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Effect of an Increased Particulate COD Load on the Aerobic Granular Sludge Process: A Full Scale Study

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Abstract: High concentrations of particulate COD (pCOD) in the influent of aerobic granular sludge (AGS) systems are often associated to small granule diameter and a large fraction of flocculent sludge. At high particulate concentrations even granule stability and process performance might be compromised. However, pilot- or full-scale studies focusing on the effect of real wastewater particulates on AGS are scarce. This study describes a 3-month period of increased particulate loading at a municipal AGS wastewater treatment plant. The pCOD concentration of the influent increased from 0.5 g COD/L to 1.3 g COD/L, by adding an untreated slaughterhouse wastewater source to the influent. Sludge concentration, waste sludge production and COD and nutrient removal performance were monitored. Furthermore, to investigate how the sludge acclimatises to a higher influent particulate content, lipase and protease hydrolytic activities were studied, as well as the microbial community composition of the sludge. The composition of the granule bed and nutrient removal efficiency did not change considerably by the increased pCOD. Interestingly, the biomass-specific hydrolytic activities of the sludge did not increase during the test period either. However, already during normal operation the aerobic granules and flocs exhibited a hydrolytic potential that exceeded the influent concentrations of proteins and lipids. Microbial community analysis also revealed a high proportion of putative hydrolysing and fermenting organisms in the sludge, both during normal operation and during the test period. The results of this study highlight the robustness of the full-scale AGS process, which can bear a substantial increase in the influent pCOD concentration during an extended period.

Keywords: aerobic granular sludge; particulate COD; full-scale wastewater treatment; nutrient removal; granule stability

1. Introduction

Aerobic granular sludge (AGS) technology is currently applied in more than 80 plants over the world treating domestic and industrial wastewater, under the tradename Nereda® (Royal HaskoningDHV, Amersfoort, The Netherlands) [1]. AGS offers various advantages in comparison to conventional activated sludge systems, including lower space and energy requirements [2]. Furthermore, the granular sludge offers a number of resource recovery options [3]. The technology is operated as a sequencing batch reactor (SBR) consisting of simultaneous anaerobic plug-flow feeding and effluent withdrawal, followed by aeration and settling [4]. The anaerobic feeding regime ensures anaerobic uptake of the readily biodegradable COD (rbCOD) by slow-growing organisms, which store this COD intracellularly and oxidise it in the subsequent aerobic phase [5]. This way, slow-growing organisms such as polyphosphate accumulating organisms (PAO) and glycogen accumulating organisms (GAO) are favoured over ordinary heterotrophic organisms (OHO), which results in smooth granule growth [6].

In addition to rbCOD, domestic wastewater and many industrial wastewaters contain a large fraction of slowly biodegradable, particulate COD (pCOD) [7–9]. This COD fraction may provide additional substrate for nutrient removal and granular sludge production, from which resources could be recovered ultimately. On the other hand, it could challenge the stability of the AGS process, due to the inability of PAO and GAO to consume more complex COD directly; pCOD needs to be extracellularly hydrolysed to rbCOD, after which it can be assimilated by the microorganisms in the sludge [10]. Hydrolysis is often regarded as rate-limiting in the degradation of particulates [11,12]. It relies on contact between the substrate and hydrolytic enzymes, which are mainly reported to be biomass-bound [13–15]. In activated sludge, the open floc structure allows good contact between sludge flocs and pCOD, rendering a continuous and homogeneous release of rbCOD throughout the floc [16]. In contrast, the dense biofilm structure of AGS limits the accessibility of pCOD to hydrolytic enzymes in the sludge. Such a mass-transfer limitation has induced irregular granule growth and deterioration of granule stability in lab-scale reactors fed with high influent particulates [17–19]. This is attributed to hydrolysis of pCOD into rbCOD during the aeration phase on the granule surface, leading to substrate concentration gradients and aerobic rbCOD consumption by fast-growing OHO [20]. Nevertheless, AGS has been cultivated successfully on domestic wastewater [21,22], high-strength industrial wastewaters [23–25] and faecal sludge-containing wastewater [26]. Even though the influent to these reactors contained a high concentration of particulates, no filamentous outgrowth was observed. However, these studies reported much higher effluent suspended solids than generally achieved in the effluent of full-scale Nereda® reactors or found a flocculent sludge fraction accompanying the AGS. Overall, the sludge bed consisted of smaller granules compared to lab reactors fed with synthetic media containing solely rbCOD in all reported lab- and pilot-scale experiments. Layer et al. [27] hypothesised that the flocculent sludge fraction benefitted the AGS process, by capturing influent pCOD and avoiding its aerobic degradation at the granule surface. Haaksman et al. [28], similarly, proposed that rbCOD leakage to the aerobic phase mainly results in flocculent sludge growth, which has to be discharged regularly in order to maintain a stable granule bed. These studies suggest that AGS can adapt well to high concentrations of pCOD in the wastewater when suitable operating conditions are employed. Moreover, they suggest that granule stability is supported by an equilibrium between different biomass fractions within the AGS reactor. However, reports on full-scale AGS treating particulate-rich wastewater are scarce and they do not focus on the removal of pCOD. Thus, the effect of pCOD on granule stability and sludge bed composition at full-scale remains elusive. Furthermore, little is known about the enzymatic activity and microbial community of full-scale AGS cultivated on high influent pCOD concentrations.

To study the effect of particulates on AGS stability, a full-scale study was conducted at wastewater treatment plant (WWTP) Epe, the Netherlands, equipped with three 4500 m³ reactors. The Nereda® reactors were operated with an increased influent COD load during a 4-month period (4 March–11 July 2019). The extra influent COD originated from the temporary discharge of untreated wastewater of a nearby slaughterhouse and consisted mainly of pCOD (71–86% of the total COD). During the experimental period, sludge growth, granule size and morphology and plant performance were monitored. Furthermore, the hydrolytic enzyme activity of the sludge was monitored to study its location (granular or flocculent sludge) and to compare the hydrolysis rates during operation with and without this additional untreated slaughterhouse wastewater. In this case, 16S rRNA gene amplicon sequencing was used to identify the main organisms in the sludge, and to detect shifts in the microbial community during increased pCOD loading. In this way, the present study aimed to assess the robustness of full-scale AGS reactors faced with an increase in influent particulate content, while exploring the involvement of different sludge fractions in the removal of particulates.

2. Materials and Methods

2.1. Description of the Plant and Additional Influent Dosing

WWTP Epe, operated by the Water Authority Vallei en Veluwe, treats the wastewater of the municipality of Epe (The Netherlands) and its surroundings. The wastewater is pre-treated with 3 mm screens and grit removal and treated in three 4500 m^3 AGS reactors with a diameter of 25 m and a depth of 9.2 m, designed and built by Royal HaskoningDHV. The reactors are operated in SBR mode with simultaneous anaerobic feeding/effluent discharge, aeration and settling. The wastewater is fed from the bottom of the reactor in a plug-flow regime, at an average upflow velocity of approximately 1 m h^{-1}. At the end of the settling phase, the slowest settling sludge is removed selectively. The influent of WWTP Epe is a mixture of domestic (70%), and industrial (30%) wastewater mainly originating from slaughterhouses. Wastewater composition is summarised in Table 1. The wastewater is treated to reach the effluent consents summarised in Table 2. The plant was designed to treat an average flow of 8000 m^3 d^{-1}, and a load of 59,000 population equivalents (PE). Nevertheless, the conditions at the time of the study have not reached the design values: the average flow is approximately 5000 m^3 d^{-1} and the load is 35,000 PE.

In the period 4 March–11 July 2019, the wastewater of a nearby slaughterhouse was discharged untreated to the municipal sewer network leading to WWTP Epe. The additional influent increased the substrate loading to the Nereda® reactors, approaching the design values. The objective was to enhance the growth of granular sludge while maintaining good pollutant removal performance. In a regular situation, the slaughterhouse treats its own wastewater using a dissolved air flotation (DAF) system. The treatment involves addition of coagulants and removal of solids by flotation, removing 80–90% of the COD of the wastewater. The treated wastewater, mostly composed of soluble COD, is discharged to the municipal sewer network and arrives at WWTP Epe, at approximately 500 m distance. During the test period, in-house treatment was stopped and concentrated raw wastewater was discharged, with a high content of suspended solids (see Table 1). The wastewater was continuously discharged during 12 h per day, with an average flow of 300 m^3 d^{-1} and a maximum flow of 50 m^3 h^{-1}.

Table 1. Influent composition of WWTP Epe during normal operation and during the test period. Values are expressed as average ± standard deviation. Routine measurements: normal operation n = 79; test period n = 21. Occasional miscellaneous analysis: normal operation n = 6; test period n = 3. Asterisks denote significant changes (* = $p < 0.05$; *** = $p < 0.001$).

	Normal Operation	Test Period	Significance
Routine measurements			
tCOD [g m^{-3}]	840 ± 254	1456 ± 692	***
TSS [g m^{-3}]	317 ± 123	633 ± 361	***
BOD$_5$ [g m^{-3}]	341 ± 109	537 ± 249	***
TN [g m^{-3}]	77 ± 21	101 ± 39	***
TP [g m^{-3}]	8 ± 2	13 ± 5	***
Q [m^3/d]	4696 ± 2099	5229 ± 3418	
COD/N [g/g]	11 ± 3	14 ± 4	***
COD/P [g/g]	101 ± 15	117 ± 24	***
Occasional miscellaneous analysis			
tCOD [g m^{-3}]	864 ± 274	1713 ± 572	*
sCOD [g m^{-3}]	339 ± 129	386 ± 49	
Acetate [g COD m^{-3}]	82 ± 59	43 ± 37	
Propionate [g COD m^{-3}]	14 ± 12	16 ± 17	
Lipids [g COD m^{-3}]	45 ± 20	290 ± 77	*
Total carbohydrates [g COD m^{-3}]	274 ± 155	538 ± 125	
Total proteins [g COD m^{-3}]	90 ± 11	162 ± 53	
Soluble carbohydrates [g COD m^{-3}]	18 ± 1	21 ± 2	
Soluble proteins [g COD m^{-3}]	20 ± 7	33 ± 23	

Table 2. Average effluent concentration of the main wastewater pollutants, in the months before the test period and during the test period. Standard deviation values are given between parentheses.

	COD [g m^{-3}]	BOD$_5$ [g m^{-3}]	TN [g m^{-3}]	NH$_4^+$-N [g m^{-3}]	NO$_X$-N [g m^{-3}]	PO$_4$-P [g m^{-3}]	TP [g m^{-3}]	TSS [g m^{-3}]
Effluent consent		7	5				0.3	30
Before test period	26 (±6)	1.5 (±0.5)	4.0 (±2.0)	0.3 (±0.2)	2.4 (±1.8)	0.04 (±0.01)	0.14 (±0.04)	5.2 (±1.6)
During test period	32 (±9)	2.4 (±1.3)	3.5 (±1.4)	0.3 (±0.3)	1.5 (±0.9)	0.07 (±0.09)	0.23 (±0.12)	5.5 (±1.2)

2.2. Sampling and Sample Handling

For this study, reactor mixed liquor samples and influent wastewater samples were collected. The samples were collected during two phases: before the test period (in a stretch of 5 months), and during the final week of the test period.

2.2.1. Influent Wastewater

Influent wastewater was collected using a flow-proportional 24-hour sampler, after screening and grit removal. The samples were stored at 4 °C in the sampler chamber, and then transported to the lab for analysis. Part of the wastewater was filtered using a vacuum filter device to characterise the particulate size-fractions. Sequential filtering was performed using filters with the following pore sizes: 100 µm, 10 µm, 1 µm and 0.1 µm (Product details: 100 µm: stainless steel mesh (Anglo Staaal, Borne, The Netherlands); 10 µm: Cyclopore®, polycarbonate [PC], 1 µm: Whatman® GF/B, glass fibre; 0.1 µm: Cyclopore®, PC. Whatman, Buckinghamshire, UK). The filtrate of each step was collected and stored for analysis. A small sample was also filtered through 0.45 µm for soluble COD (sCOD) analysis (Durapore® filters, PVDF. Merck, Darmstadt, Germany). Samples were preserved at 4 °C for short-term storage, and −20 °C for long-term storage.

2.2.2. Reactor Mixed Liquor

Reactor mixed liquor was sampled to study hydrolytic activity and sludge characteristics. Mixed liquor samples were collected during the aeration phase, at least 40 min after the beginning of aeration to ensure a completely mixed sample. Sieve fractions of the mixed liquor were obtained by pouring the mixed liquor through a stack of sieves of 0.045, 0.2 and 1 mm, and gently rinsing with tap water. The fraction smaller than 0.045 mm was centrifuged and the supernatant was kept. The following fractions were collected: mixed sludge; bulk (<0.045 mm fraction, centrifuged); flocs (0.045–0.2 mm); and granules (>1 mm). The fraction between 0.2 and 1 mm was excluded from analysis due to its high amount of debris and heterogeneous composition.

2.3. Analytical Methods

Several measurements were performed on the influent and its size fractions, as well as the two samples of slaughterhouse wastewater. TSS and VSS were measured according to the Standard Methods [29]. Volatile fatty acids (VFA) acetate and propionate were quantified using high performance liquid chromatography (HPLC) with an Aminex HPX-87H column from Bio-Rad (Hercules, CA, USA). tCOD was measured using photochemical test kits from Hach (Dusseldorf, Germany). sCOD was determined by measuring COD in the wastewater filtered through 0.45 µm. Particulate COD (pCOD) was calculated as tCOD − sCOD. Proteins were quantified using the modified Lowry method [13]. Carbohydrates were quantified using the anthrone-sulfuric acid method [30]. Lipids were measured by Merieux Nutrisciences (Resana, Italy) using the gravimetric method.

Sludge was inspected using a Keyence VHX-700F digital microscope (Mechelen, Belgium). The TS and VS of the sludge used in activity assays were determined according to the standard Methods [29].

2.4. Hydrolytic Enzyme Activity Tests

Lipase and protease enzyme activities of the sludge were assessed in the mixed sludge, granules, flocs and bulk liquid. The enzyme assays were performed within 8 h after sampling. The procedure of the assays is described in detail in Toja Ortega et al. (in press) [31]. In short, the sludge was incubated with chromogenic substrates, in anaerobic vials with a sampling port. The assay was conducted at 20 °C, pH 7.5 and at fully mixed conditions, using a Fisherbrand Seastar orbital shaker (Thermo Fisher Scientific, Waltham, USA) running at 120 rpm. The vials were flushed with N_2 gas for 2 min to provide anaerobic conditions. The substrates used were azocasein for the protease assays, and p-nitrophenyl-palmitate (pNP-palmitate) for the lipase assays (Sigma-Aldrich, Darmstadt, Germany). The assay vials were sampled every 15–30 min and the samples were filtered through 0.45 μm with a syringe filter to remove biomass. 1 mL sample was mixed with 1 mL TCA to stop enzyme activity. Samples were stored at −20 °C until analysed, and then thawed, centrifuged and filtered through 0.45 μm. The filtrate was mixed on a 1:1 proportion with NaOH 2M and its absorbance was measured, at 410 nm in the lipase assay and 440 nm in the protease assay. The increase in absorbance over time was translated to substrate hydrolysis rate, as described in Toja Ortega et al. [31]. The sludge-specific activity was calculated considering the sludge concentration in the vials. Finally, the total activity contained in the reactor was calculated by multiplying the specific activity of each sludge fraction by its abundance in the reactor.

2.5. Routine Measurements

Routine influent and effluent measurements were conducted by a certified lab and provided by Water Authority Vallei en Veluwe. These included: COD, biological oxygen demand (BOD_5), total nitrogen (TN), total phosphorus (TP), total suspended solids (TSS), ammonia nitrogen (NH_4-N), nitrate/nitrite nitrogen (NO_X-N) and flow. As were the excess sludge production, long-term reactor sludge concentration and granule size distribution measurements.

In addition to influent and effluent composition, key performance indicators (KPI) were used to follow reactor performance during the SBR cycle. Online samplers measure nutrient profiles during the AGS cycle (NH_4-N, NO_3-N, PO_4-P, dissolved oxygen [DO]). Measurement data were retrieved from the Aquasuite Nereda® controllers ® (Royal HaskoningDHV, Amersfoort, The Netherlands) and provided by Royal Haskoning DHV.

2.6. Microbial Population Analysis

2.6.1. Sludge Processing and DNA Extraction

The mixed sludge and sludge sieve fractions were homogenised using a Potter-Elvehjem tissue grinder to ensure a representative sludge sample for DNA extraction. The homogenised sludge was transferred to 1.5 mL Eppendorf tubes (Eppendorf, Hamburg, Germany) and centrifuged in a microcentrifuge (Eppendorf, Hamburg, Germany) at 14,000 g for 5 min. Around 30 mg of pellet were added to extraction tubes from the FastDNA spin kit for soil (MP Biomedicals, Irvine, CA, USA). The DNA was extracted following the protocol optimised by Albertsen et al. for activated sludge samples [32]. Each sample was extracted three times, to improve the recovery of the DNA of all microorganisms in the samples [33,34]. The concentration of the extracted DNA was measured using a Qubit dsDNA HS assay kit (Thermo Fisher Scientific, Waltham, MA, USA).

2.6.2. 16S rRNA Gene Amplicon Sequencing and Data Analysis

The 16S rRNA gene was amplified and paired-end sequenced in an llumina NovaSeq 6000 platform by Novogene (Beijing, China). The hypervariable regions V3–V4 were amplified and sequenced, using the primer set 341F [5′–CCTAYGGGRBGCASCAG–3′] and 806R [5′–GGACTACNNGGGTATCTAAT–3′]. The raw reads were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) on BioProject PRJNA746138.

The trimmed and merged sequences provided by Novogene were processed using QIIME2, version 2020.2 [35]. The sequences were quality-filtered using Deblur [36], trimming the sequences the 3′ end at position 403 (parameter p-trunc-len). The remaining sequences were assembled into a phylogenetic tree to perform diversity analyses, using the q2-phylogeny plugin. Beta diversity metrics (Bray–Curtis and Unweighted Unifrac) were generated, and differences in beta diversity between sludge types and experimental conditions were analysed using PERMANOVA [37]. A *p*-value of 0.05 was used as cut-off for significance. Finally, taxonomic affiliation of the sequences was determined by aligning the sequences to the MiDAS 3.6 database [38]. Sample subsetting, visualisation and further statistical analysis was performed in R, using the Phyloseq and Ampvis2 packages [39,40]. Abundant taxa were defined as taxa with 1% abundance of more. Per sludge type, significant differences in abundant taxa before and after the test period were determined through t-tests. A Bonferroni-corrected *p*-value was used, of 0.01 divided by the number of abundant taxa per sludge type.

3. Results

### 3.1. Additional Influent Dosing and Changes in Influent Composition

During the test period, the influent COD concentration almost doubled (from 840 to 1460 g m^{-3}). The pCOD, in particular, increased from 525 to 1327 g m^{-3}, along with the TSS content, which increased from 317 to 633 g m^{-3}. Table 1 summarises the characteristics of the influent to the wastewater treatment plant of Epe, before and during the addition of the untreated slaughterhouse wastewater. The VFA content in the influent was very variable, and the average VFA concentrations before and after the test period did not significantly change ($p = 0.39$). The sCOD measurements did not indicate an increase in soluble compounds in the wastewater either. Furthermore, particle size measurements of the influent showed that the extra COD during the test period came in the fractions between 1 and 100 μm, especially in the size fraction of 10 to 100 μm (Supplementary Information S1). The smallest COD fraction (<0.1 μm) had an average concentration of 260 ± 92 g m^{-3}. before the test period, and 331 ± 61 g m^{-3} during the test period. However, the differences on this size fraction between both situations are not statistically significant ($p = 0.11$). From the macromolecules quantified (proteins, carbohydrates and lipids), lipid concentration increased most, from 45 to 290 g m^{-3}.

In short, there was a significant increase in the influent concentration of tCOD ($p < 0.001$) and of TSS ($p < 0.001$). sCOD and VFA concentrations, in contrast, were not significantly affected by the addition of the untreated slaughterhouse wastewater, since the location is only 500 m from the WWTP. Apparently, there was not enough residence time in the sewer for fermentation of this wastewater, despite the warm spring season. Even though sCOD did not increase, the BOD$_5$ was higher during the test period, indicating the presence of particulate substrates with high biodegradability in the added untreated slaughterhouse wastewater.

3.2. Sludge Production

The biomass concentration of the reactors increased during the 4 months of changed influent. On the year before the test, the average TS concentration of the reactors was 5.8 ± 1.7 g TS/L. At the end of the test period, the average sludge concentration was 8.3 ± 0.6 g TS/L. However, it should be noted that already the months before the test period the sludge concentration had started to increase (Supplementary Information S2). The granule bed was composed of different sludge morphologies (size fractions). The measured percentage of each sludge size fraction over the total sludge oscillated during the experimental period. Overall, the trend showed an increased large granule fraction (from 56 to 67%) and a decreased smaller granule fraction (from 23 to 18%) and floc fraction (from 20 to 15%). However, such tendency could not be attributed to the test period alone, because the change in sludge bed composition occurred during a longer time period and not only during the test itself (Supplementary Information S2). Furthermore, protozoa

growth was observed on granule surfaces (Figure 1), but this did not affect the settleability of the sludge; SVI$_5$ was maintained around 40–45 mL/g VS.
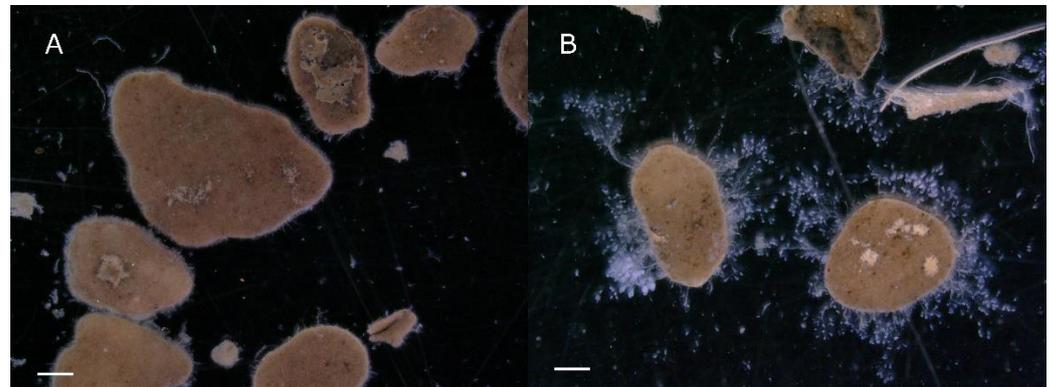
Figure 1. Micrographs of large granules (>1 mm): (**A**) on 23 January 2019 (normal operation); and (**B**) on 9 July 2019 (at the end of the test period). Size bar: 0.5 mm.
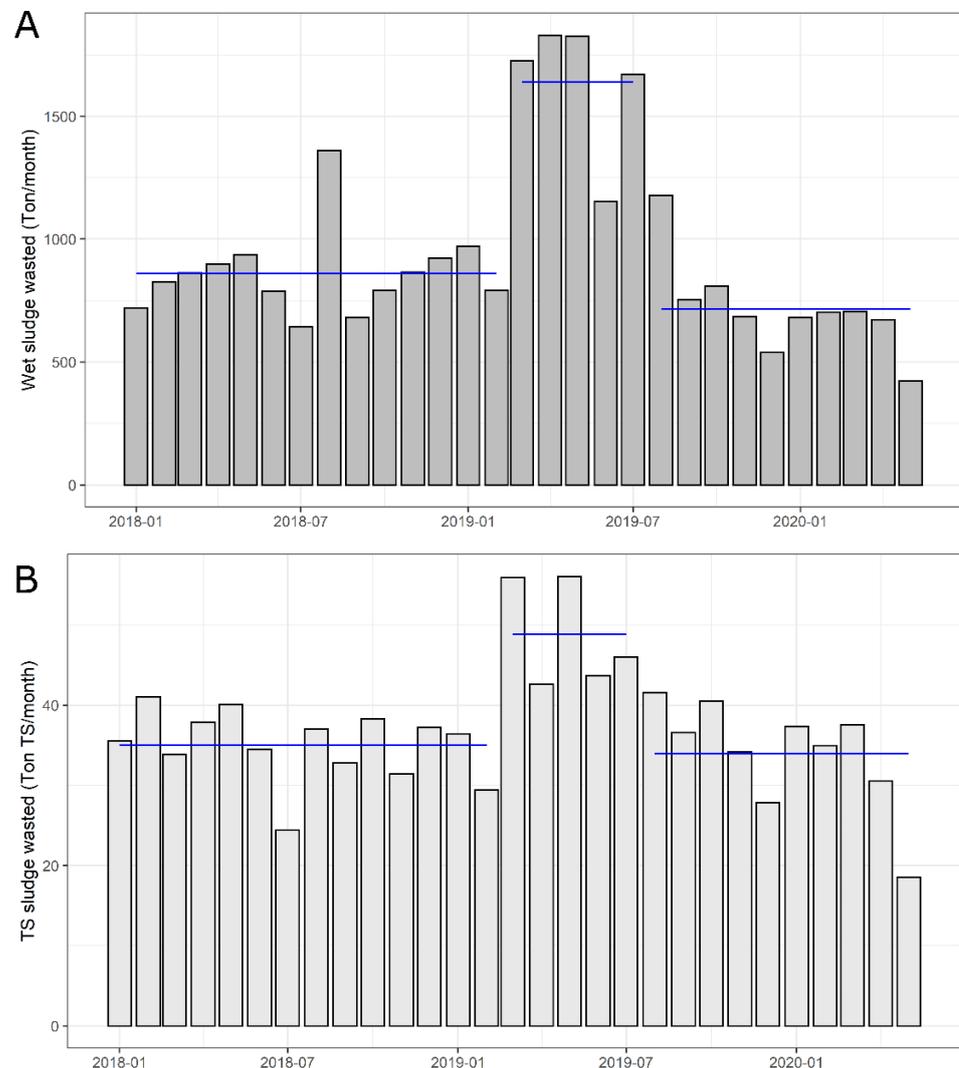
During the test period, the volume of the wasted sludge increased (Figure 2). The amount of wet sludge withdrawn doubled during the test period, from 870 to 1640 tonnes per month. The TS measurements of the waste sludge were lower during the test period than during normal operation (40 $\pm$ 10 g TS/L versus 31 $\pm$ 8 g TS/L). Thus, in terms of dry weight the increase in wasted sludge was less pronounced: 48.8 tonnes TS per month instead of the normal 34.7 tonnes TS per month, or around 13 tonnes TS per month higher. These data show that the additional influent particulates were at least partly degraded during the process. If the particulates were removed non-degraded with the excess sludge, the sludge production would have been considerably higher during the test period. Considering the increase of 0.3 g TSS/L in the influent, sludge wasting would increase 50 tonnes TS/month instead of the observed 13 tonnes TS/month. The sludge production data should be interpreted with caution, though. The measurement of the dry solids content of the excess sludge was not very accurate, due to difficulties associated with full-scale monitoring. The operation of the sludge thickening unit was fluctuating, resulting on a variable spill concentration. Therefore, the TS measurements on weekly grab samples of the thickened sludge might not be fully representative of the TS concentration of the spill over the whole period. Nevertheless, such a large gap between the expected and observed spill production still indicates that a fraction of particulate COD was likely to be consumed by the sludge. For a more detailed mass balance, check Supplementary Information S5.

3.3. Hydrolytic Enzyme Activity Tests

Hydrolytic enzyme activity assays showed that both granules and flocs have the ability to hydrolyse proteins and lipids (Figure 3). No protease and lipase activity was detected in the bulk liquid.

Before adding the particulate-rich influent stream, the specific protease activity (i.e., per gram of VS) of granules was about half of that of flocs. Nevertheless, due to the higher percentage of granular than flocculent sludge in the reactor, the total contribution to protease activity of the granule fraction in the reactor was as high as that of the flocculent fraction (Figure 3B). It is noteworthy that the large granule and floc activities do not add up to the total activity in the mixed sludge. This can, at least partly, be explained by the activity of the small granule fraction (0.2–1 mm) which was not measured individually but is also part of the mixed liquor sample [31]. Increasing the influent pCOD did not significantly affect the biomass-specific protease activity of the mixed liquor and the granule fraction, and flocculent sludge activity decreased (Figure 3A). The total activity at reactor scale seemed to increase due to higher sludge concentration after the test period, although the

large variation in activity in the mixed sludge fraction makes the difference statistically just not significant ($p = 0.056$) (Figure 3B). The total protease activity contributed by large granules did increase significantly ($p = 0.03$).

Figure 2. Monthly waste sludge production. (**A**) Tons of wet sludge produced (**B**) Tons of TS produced, based on the measured TS content of the waste sludge (40 g/L during normal operation and 31 g/L during the test period). The blue lines indicate the average sludge production in the periods before, during and after the test period.

Regarding the hydrolysis of lipids, before the addition of the extra untreated slaughterhouse effluent the lipase activity was considerably higher in flocs than in granules (approximately 9-fold) (Figure 3A). Due to such a large difference in specific activity, also at a reactor level, the flocs contributed more to lipid hydrolysis than granules (three-fold). After the influent change, the specific lipase activity in flocculent sludge increased ($p = 0.02$) and was approximately 10-fold of the specific lipase activity of granules. The biomass-specific lipase activity of granules, as with protease, did not change. On reactor-scale, the total mixed liquor lipase activity increased significantly due to the increased biomass concentration in the reactor ($p = 0.009$). Regarding the contribution of each biomass fraction, the total lipase activity of granules increased due to the increased granular sludge concentration ($p = 0.007$), so the activity of flocs became only twice as high as that of large granules at reactor scale. In any case, flocs seemed to be strongly involved in lipid hydrolysis, although granules also revealed measurable activity.
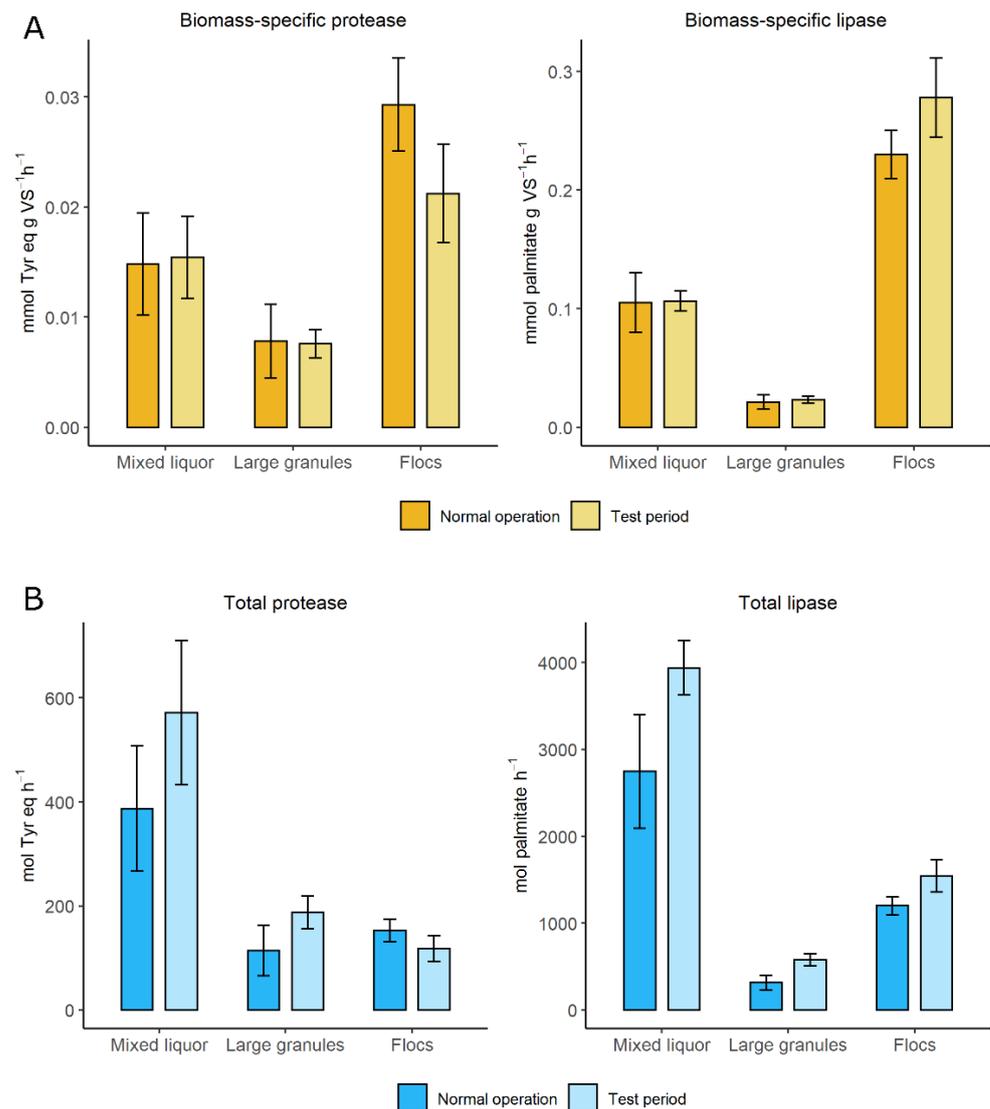
Figure 3. Hydrolytic activity of mixed sludge, granules and flocs. (**A**) Specific hydrolytic activities of the mixed liquor (Mix), and the sieve fractions large granules (>1 mm) and flocs (<0.2 mm); (**B**) Total hydrolytic activities at reactor-scale in the mixed liquor, and the total activity contributed by large granules and flocs. The fraction in the range 0.2 to 1 mm was not analysed but can be estimated by the difference between the mixed liquor and the sum of the other two fractions. Each bar represents the average of: 2 reactor samples and duplicate vials before the influent change ($n = 4$); 3 reactor samples and duplicate vials at the end of the test period ($n = 6$).

3.4. Reactor Performance and Nutrient Cycles

Effluent quality did not deteriorate during the test period (Table 2). Regarding reactor performance, several key performance indicators (KPI) were higher during the test period, including phosphate uptake rate, total phosphate release and ammonia uptake rate (nitrification) (Supplementary Information S3). However, a similar trend was observed in the spring–summer period (April–July) of the years 2018 and 2020. Furthermore, the changes in the different KPI rate values can be theoretically coupled to temperature increase, so the increase was more likely attributed to seasonal variations than to the influent composition. Denitrification rate seems to be an exception. Denitrification rate was higher during the test period (Figure 4), and no similar increase was observed in the previous summer, except in reactor 2. In the following summer (2020) denitrification rate was higher than in the winter, but the change was not as pronounced as during the test period. Furthermore, changes

in denitrification rate in previous years clearly followed increases in temperature, while during the test period the increase in denitrification rate was uncoupled from the increase in temperature. Nitrate uptake rates were determined during post-denitrification phases in all cycles. Simultaneous nitrification denitrification (SND) was not monitored through the KPI, because intermittent aeration was used, which did not allow a straightforward comparison of the produced and consumed nitrate.
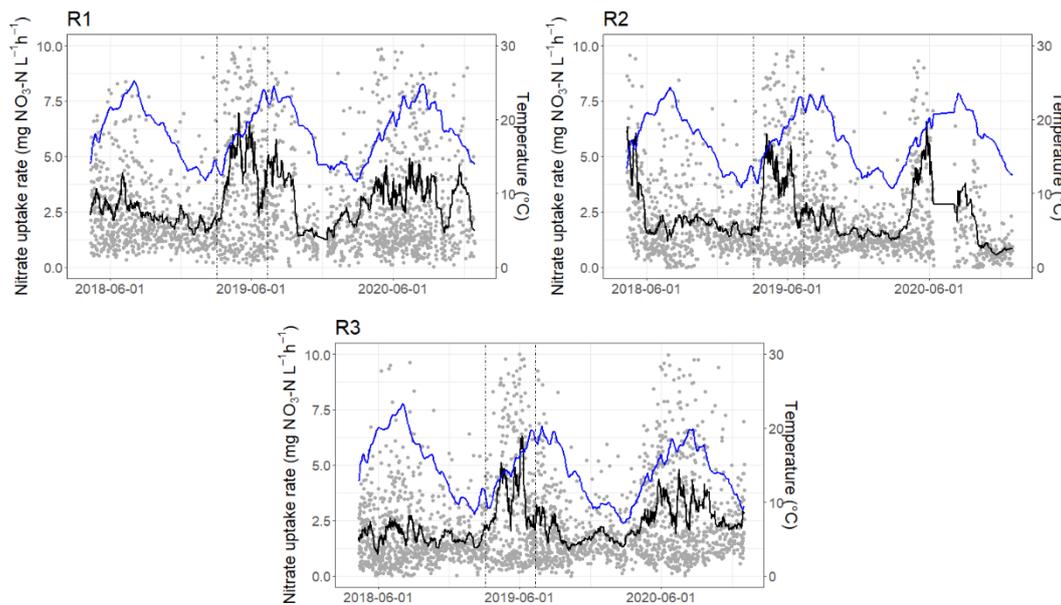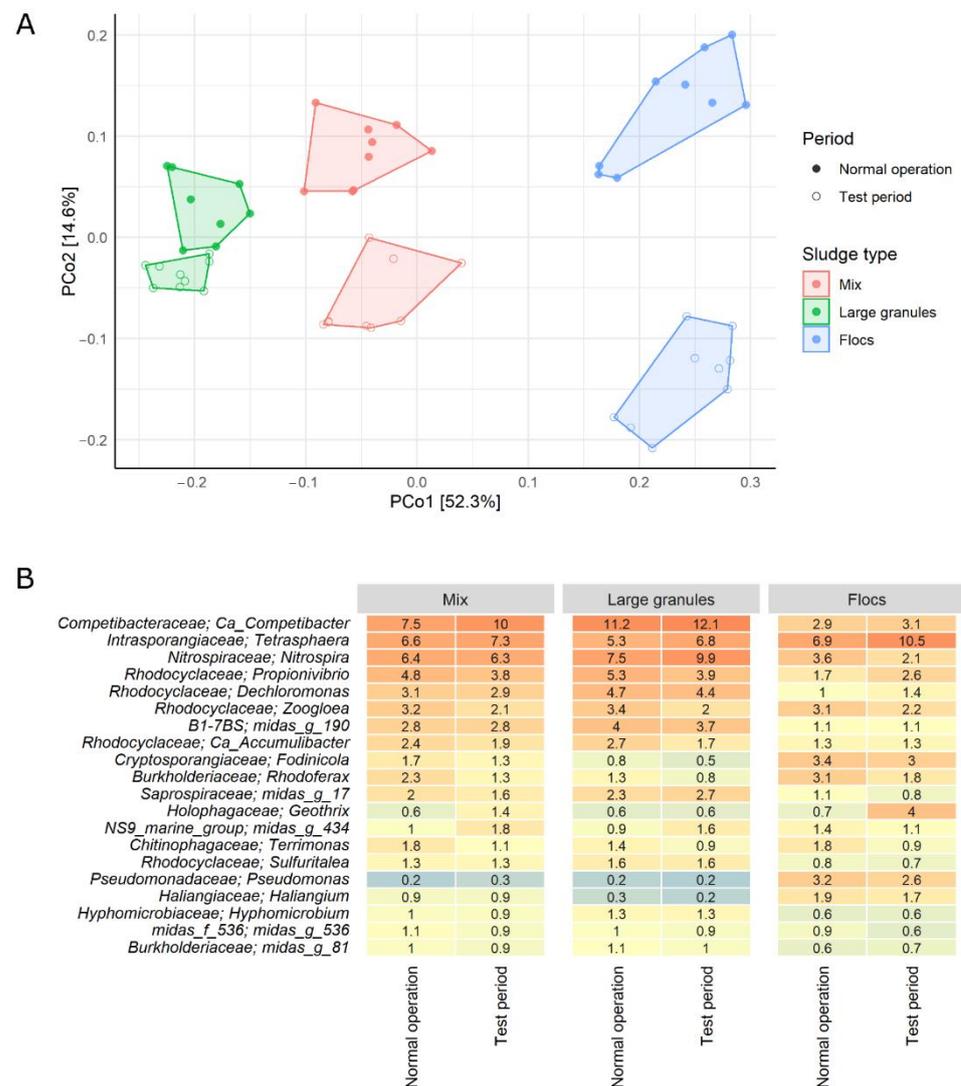

Figure 4. Denitrification rates measured during the post-denitrification period. R1, R2 and R3 indicate Nereda® reactors R1, R2 and R3 of WWTP Epe. Each dot represents the average rate in one SBR cycle. The continuous black line represents the moving average, with a sliding window of 7 days. The blue line indicates the temperature in the reactors. The test period is delimited by dashes.

3.5. Microbial Community Composition: 16S rRNA Sequencing Results

The microbial community of the mixed liquor, large granules and flocs differed significantly, in terms of beta-diversity ($p = 0.001$). Significant differences were observed too between the samples taken during normal operation and during the test period. The shift in microbial composition during the test period was most pronounced in flocculent sludge, and less in large granules. The distances between the analysed samples are presented in Figure 5A.

The 20 most abundant genera in the sludge are depicted in Figure 5B. Different genera of putative PAO and GAO were abundant in the sludge, such as Ca. *Accumulibacter*, *Tetrasphaera*, *Propionivibrio*, *Dechloromonas* and Ca. *Competibacter*. In the mixed sludge, a large proportion of the sequenced reads belonged to Ca. *Competibacter*, and the proportion increased during the test period from 7.5 to 10% of the reads. The genus *Tetrasphaera* was also abundant but did not significantly change over the test period in the mixed sludge. This putative PAO genus was predominant in the flocculent sludge fraction, where its proportion significantly increased during the test period. Large granules also had a considerably high percentage of *Tetrasphaera* (5.3–6.8%) and Ca. *Accumulibacter* (1.7–2.7%).

The genus *Nitrospira* was also at high relative abundance in the sludge. Only a marginal percentage of *Nitrotoga* (0.002%) was detected, making *Nitrospira* the dominant nitrite oxidising bacteria (NOB). *Nitrospira* was mainly associated to large granules, where it also became more abundant during the test period. The AOB *Nitrosomonas* was detected but at low relative abundance (0.015%).

A

B

	Mix		Large granules		Flocs	
	Normal operation	Test period	Normal operation	Test period	Normal operation	Test period
Competibacteraceae; Ca_Competibacter	7.5	10	11.2	12.1	2.9	3.1
Intrasporangiaceae; Tetrasphaera	6.6	7.3	5.3	6.8	6.9	10.5
Nitrospiraceae; Nitrospira	6.4	6.3	7.5	9.9	3.6	2.1
Rhodocyclaceae; Propionivibrio	4.8	3.8	5.3	3.9	1.7	2.6
Rhodocyclaceae; Dechloromonas	3.1	2.9	4.7	4.4	1	1.4
Rhodocyclaceae; Zoogloea	3.2	2.1	3.4	2	3.1	2.2
B1-7BS; midas_g_190	2.8	2.8	4	3.7	1.1	1.1
Rhodocyclaceae; Ca_Accumulibacter	2.4	1.9	2.7	1.7	1.3	1.3
Cryptosporangiaceae; Fodinicola	1.7	1.3	0.8	0.5	3.4	3
Burkholderiaceae; Rhodoferax	2.3	1.3	1.3	0.8	3.1	1.8
Saprospiraceae; midas_g_17	2	1.6	2.3	2.7	1.1	0.8
Holophagaceae; Geothrix	0.6	1.4	0.6	0.6	0.7	4
NS9_marine_group; midas_g_434	1	1.8	0.9	1.6	1.4	1.1
Chitinophagaceae; Terrimonas	1.8	1.1	1.4	0.9	1.8	0.9
Rhodocyclaceae; Sulfuritalea	1.3	1.3	1.6	1.6	0.8	0.7
Pseudomonadaceae; Pseudomonas	0.2	0.3	0.2	0.2	3.2	2.6
Haliangiaceae; Haliangium	0.9	0.9	0.3	0.2	1.9	1.7
Hyphomicrobiaceae; Hyphomicrobium	1	0.9	1.3	1.3	0.6	0.6
midas_f_536; midas_g_536	1.1	0.9	1	0.9	0.9	0.6
Burkholderiaceae; midas_g_81	1	0.9	1.1	1	0.6	0.7

Figure 5. Microbial community composition of the sludge from WWTP Epe derived from 16S rRNA sequencing results. (**A**) Principal coordinates analysis (PCoA) plot based on the Bray-Curtis distance matrix between samples. (**B**) 20 most abundant genera and their families, in the mixed liquor sludge (Mix) and the large granule and floc fractions.

Microorganisms with a central role in hydrolysis and fermentation have been studied less than PAO, GAO and nitrifiers. This is partly due to the large array of organisms that can perform these functions. Several fermenting microorganisms were identified by Layer et al. in AGS reactors fed with complex wastewaters [27]. The families *Saprospiraceae* and *Chitinophagaceae*, and the phylum *Chloroflexi* found in their study were at high relative abundances in Epe too (4.8–5.1%, 2.9–3.8% and 2–3.5%, respectively). Many members of these taxa can perform hydrolysis and fermentation [41]. Between the abundant genera (>1% relative abundance) in Epe, there were several putative fermenters and hydrolysers: *Rhodoferax* (1.3–1.7%), *Fodinicola* (1.3–2.3%), *Terrimonas* (1.2–1.8%) and *Geothrix* (0.7–1.4%). *Rhodoferax* are denitrifying organisms and putative fermenters, which seem to be able to utilise carbohydrates, amino acids and short chain fatty acids. *Fodinicola*, in turn, are aerobic filamentous organisms that can hydrolyse proteins and carbohydrates. *Terrimonas*, too, are likely strictly aerobic hydrolysers. Last, *Geothrix* might play a role in lipid metabolism, as they can metabolise both short-chain and long-chain fatty acids, using nitrate as electron acceptor [42]. However, the metabolism of these genera has not been systematically studied in situ and therefore it is difficult to infer their function in our system.

4. Discussion

4.1. Changes in Hydrolytic Activity of the Sludge

From the enzyme activities measured, none was detected in the bulk liquid. All lipase and protease activity were associated to the biomass, both to granules and to flocs. This observation is in line with previous biofilm and activated sludge studies that found hydrolytic activity to be predominantly associated to the biomass [13,17,43]. Flocs had higher specific lipase and protease activity than granules, as observed in WWTP Garmerwolde [31], most markedly in the case of lipase. The increased particulate load period had an effect on the biomass-specific activities of flocs, with a decrease in protease activity and an increase in lipase activity. This could be related to the increased influent lipid concentration in the test period, whereas the protein concentration did not change as sharply. That only the biomass-specific hydrolytic activities of flocs changed over the test period indicates that floc activity is more rapidly influenced by changes in the influent than granules. Furthermore, it seems from our results that flocculent sludge is actively involved in lipid hydrolysis and can increase the production of enzymes during a higher exposure to lipid substrates.

The change in influent composition did not affect the biomass-specific activity of granules. The total enzyme activity in the reactors mostly increased because of overall granule growth. The newly produced granules had the same biomass-specific activity, so the total hydrolytic activity increased. Under normal operation, the granular sludge already had, theoretically, enough potential to hydrolyse all the proteins and lipids from the more concentrated influent within one SBR cycle (Supplementary Information S4). However, it would be unrealistic to assume that the rates measured in laboratory assays, based on kDa-size substrates with good biodegradability, are directly translatable to full-scale conditions. Nevertheless, the enzymatic activity of the granules does not seem to be a limiting factor for complete hydrolysis of influent pCOD. Similar results were obtained at the AGS reactors at WWTP Garmerwolde [31]. The hydrolysis rate apparently does not depend so much on enzyme content of the granules but on mass-transfer of the substrate into the biofilm (granule). In biofilm reactors, hydrolysis can be described using an areal hydrolysis rate (g TOC/m^2/h), which accounts for the biofilm area in the reactor [44].

4.2. Microbial Community Composition and Shifts

4.2.1. Microorganisms Involved in P and N Removal

In this section the most abundant taxa are discussed under the assumption that the relative abundances in the sequenced 16S rRNA genes reflect the abundance of their respective microorganisms in the sludge. This might not hold true in all cases, due to biases associated to 16S rRNA gene amplicon sequencing, including biases in the extraction and amplification of the DNA and differences in DNA copy number among microbial groups. For example, the relative abundances of Ca. *Accumulibacter* and *Tetrasphaera* are underestimated when determined by 16S sequencing compared to FISH, while *Dechloromonas* seems to be overestimated [45]. However, the data can provide a glimpse of the main microbial components in the sludge. The sequencing results revealed a large proportion (~20% of the reads) of microorganisms related to enhanced biological phosphorus removal (EBPR) and nitrogen removal [45,46]. This is in line with the good nutrient removal performances that were achieved in the plant. The NOB *Nitrospira* was one of the most abundant microorganisms in the sludge, comprising 6.3–6.4% of the reads in the mixed sludge. Numerous studies have reported *Nitrospira* as the main NOB in full-scale EBPR plants, probably due to its high substrate affinity, which allows them to thrive under low oxygen and nitrite concentrations [47,48]. The only detected AOB was *Nitrosomonas*, with low relative abundance (0.015%). Finding AOB at low relative abundances in 16S rRNA amplicon sequencing data does not necessarily imply a low AOB activity in the sludge [49]. That was also the case in this study where no issues with nitrification were identified.

The classical PAO genus Ca. *Accumulibacter* was present in relatively high abundance (1.9–2.4%) in the mixed sludge. In general, at more complex influent composition fermentative PAO and GAO populations seem to be favored [27,50–52], which is in line with the

observations for the sludge in Epe. The fermentative PAO *Tetrasphaera* was found in high relative abundance, accounting for 6.6–7.3% of the reads in the mixed sludge samples. *Tetrasphaera* is often found in AS and AGS fed with complex wastewaters. They can use a wider range of substrates compared to Ca. *Accumulibacter*, including sugars and amino acids which they can utilise through a fermentative metabolism [45]. The fermentative *Dechloromonas* was also abundant in the mixed sludge samples. Members of this genus seem to possess a PAO metabolism, but others behave as GAO [50]. Therefore, their contribution to EBPR in our system is unclear. Overall, GAO were abundant in the sludge from Epe, the main genera being Ca. *Competibacter* (7.5–10%) and the fermentative GAO *Propionivibrio* (3.8–4.8%). The high COD/P ratio of the wastewater (>100 mg COD/mg P) likely favored the growth of GAOs. GAOs in most cases are not harmful to the EBPR process and indicate an excess of COD [50]. In our study too, effective EBPR was observed regardless of the high GAO abundance.

Similar to previous studies, slow-growing microorganisms (PAO, GAO and nitrifiers) were enriched in the granular sludge in comparison with flocs, due to the longer solids retention time (SRT) of granules [51,53]. Furthermore, granules have preferential access to influent substrates during the feeding phase, due to their localisation at the bottom of the reactor resulting from their higher settling velocity compared to smaller granules and flocs [54]. This further enhances the access of PAO and GAO to the rbCOD in the influent. Interestingly, *Tetrasphaera* was more concentrated in flocculent sludge, and even increased during the test period, while previous studies reported higher *Tetrasphaera* abundances in granules than in flocs [51]. The reasons for the discrepancy with our study might be related to the differences in substrate utilisation of both PAO. Ca. *Accumulibacter*, in granules, have preferential access to the VFA in the influent. Meanwhile, flocculent sludge has the ability to entrap more particulate substrates throughout the cycle, and also has a higher hydrolytic capacity, making monomers such as amino acids and monosaccharides available to *Tetrasphaera* throughout the cycle. However, this is a mere speculation that should be tested in more AGS plants and using more quantitative methods than 16S rNA sequencing.

4.2.2. Microbial Community Changes during the Test Period

The changes in microbial diversity during the test period were more pronounced in flocculent sludge than in large granules. The shorter SRT of flocculent sludge, and the higher immigration rates to this sludge fraction, can explain this observation [51]. The new influent did not have a strong impact on the microbial communities in the more rigid and older granules, which relates to the stable performance of the plant and the minimal changes in the hydrolytic activity of granules. The larger shift in microbial community of flocs is also in line with the activity changes measured in this fraction.

In terms of changes in individual taxa, the GAO Ca. *Competibacter* proliferated during the test period, possibly due to the increased COD/P ratio. No clear increase was observed among the putative hydrolyser and fermenter taxa, even if the reactors received higher particulate contaminant concentrations. The abundance of *Chloroflexi* and *Geothrix* increased, but *Saprospiraceae* and *Fodinicola* decreased, while *Tetrasphaera*, *Propionivibrio*, *Dechloromonas* and *Rhodoferax* did not change significantly. The sludge contained various organisms capable of metabolising different types of organic matter already during normal operation, and at increased particulate loads there were shifts among these groups but no sharp increase in their relative abundances. The lack of a clear increase aligns with the results from the hydrolytic tests, as the specific hydrolytic activity of the sludge did not change either. Lipid metabolism in flocculent sludge was an exception; the specific lipase activity of flocculent sludge increased. In flocculent sludge, the abundance of *Geothrix* increased from 0.7 to 4.2%, which might be related to the increase in lipase activity. However, the metabolism of *Geothrix* in aerobic wastewater treatment systems has not been studied. Further analysis would be insightful to understand their role in this ecosystem. Filamentous microorganisms related to LCFA metabolism (*Ca. Microthrix*, *Gordonia*) were not abundant in the sludge, and Ca. *Microthrix* even decreased during the test period (from

0.5 to 0.09%). No foaming or bulking was observed in the reactors even if the lipid load increased considerably.

Protozoa were not studied through sequencing, as only the bacterial and archaeal 16S gene was targeted. However, microscopy revealed a protozoa bloom in the sludge during the test period (Figure 3). Protozoa are likely to take up particulates, although their contribution to pCOD removal was not quantified [10,55]. They did not deteriorate the settleability of the sludge and might contribute to granule stability by metabolising particulates at the granule surfaces.

4.3. Impact of Particulates on Wastewater Treatment and Granular Sludge Growth

The treatment performance during the test period remained stable, in terms of COD, P and N removal. Effluent suspended solids did not increase either. Furthermore, during the test period the denitrification rates were higher than usual (on average, 4.0 mg NO_3-N L^{-1} h^{-1} compared to 3.1 mg NO_3-N L^{-1} h^{-1} during normal operation). Previous studies showed that the particulates could significantly contribute to denitrification in AS [56,57], as well as in biofilm reactors [58,59]. In those studies, denitrification relied partly on intracellularly stored substrates, but mostly on particulates that remained entrapped in the sludge and kept hydrolysing during the anoxic phase. In AGS reactors, this would mainly benefit post-denitrification, since simultaneous nitrification-denitrification (SND) requires the anaerobic storage of substrates and subsequently the coexistence of different redox zones during aeration. Particulates would have to be anaerobically hydrolysed and fermented by granules and diffuse below the granule surface in order to be stored as PHA and contribute to SND. This is likely a slow process due to mass-transfer limitation. Even if the anaerobic uptake of particulates by granules would be limited, SND can be enhanced by applying optimised aeration strategies. Layer et al. proposed to apply alternating aeration (intermittent switching on and off) or 2-step aeration (a pulse of high aeration followed by low DO for the rest of the reaction phase) to improve SND when AGS was fed with complex wastewater [60]. This way, with an increased particulate COD load such as the one in the present study it is likely possible to enhance SND, as well as to reduce the post-denitrification phase length and achieve lower effluent N.

During the test period, sludge growth was observed in the reactors at WWTP Epe. The total sludge concentration of all three reactors increased (from 5.7 to 7.6 g TS/L in R1, 5.8 to 8.5 g TS/L in R2 and 5.9 to 8.7 g TS/L in R3), as well as the granule proportion (from 58 to 67%). Thus, granule growth was observed following the increase in influent COD. After the test period, the sludge concentration remained high but did not increase further. Nevertheless, the sludge concentration in the reactors, as well as the proportion of large granules, had started to increase before the test period and there were large oscillations in the measurements (Supplementary Information S2). This makes it difficult to conclude whether granular sludge growth was supported by the added particulates. Part of these particulates were consumed in the reactor, but it is not clear if this was accomplished by the granules or by the flocculent sludge fraction. Nonetheless, the study does show that the sustained increased load of biodegradable influent particulates did not negatively impact granule growth. This holds for an influent particulate concentration of approximately 1.3 g COD/L (77% of the COD). The granule size did not decrease, as opposed to previous studies where at more complex wastewater feed the sludge bed was composed of smaller granule sizes [21,27]. The proportion of flocculent sludge did not increase either, probably due to increased sludge wasting (Figure 2). Furthermore, the higher influent particulate concentration did not result in filamentous outgrowths in the granules or disruption of granule stability as seen in previous studies [18,19]. Only a higher concentration of protozoa was observed on granule surfaces. According to our results, a moderate increase of influent suspended solids does not necessarily deteriorate granule integrity or size distribution. There might be a few reasons for this: (1) The granules can anaerobically hydrolyse part of the pCOD, as indicated by their relatively high hydrolytic activities. The available pCOD during aeration is therefore lowered; (2) protozoa at the

granule surfaces might take up a considerable amount of particulates aerobically, limiting the creation of substrate gradients at the granule surfaces [55]; and (3) the flocculent sludge can capture and hydrolyse particulates, as indicated by its high specific hydrolytic activity, an observation in line with the hypothesis of Layer et al. [27]. An adjusted sludge wasting maintains the sludge bed composition avoiding a buildup of the flocculent sludge fraction and particulate material in the reactor.

5. Conclusions

We studied a full-scale Nereda® plant faced with an increased influent pCOD concentration during a 3-month period. Doubling the influent pCOD concentration did not interfere with the smooth granule morphology and did not compromise nutrient removal efficiency. In line with this observation, the sludge exhibited high hydrolytic activity and a high proportion of putative hydrolysing and fermenting organisms. The exact contribution of pCOD to sludge growth and nutrient removal could not be clarified and deserves further research. Nonetheless, the results of this study point towards good performance stability of the AGS process operated with an increased influent particulate concentration. An increased COD could permit the achievement of more efficient N removal and meeting more stringent effluent discharge requirements, without adding external C sources or post-treatment steps. Therefore, adding a COD-rich wastewater stream, even with a high particulate content, can be a good solution when a higher COD load to AGS reactors is desired.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/pr9081472/s1, Figure S1: Distribution of the influent COD in different size fractions, during normal operation and during the test period, Figure S2: Mixed liquor suspended solids (MLSS) concentration of the reactors at WWTP Epe, and the concentration of different granule size fractions, Figures S3.1–S3.5: Key performance indicators from reactors 1–3 of the WWTP Epe, Tables S4.1–S4.3: Calculation of total hydrolytic activity during feeding, Table S5: Comparison of estimated and observed sludge production.

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