5123 | 171.2 | 14.4 | 5.0 | 0.3 | 6.8 |
| 56 | 409.2 | 14.8 | 80.6 | 13.8 | 3529 | 25.4 | 8.5 | 2.7 | 0.4 | 3.7 |
| 62 | 411.8 | 14.9 | 81.2 | 13.8 | 4107 | 76.0 | 7.7 | 5.0 | 0.2 | 3.5 |
| 69 | 226.7 | 9.8 | 21.8 | 4.3 | 3745 | 53.0 | 32.5 | 6.5 | 0.2 | 2.8 |
| 76 | 345.7 | 12.9 | 102.9 | 11.6 | 3962 | 83.8 | 14.5 | 8.1 | 0.4 | 3.0 |
| 83 | 473.8 | 33.6 | 55.5 | 34.3 | 5128 | 33.0 | 35.1 | 3.8 | 0.8 | 2.8 |
| 90 | 133.0 | 8.1 | 55.5 | 3.6 | 5237 | 150.7 | 53.1 | 5.0 | 0.2 | 2.7 |
| 97 | 146.9 | 5.3 | 87.3 | 10.9 | 4617 | 71.9 | 94.1 | 5.1 | 0.2 | 3.2 |
| 104 | 155.3 | 9.9 | 116.8 | 5.0 | 2915 | 75.9 | 88.4 | 6.1 | 0.2 | 5.5 |
| 113 | 120.7 | 4.1 | 54.2 | 2.5 | 3426 | 51.7 | 65.2 | 4.2 | 0.3 | 7.3 |
| 120 | 153.0 | 14.9 | 106.8 | 11.5 | 5891 | 124.4 | 44.9 | 6.1 | 0.2 | 6.8 |
| 127 | 140.6 | 1.8 | 154.3 | 10.1 | 4892 | 126.5 | 35.1 | 7.1 | 0.3 | 12.9 |
| 139 | 122.7 | 15.9 | 95.8 | 7.3 | 4725 | 228.5 | 32.5 | 4.6 | 0.2 | 9.0 |
| 146 | 130.2 | 10.4 | 18.2 | 9.7 | 4812 | 75.3 | 24.2 | 8.2 | 0.2 | 7.1 |
| 153 | 144.1 | 7.3 | 26.9 | 4.2 | 5103 | 82.5 | 11.2 | 4.0 | 0.2 | 7.0 |
| 160 | 75.0 | 3.8 | 22.2 | – | 5236 | 114.8 | 26.4 | 5.9 | 0.2 | 7.1 |
| 166 | 136.3 | 8.5 | 18.5 | 0.8 | 5348 | 89.5 | 16.4 | 5.6 | 0.4 | 6.5 |
| 174 | 143.4 | 5.2 | 21.5 | 3.7 | 4759 | 120.4 | 28.3 | 1.6 | 0.4 | 7.6 |
| 182 | 141.6 | 7.4 | 10.8 | 1.5 | 4120 | 84.2 | 59.4 | 1.5 | 0.2 | 8.0 |
| 189 | 118.0 | 9.4 | 5.4 | 2.0 | 4289 | 92.5 | 71.2 | 7.3 | 0.1 | 9.2 |
| 194 | 139.8 | 6.8 | 10.2 | 0.7 | 5206 | 120.2 | 96.2 | 6.2 | 0.2 | 9.2 |

Table 2

Operational conditions of the selection SBR.

| Period | Influent C/N | F/M | SRT | vOLR | vNLR | MLSS | MLVSS | T |
|--------|--------------|----------------|-------------|--------------------------|------------------------|-------------|-------------|------------|
| | g COD/g N | kgBOD/kgSS day | days | kgCOD/m ³ day | kgN/m ³ day | g/L | g/L | °C |
| I | 9 | 0.10 ± 0.03 | 61.8 ± 36.3 | 0.15 ± 0.03 | 0.02 ± 0.01 | 1.55 ± 0.31 | 1.17 ± 0.22 | 17.4 ± 1.3 |
| II | 4 | 0.21 ± 0.05 | 49.1 ± 14.7 | 0.23 ± 0.06 | 0.06 ± 0.02 | 1.12 ± 0.22 | 0.84 ± 0.16 | 20.1 ± 3.6 |
| III | 4.5 | 0.23 ± 0.07 | 66.2 ± 29.1 | 0.26 ± 0.07 | 0.06 ± 0.02 | 1.15 ± 0.18 | 0.86 ± 0.13 | 26.4 ± 2.3 |

F/M: food to microorganisms ratio; vOLR: volumetric organic loading rate; vNLR: volumetric nitrogen loading rate; SS: suspended solids.

literature [31]. The Raspberry was connected to a multimeter (WTW Multi 3630 IDS) to read the DO concentration inside the reactor and to a programmable logic controller (Crouzet M3 XD26) to control the utilities [30]. 1 L of fermented WAS liquid was fed per pulse. Once the reactor reached its maximum volume, the biomass was left to settle for 20 min, after which the supernatant was removed. Three accumulations were carried out and each batch was stopped once the PHA concentration did not vary significantly. The accumulation started after 35 days of S-SBR operation once the pseudo-steady-state was achieved.

2.4. Analytical methods

The fermentation process was sampled for soluble chemical oxygen demand (SCOD), VFA, ammonium (NH₄⁺-N), and phosphate (PO₄³⁻-P) twice a week. Further, for the biomass characterization, the parameters of total chemical oxygen demand (TCOD), extracellular polymeric substances (EPS), soluble microbial products (SMP), MLSS and MLVSS were analysed at the start and the end of the fermentation process.

S-SBR was monitored twice weekly by sampling the influent, mixed liquor inside the reactor, and effluent. SCOD, NH₄⁺-N, PO₄³⁻-P, nitrate

(NO₃-N) and nitrite (NO₂-N) were monitored in the influent and effluent. TSS and VSS were monitored in the mixed liquor and the effluent. EPS, SMP and dissolved N₂O were monitored in the mixed liquor. Gaseous N₂O samples were collected by directly sampling the reactor's headspace volume (40 L), which was kept closed [34]. The air flow rate was measured using an anemometer and accounted for 14.1 ± 2.8 L h⁻¹.

The A-SBR was monitored by collecting mixed liquor samples to determine the MLSS, MLVSS, and PHA concentration as the sum of polyhydroxy butyrate (PHB) and polyhydroxy valerate (PHV) [11].

SCOD, TCOD, NH₄⁺-N, PO₄³⁻-P, NO₃⁻-N, NO₂⁻-N, EPS, SMP, MLSS and MLVSS were analysed using standard methods [35]. VFA concentrations were measured, as reported by using a Gas Chromatograph (GC) (Agilent 8860) equipped with a flame ionization detector (FID) and a DB FFAA column (30 m x 0.25 mm x 0.25 μm) [36]. PHA concentration was measured using a GC with an FID and a Restek Stabilwax column (30 m x 0.53 mm x 1.00 μm) [37]. Gaseous and dissolved N₂O concentrations were measured using a GC with an electron capture detector and a Porapak-Q 80/100 mesh column (6 ft x 1/8 in x 2.1 mm).

PHA content is expressed as the weight % on VSS weight. PHA productivity was calculated as the gram of PHA produced over time (g PHA/day), while the PHA yield per given VFA is expressed as g COD_{PHA}/g COD_{VFA}. The N₂O emission factor was calculated as reported by Tsuneda et al. (2005) [38]. The VFA produced were correlated with the WAS features reported in Table 1 according to Equation (1):

Correlation Index (x,y):

$$\frac{\sum(x - \bar{x})(y - \bar{y})}{\sqrt{\sum(x - \bar{x})^2 \sum(y - \bar{y})^2}} \quad (1)$$

3. Results and discussion

3.1. WAS acidogenic fermentation

WAS fermentation was carried out to produce the VFA stream used for the S-SBR and A-SBR. The fermentation was carried out for a total of 194 days, and each fermentation batch was carried out for 5 days plus 2 additional days for sludge filtration. The details of the WAS fermented

liquid are reported in Table 3. The SCOD concentration varies between 200–600 mg/L, reaching the highest value on day 139 at 669.4 mg/L. The NH₄⁺ production followed an increasing trend through the experimental period, reaching a peak of 146.3 mg N/L on day 194. The obtained ammonium concentration was mainly stable above 100 mg N/L between days 83 to 160, which resulted in a progressive C/N reduction. Three different periods can indeed be identified, based on the fermented liquid features: Period I, having a C/N of 9 g COD/g N between days 7–69, Period II between days 70–127 with C/N of 4 g COD/g N and finally Period III, from days 128 to the end of the experimental period with a C/N of 4.5 g COD/g N. Except for the first two weeks, PO₄³⁻-P concentration was ≥ 10 mg P/L, with three peaks around days 21–62 of 31.5, 31.7 and 62 mg P/L. The VFA/SCOD ratio remained relatively constant throughout the experiments, averaging 0.34 ± 0.04. This result indicates that the layouts adopted in the main wastewater treatment line did not affect the VFA production rate and that no higher value could be achieved without appropriate sludge treatment [39]. However, different VFAs were obtained during the operation, meaning that different parameters affect the VFA yield and VFA distribution. Future studies may also investigate this aspect related to the process. COD solubilization accounted for an average of 6.6 ± 2.9 %, with two peaks of 12.2 % and 12.4 % for days 104 and 116, respectively. Since no pre-treatments and no pH or T control have been carried out, the results align with what could be expected from WAS' fermentation. However, low COD solubilization indicates that higher VFA production could be achieved with optimal conditions [40].

Despite the VFA/SCOD ratio remaining relatively constant throughout the process, the individual VFA concentrations showed significant variation. The main ones produced were acetic, propionic and butyric acid, with an average of 91.2 ± 44.0 mg COD/L, 21.8 ± 12.3 mg COD/L and 13.7 ± 12.3 mg COD/L, respectively. Acetic acid accounted for 68.5 ± 15.1 % of the total VFA produced, while propionic and butyric acid accounted for 18.4 ± 10.9 % and 10.7 ± 7.6 %, respectively. The remaining 2.4 % was divided into isobutyric acid (0.8 ± 12.3 %) and valeric acid (1.6 ± 3.0 %). A correlation index was calculated to determine which aspect influenced the individual VFA production, as shown in Fig. 2. The correlation was conducted to assess if the F/M and/or SRT applied in the main wastewater treatment line can affect the

Table 3
Features of the WAS fermented liquid used as the substrate to produce PHA.

| Days | SCOD | NH ₄ ⁺ -N | PO ₄ ³⁻ -P | VFA | Acetic Acid | Propionic Acid | Butyric Acid | VFA/SCOD | C/N |
|------|-------|---------------------------------|----------------------------------|----------|-------------|----------------|--------------|----------|-----------|
| | mg/L | mg N/L | mg P/L | mg COD/L | mg COD/L | mg COD/L | mg COD/L | | g COD/g N |
| 7 | 224.2 | 21.5 | 4.1 | 77.8 | 48.4 | 18.4 | 4.9 | 0.35 | 10.5 |
| 14 | 258.5 | 34.6 | 6.5 | 86.7 | 44.1 | 29.6 | 8.5 | 0.34 | 7.5 |
| 21 | 317.0 | 42.5 | 31.5 | 118.7 | 50.1 | 46.8 | 15.6 | 0.37 | 7.5 |
| 27 | 180.2 | 38.7 | 19.6 | 62.9 | 23.3 | 18.4 | 12.7 | 0.35 | 4.7 |
| 34 | 263.7 | 36.5 | 24.5 | 72.7 | 29.1 | 28.5 | 6.2 | 0.28 | 7.2 |
| 43 | 261.4 | 40.2 | 31.7 | 200.4 | 163.6 | 36.8 | – | 0.36 | 6.5 |
| 56 | 355.5 | 23.0 | 16.0 | 115.5 | 85.2 | 26.0 | 4.2 | 0.32 | 15.4 |
| 62 | 283.8 | 21.6 | 30.2 | 85.0 | 54.6 | 18.6 | 6.8 | 0.30 | 13.1 |
| 69 | 239.4 | 25.9 | 25.7 | 91.4 | 49.4 | 33.9 | 8.0 | 0.38 | 9.3 |
| 76 | 257.7 | 47.6 | 18.8 | 90.9 | 68.8 | 16.6 | 5.5 | 0.35 | 5.4 |
| 83 | 330.8 | 135.9 | 23.6 | 92.7 | 82.4 | 10.3 | 0.0 | 0.28 | 2.4 |
| 90 | 345.9 | 93.9 | 18.6 | 133.2 | 100.5 | 19.5 | 9.3 | 0.38 | 3.7 |
| 97 | 315.1 | 105.4 | 15.2 | 96.2 | 80.7 | 11.9 | 3.6 | 0.31 | 3.0 |
| 104 | 430.4 | 124.3 | 16.5 | 146.3 | 117.3 | 4.4 | 24.6 | 0.34 | 3.5 |
| 113 | 476.7 | 135.2 | 12.5 | 117.1 | 82.9 | 2.9 | 21.6 | 0.25 | 3.5 |
| 120 | 578.6 | 176.1 | 16.8 | 187.8 | 117.0 | 12.7 | 58.1 | 0.32 | 3.3 |
| 127 | 531.0 | 99.4 | 15.0 | 166.6 | 142.8 | 23.8 | – | 0.31 | 5.3 |
| 139 | 669.4 | 136.7 | 12.4 | 238.4 | 179.5 | 43.4 | 15.5 | 0.36 | 4.9 |
| 146 | 369.0 | 130.1 | 13.0 | 111.6 | 69.9 | 18.6 | 23.1 | 0.30 | 2.8 |
| 153 | 514.3 | 121.4 | 9.9 | 157.5 | 90.0 | 41.7 | 25.8 | 0.31 | 4.2 |
| 160 | 354.3 | 111.3 | 12.7 | 118.2 | 69.9 | 26.7 | 21.6 | 0.33 | 3.2 |
| 166 | 201.1 | 38.3 | 8.5 | 66.5 | 50.6 | 3.4 | 12.5 | 0.33 | 5.3 |
| 174 | 478.6 | 70.3 | 15.6 | 200.1 | 142.0 | 33.9 | 24.3 | 0.42 | 6.8 |
| 182 | 446.1 | 78.6 | 18.3 | 164.4 | 147.6 | 8.7 | 8.1 | 0.37 | 5.7 |
| 189 | 468.4 | 146.3 | 16.2 | 163.9 | 138.5 | 12.7 | 12.7 | 0.35 | 3.2 |
| 194 | 516.9 | 148.2 | 15.9 | 183.8 | 142.0 | 18.6 | 23.2 | 0.36 | 3.5 |

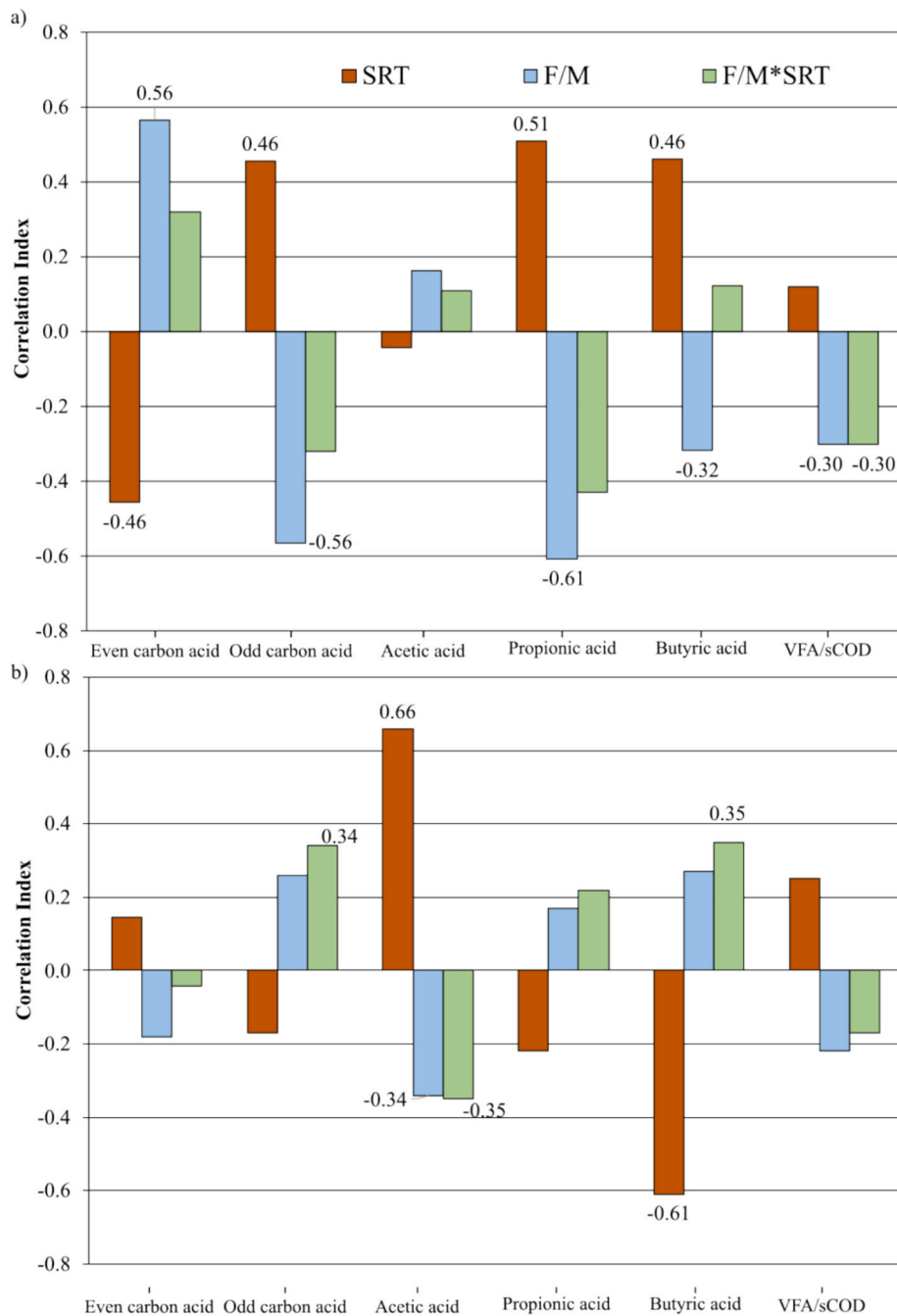


Fig. 2. VFA to main wastewater treatment line operational parameters correlation. The WAS used to produce the VFA was withdrawn from IFAS-MBR-IA (a) and IFAS-MBR-UF-IA (b) configurations.

production of different VFA.

F/M showed the strongest correlation for IFAS-MBR-IA, as shown in Fig. 2a. Considering that the F/M of the sludge from IFAS-MBR-IA (day 1 to day 97) was between 0.15 and 0.76 kg BOD/kg SS day, results show that high F/M favours an even number of carbon VFA production, mainly as acetic acid. Lower F/M, which is close to 0.2, will favour odd carbon VFA production, especially propionic acid. An opposite trend was registered for SRT since an SRT of around 3 days moderately correlates with even carbon acetic acid production. However, higher SRT around 6–8 days favours propionic and butyric production with a moderate positive correlation, namely 0.51 and 0.46, respectively. The same trend was reported for IFAS-MBR-UF-IA (days 98–194) despite the decreased correlation observed, as shown in Fig. 2b. In this scenario, SRT showed the highest correlation, positive for acetic acid (0.66) and

negative for butyric acid (–0.61). Lower correlations were observed for F/M and F/M*SRT, mainly for acetic (–0.34 and –0.35, respectively) and butyric acid, namely 0.27 and 0.35, respectively.

During the IFAS-MBR-UF-IA operation, the SRT applied was between 5.5 and 12.9 days, considerably higher than the one adopted in IFAS-MBR-IA, around 2.7 – 8.0 days. Considering the different SRT values, it can be said that butyric acid production was enhanced when an SRT of around 6–8 days was applied. Moreover, butyric acid was the only fatty acid showing a coherent correlation between the two layouts since acetic and propionic acid did not follow the trend in the relative layout. This result contrasts with previous literature, which reported an increased propionic acid production at higher SRT despite a different layout applied in other studies [41–43]. Moreover, no strong correlation was found between SRT and the VFA/SCOD ratio, contrary to what is

reported in the literature [36,44]. Still, it has to be highlighted that different configurations were applied in the wastewater treatment line, different conditions were applied during the fermentation, and that the SRT reported in Table 1 and used as a reference for the correlation calculation referred to the suspended biomass in the main wastewater treatment line layouts, neglecting the effect of the attached one. Since the layouts were complex, the results showed they could hardly be compared to the few ones reported in the literature. The correlations reported in this paragraph are preliminary investigations carried out to evaluate the possibility of operating the main wastewater treatment line in view of optimizing the VFA production. If the SRT correlation is proved by future studies adopting different configurations, it could provide valuable insights towards enhanced butyric acid production.

3.2. Contaminant removal and GHG emission

COD, ammonia and phosphate removal efficiencies are reported in Fig. 3. Overall, SCOD removal efficiency was always above 90 %, except for the first analysis on day 14 (84.7 %). Half of the experiment's SCOD effluent concentration was within the BOD discharge limits (< 10 mg/L), while during days 106–143 the sharp increase in influent COD concentration resulted in an average 17.8 mg/L effluent COD concentration. Traina et al. [22] evaluated the MBR efficiency in an enrichment reactor fed with agro-food industrial wastewater. Despite the higher COD concentration adopted, compared to this study (on average 4500 mg/L and 410 mg/L, respectively), COD effluent concentration was within the legislation limits. However, around 80 % of the COD was constituted by VFA, indicating a higher concentration of readily biodegradable substances than the one reported in this study, which eases COD removal since a higher VFA concentration favors PHA production over biomass growth [45].

Ammonia removal efficiency was always above 75 % during the experiments, achieving an average of 89.9 ± 6.7 %. The system did not show the effect of the C/N shifts over the periods since ammonia removal accounted for 89.4 ± 6.9 %, 90.1 ± 8.5 % and 90.0 ± 5.5 %, for periods I, II and III, respectively. However, only during period I the TN effluent concentration was within the legislation limits (6.7 ± 3.9 mg N/L), while it exceeded the limit during period II (20.9 ± 10.4 mg N/L) and period III (20.3 ± 5.3 mg N/L). The difference was mainly due to the ammonia concentration increase during periods II and III compared to period I since the nitrification efficiency was also not affected by the C/N shifts, achieving 88.4 ± 8.1 %, 91.3 ± 8.5 % and 90.3 ± 5.5 %, respectively for periods I, II and III. The system maintained a robust ammonia removal efficiency over a 200-day experimental period, indicating a stable biological activity. The C/N ratio shifts did not affect the system performance mainly because of the slow-rated shifts, which did not interfere with the occurring nitrification [46]. Neither the phosphate removal efficiency was affected by the C/N ratios, achieving, on average 49.9 ± 14.2 %. Despite the stable operation, phosphate effluent concentration was always above the legislation limits except for days 14 and 91. This result highlights the inability of the ADF in phosphorus removal, suggesting that further processes must be considered to respect the legislation limits [47].

Compared to previous studies by the authors [43,48], the MBR resulted in lower effluent SCOD, $\text{NH}_4^+\text{-N}$ and $\text{PO}_4^{3-}\text{-P}$ concentrations. Despite the promising results, in view of integrating the PHA production process within the WWTP operation, contaminant removal has to be improved, primarily by referring to the more stringent legislation limits that are going to be applied. Focusing on achieving a stable PHA production while producing water eligible for reuse, future studies could focus on further treating the PHA process' effluent or applying different strategies to improve the effluent water quality.

The monitored N_2O concentrations and emission factors are reported in Fig. 4a. The total N_2O concentration never exceeded 0.52 mg $\text{N}_2\text{O-N/L}$ and the influent N emission factor never exceeded 0.54 %. Only the first period was characterized by a higher emission factor (on average

0.39 ± 0.12 %) compared to the following periods, which achieved 0.18 ± 0.09 % and 0.14 ± 0.06 %, respectively. This result is also shown by the effluent N fractions reported in Fig. 4b. The slightly higher N_2O emission in period I may also be related to an initial acclimatation phase, as shown by the higher amount of metabolic effluent N in the first period (22.7 ± 7.1 %) compared to the following, 13.8 ± 5.0 % and 15.5 ± 3.3 %, respectively. However, the C/N ratios achieved in this work are higher than the low values considered as the threshold for triggering N_2O emission ($\text{C/N} < 2$ — 3.5 g COD/g N), which resulted in a consistently low N_2O emission over the 200 days of experiments [49,50]. The emission factor achieved in periods II and III was slightly higher than that reported in the literature for MBR configurations [51,52]. However, these studies reported an initial TN concentration of, maximum, 60 mg/L, while during periods II and III, the $\text{NH}_4^+\text{-N}$ concentration accounted for 99.4 ± 36.9 mg N/L and 95.5 ± 25.4 mg N/L, respectively. The substrate adopted may also affect the low N_2O emission achieved in this study. As demonstrated in the literature, acetic acid significantly lowers N_2O emissions compared to methanol or glucose [53,54]. The higher portion of acetic acid in the feeding substrate during periods II and III may relate to the lower emission factor measured during those periods compared to the first [55]. The N_2O emissions highlight the system's stability and high performance over a long-term experimental period, pointing out that GHG emissions generally cannot be negligible in the PHA production process.

3.3. PHA production

The amount of PHA obtained at the end of the three accumulations did not vary significantly, achieving 38.3, 35.4 and 33.8 % w/w, respectively, as shown in Fig. 5. In the first accumulation batch the highest concentration of PHV was completed (5 % w/w), followed by 1 % w/w and 2.2 % w/w in the following batch. The slight difference in PHB and PHV concentration is related to the odd carbon VFA concentration (propionic and valeric acid), which accounted for 28 % of the total VFA concentration in the first accumulation while only for 10.5 % and 14.1 % for the following, respectively [56]. A slight decrease in the PHA productivity peak was registered during the accumulation, respectively 0.65, 0.47 and 0.25 g PHA/day (65, 47 and 25 g PHA/ m^3 day) for the three batches. Despite the higher amount of VFA for batch 2 and 3 (on average 155.1 and 145.8 mg COD/L, respectively) compared to the first batch (on average 106.9 mg COD/L), the amount of PHA produced is slightly lower. This result is related to the lower C/N ratio registered during batch 2 and 3, which negatively affected the PHA production while favouring biomass growth [57,58]. Moreover, the low C/N ratio effect is also shown by the PHA storage yield, which decreased from 0.42 g $\text{COD}_{\text{PHA}}/\text{g COD}_{\text{VFA}}$ in batch 1 to 0.32 and 0.28 g $\text{COD}_{\text{PHA}}/\text{g COD}_{\text{VFA}}$ in batch 2 and 3, respectively [59].

As reported in Table 4, the amount of PHA produced in this work is slightly lower than that of pilot-scale literature studies adopting ADF-enriched sludge. Still, several process parameters must be considered when discussing the amount of PHA produced. The storage yield obtained in this study is comparable to those reported by Morgan-Sagastume et al. (2014) [60] and Valentino et al. (2019a) [12]. Moreover, it is only slightly lower compared to other WAS-based feedstocks, such as the one reported by Lorini et al. (2022) [9], Valentino et al. (2019b) [61] and Moretto et al. (2020) [13]. Despite the lower amount of PHA produced, the obtained storage yields are proof of the process efficiency, highlighting that it is possible to enrich strong PHA producers' cultures by adopting WAS as feedstock. Moreover, no pre-treatment has been carried out on the WAS adopted in this study. The lower PHA production is also related to accumulation and enrichment parameters. The C/N ratio of the substrate used in this study does not correspond to the optimal range for PHA production, as discussed before [57,62]. The C/N ratio is poorly reported in the literature since it's a parameter that is usually externally modified. However, in view of introducing the PHA production process within the WWTP operation, C/

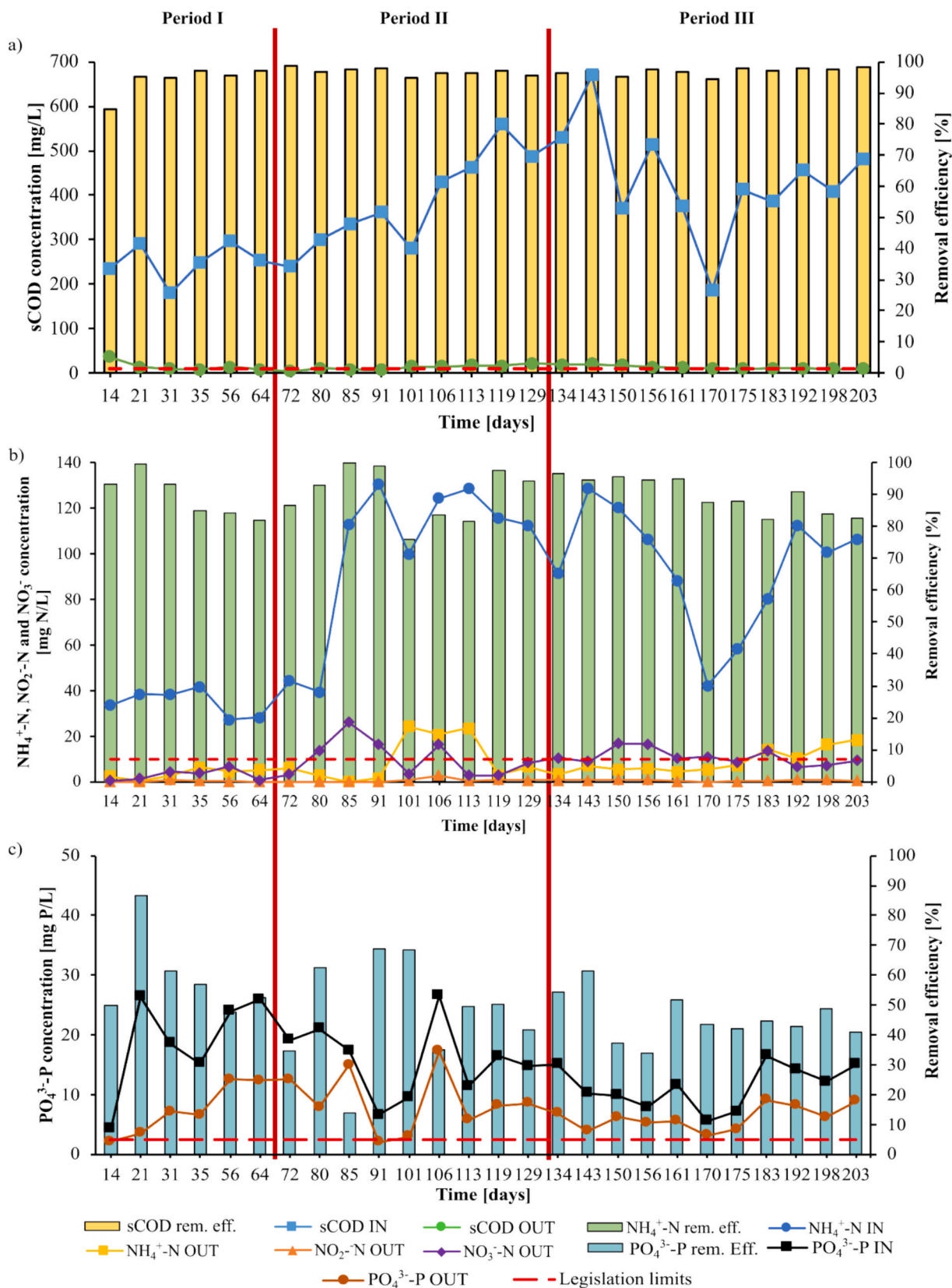


Fig. 3. Removal efficiency on SCOD (a), $\text{NH}_4^+\text{-N}$ (b) and $\text{PO}_4^{3-}\text{-P}$ (c) monitored during the enrichment process. Legislation limits refer to 2020/741/EU and Italian national decree 39/2023.

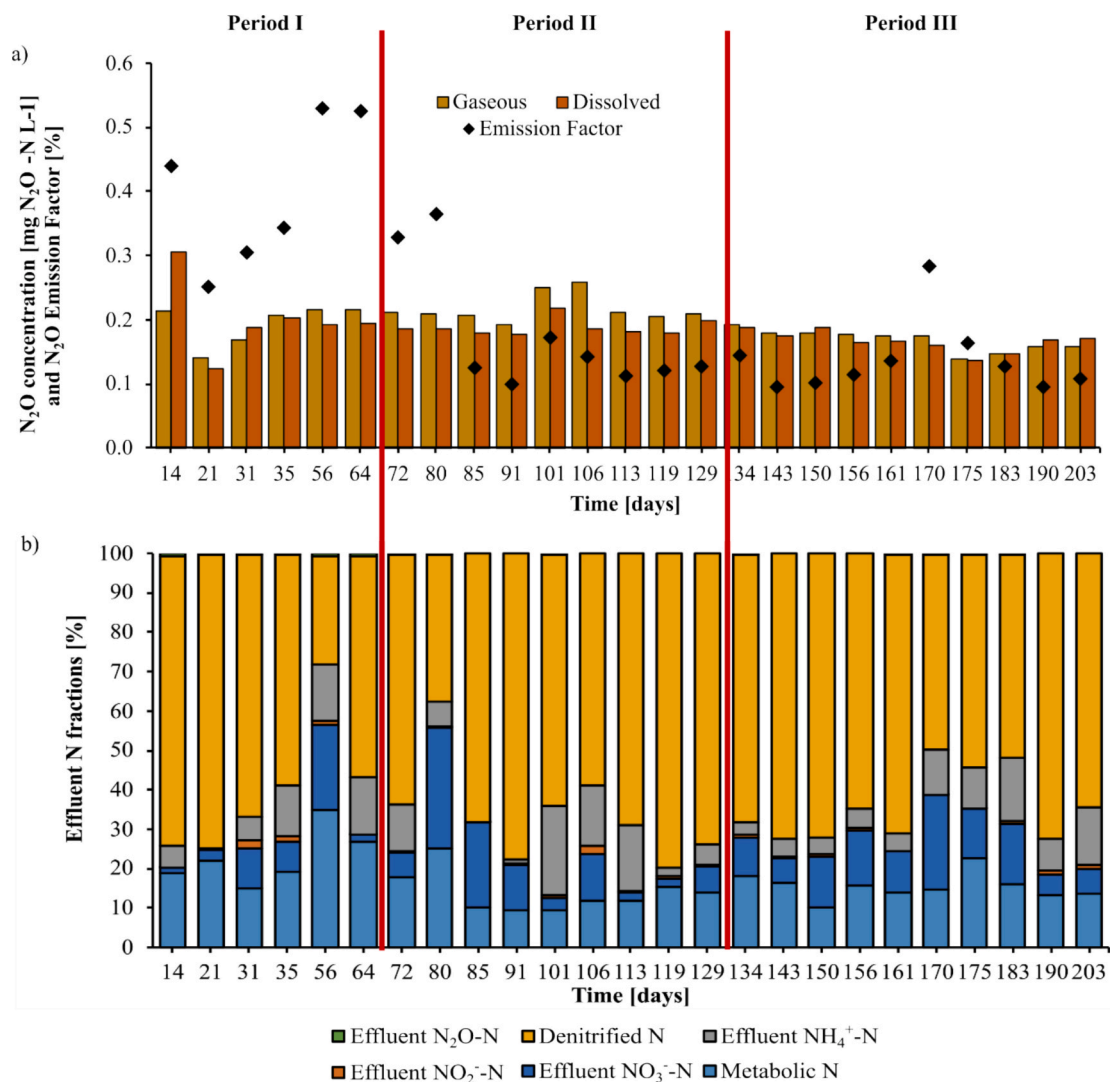


Fig. 4. Nitrous oxide emission (a) and effluent nitrogen fractions (b) monitored during the enrichment process.

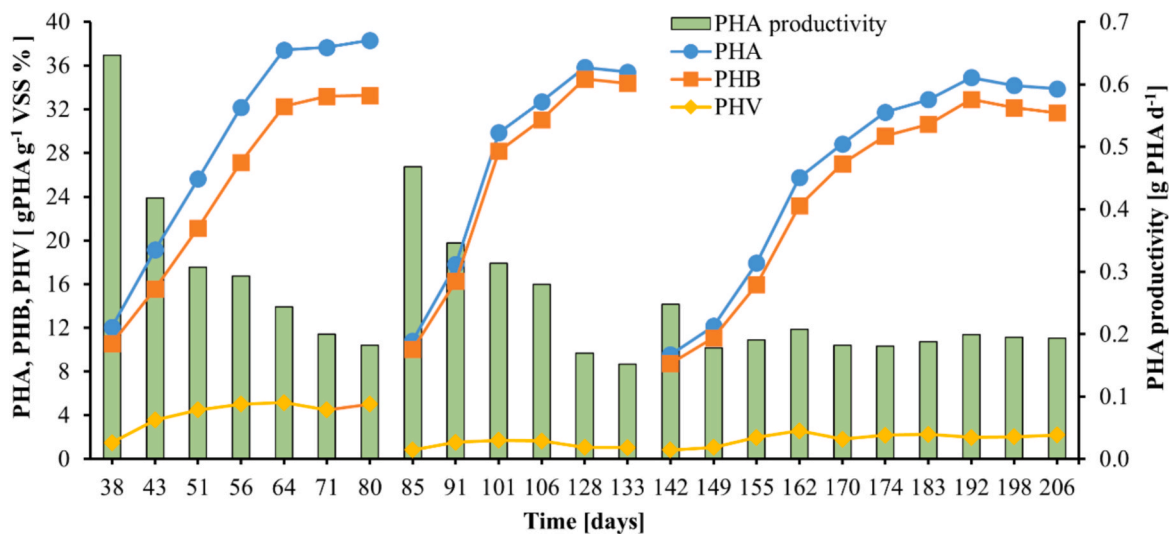


Fig. 5. PHA concentration and productivity obtained during the accumulation process.

Table 4

Average values monitored during the selection and accumulation and comparison with relevant literature studies.

| PHA | HV:HB ratio | Storage Yield | Accumulation C/N | Enrichment SRT | Enrichment vOLR | VFA source | Reference |
|--------------|-------------|--|------------------|----------------|--------------------------|---|------------------------------------|
| gPHA/g VSS % | | gCOD _{PHA} /gCOD _{VFA} | g COD/g N | day | kgCOD/m ³ day | | |
| 52 | 0.5 | 0.48 | 8.3 | 1–2 | 3 | Fermented thickened WAS and biomass enriched with fermented cheese whey permeate | Morgan-Sagastume et al., 2014 [60] |
| 18–23 | – | 0.20–0.23 | – | 1.5 | 3.08 | Synthetic substrate | Valentino et al., 2015 [67] |
| 38–49 | 0.12–0.15 | 0.33–0.44 | 33.5 | 1 | 2–3.5 | Co-fermented Organic Fraction of Municipal Solid Waste (OFMSW) and WAS | Valentino et al., 2019a [12] |
| 47–62 | 0.11 | 0.55 | 15 | – | 2.0–2.2 | WAS thermally pre-treated thermophilic fermentation | Lorini et al., 2022 [9] |
| 35–54 | 0.04–0.89 | 0.8 | – | – | – | Synthetic substrate | Mannina et al., 2019 [37] |
| 43–46 | 0.11–0.15 | 0.44–0.5 | – | 6 | 4 | Co-fermented OFMSW and sewage sludge | Valentino et al., 2019b [61] |
| 52 | 0.2–0.4 | 0.59 | – | 1 | 4.4 | Co-fermented OFMSW and WAS | Moretto et al., 2020 [13] |
| 30–50 | 0–0.4 | 0.2–0.6 | – | 0.3–29 | – | Fermented candy factory effluent, fermented primary sludge or synthetic substrate | Bengtsson et al., 2017 [68] |
| 34–38 | 0.03–0.15 | 0.28–0.42 | 4–9 | 49–66 | 0.15–0.26 | Fermented WAS | This study |

N ratio adjustment at full scale will require a large amount of chemicals unless proper feedstocks are used. Also, the enrichment's SRT and OLR adopted in this work are not optimal to favour PHA production [63–66]. However, the enrichment was carried out by fixing an MLSS concentration during the experimental period since the low COD concentration in the influent was not proper to use higher OLR and lower SRT. The results presented in this study demonstrate that, although no-pretreated WAS is not the optimal feedstock, it can still be utilized to produce a considerable amount of PHA while reducing the fermentation step carbon footprint.

4. Conclusions

This study demonstrated the feasibility of producing PHA at a pilot scale by fermenting WAS without pre-treatments or further process control. It also provided preliminary findings for deriving constitutive relationships to support the design, operation and management of WRRF for future implementation. An MBR was coupled with the enrichment reactor to assess the possibility of producing effluent water that is eligible for reuse. The system demonstrated good steadiness during a long-term monitoring period of over 200 days. Nitrogen removal efficiency and N₂O emissions were not influenced by the C/N ratio shifts (4–9 g COD/g N) registered, maintaining a low N₂O concentration emitted (< 0.52 mg N₂O-N/L). Also, despite the high nitrogen influent concentration for domestic wastewater sewage sludge (82.6 ± 37.4 mg N/L), N₂O only accounted for 0.21 ± 0.14 % of the influent nitrogen. High carbon and nitrogen removal efficiencies were achieved, 96.3 ± 2.6 % and 89.9 ± 6.7 %, respectively, while the low phosphorus removal (49.3 ± 14.2 %) highlights the need to apply different strategies to improve the effluent water quality. The dynamic accumulation process achieved an average of 36.3 ± 1.8 % g PHA/g VSS, with a PHV concentration up to 5 % g PHV/g VSS. The obtained storage yields, up to 0.42 g COD_{PHA}/g COD_{VFA}, highlight the process efficiency, especially in enriching a considerably strong PHA producers' culture. Future studies must focus on adopting a comprehensive/holistic approach, deepening and unveiling the interconnections among the main stream and side stream of WWTP, taking into account carbon footprint (direct and indirect), to design, operate and manage the WWTP/WRRF in a more synergic and sustainable approach.

CRedit authorship contribution statement

Antonio Mineo: Writing – original draft, Formal analysis. **Mark M. C. van Loosdrecht:** Writing – review & editing, Visualization, Conceptualization. **Giorgio Mannina:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Investigation, Conceptualization.

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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Data availability

The data that has been used is confidential.

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