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Laboratory scale aerobic bioreactor conditioning of dairy processing wastewater as feedstock for *Lemna minor* production.

Thesis presented by

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For the degree of

Master of Science

University College Cork

School of Microbiology

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Table of Contents

Declarations....3
Acknowledgements....4
Abstract....5
Chapter 1 – Literature Review....6
1.1 Dairy Industry in Ireland....7
1.2 Dairy Processing Wastewater Generation....9
1.3 Treatment of Dairy Processing Wastewater....11
1.3.1 Chemical treatment options....11
1.3.2 Biological Nutrient Removal....12
1.3.3 Microbiology of Nitrogen Removal for Biological Nutrient Removal....18
1.4 Future prospects and challenges....20
Chapter 2 – AO reactor set-up and performance....22
2.1 Introduction....23
2.2 Materials and methods....25
2.3 Results and Discussion....30
2.3.1 Floc formation and MLSS with synthetic wastewater....30
2.3.2 Chemical analysis of performance with synthetic wastewater....33
2.3.3 Floc formation and MLSS with real-time wastewater....44
2.3.3 A/O filament identification....47
2.3.4 Chemical analysis of performance with real-time wastewater....53
2.4 Conclusion....61
Chapter 3 – IASBR set-up and performance....63
3.1 Introduction....64
3.2 Materials and methods....66
3.2.1 IASBR set-up and operation....66
3.2.2 Chemical analysis and other tests....67
3.3 Results and Discussion....69
3.3.1 Floc formation and MLSS....69
3.3.2 IASBR filament identification....72
3.3.3 Chemical analysis of performance....77
3.4 Conclusion....88
Chapter 4: Integration of *Lemna minor* cultivation system with IASBR wastewater treatment....89

4.1 Introduction 90
4.2 Materials and Methods 93
4.3 Results and discussion 96
4.3.1 *Lemna* growth experiments. 96
4.4 Conclusion 127
Chapter 5 – Overall Conclusions and Future Prospects. 128
Bibliography 132

Declarations

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism.

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Róisín Broughton

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Lastly, I would like to thank my family and friends, particularly my husband, for putting up with me during my periods of stress, but also for supporting me and having faith in me to succeed.

I wouldn't be where I am today without you all.

Abstract

Dairy processing wastewater is a nutrient-rich resource, containing chemical oxygen demand (COD) ranging from 1,150 – 68,814 mg/L, nitrogen (N) from 14 – 1,462 mg/L and phosphorus (P) from 7.2 – 650 mg/L, depending on the product stream. This resource has potential to be utilised for cultivation of *Lemna minor* as a value-added product, in line with EU circular economy principles. The purpose of this project was to condition dairy processing wastewater for downstream application as growth media for *Lemna minor*. Effluent from both systems were tested for nutrient removal, and IASBR effluent was more suitable for the coupled system, with removal efficiencies of >90% COD, 4 – 94% NH_4^+ and 30 – 80% P respectively. The coupled system yielded poor nutrient removal and little to no plant growth, with plants reaching senescence by day 28 of the trial. It can be concluded that *Lemna minor* cultivation failed as a result of both A/O and IASBR operational issues (including starting pH) and insufficient seeding of cultivation tanks. It is therefore recommended to focus future studies on the suitability of anaerobic treatment technologies for conditioning of dairy processing wastewater for *Lemna minor* cultivation. Future work with coupled wastewater treatment/duckweed cultivation systems should also involve investigation into plant-symbiont interactions, as well as profiling of wastewater microbial communities and their potential impacts on duckweed growth.

Chapter 1 – Literature Review

1.1 Dairy Industry in Ireland

The Dairy Industry has long been an important part of the Irish economy, generating almost €2 billion in revenue in 2018. Milk is processed into a large variety of dairy products and ingredients for both domestic and international markets (Figures 1.1 & 1.2). Products traditionally processed from milk include liquid milk for consumption, butter, cheese, yoghurt and ice-cream. More recently, milk/whey powders and milk proteins such as casein are generated as saleable products.

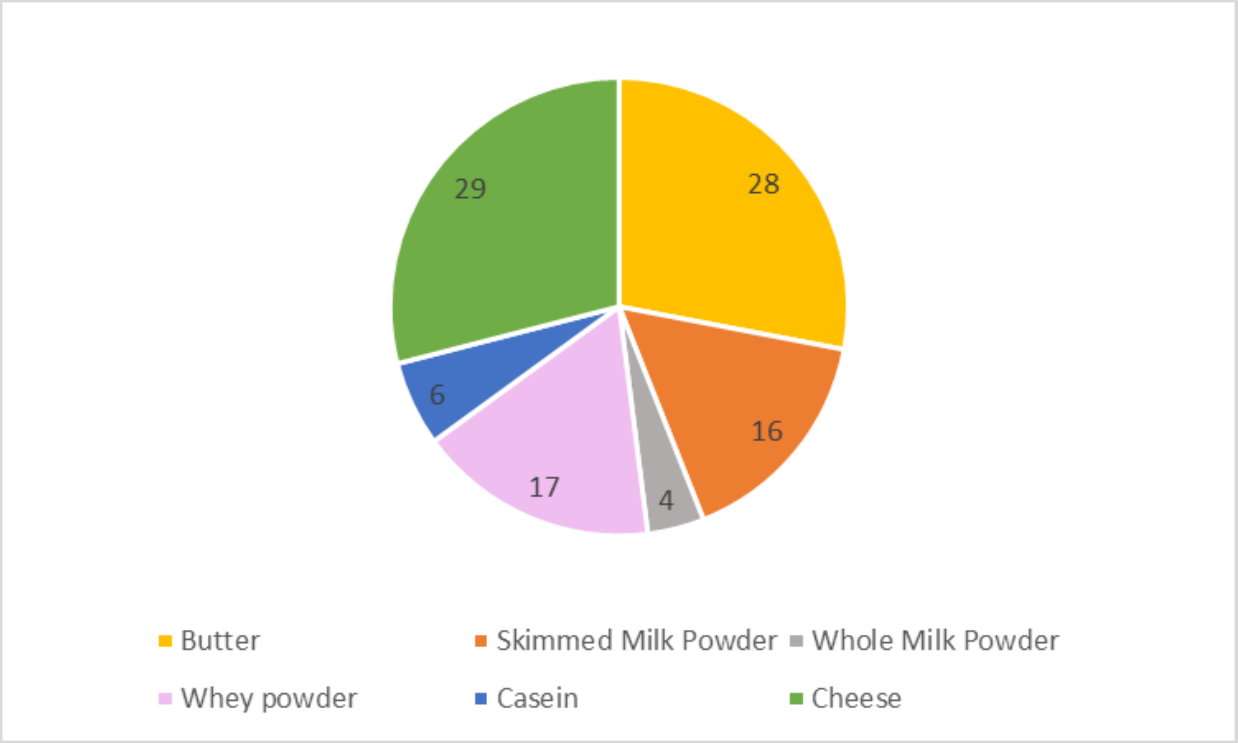


Figure 1.1: Main dairy products produced (%) in Ireland in 2015/16, (CSO Eurostat).

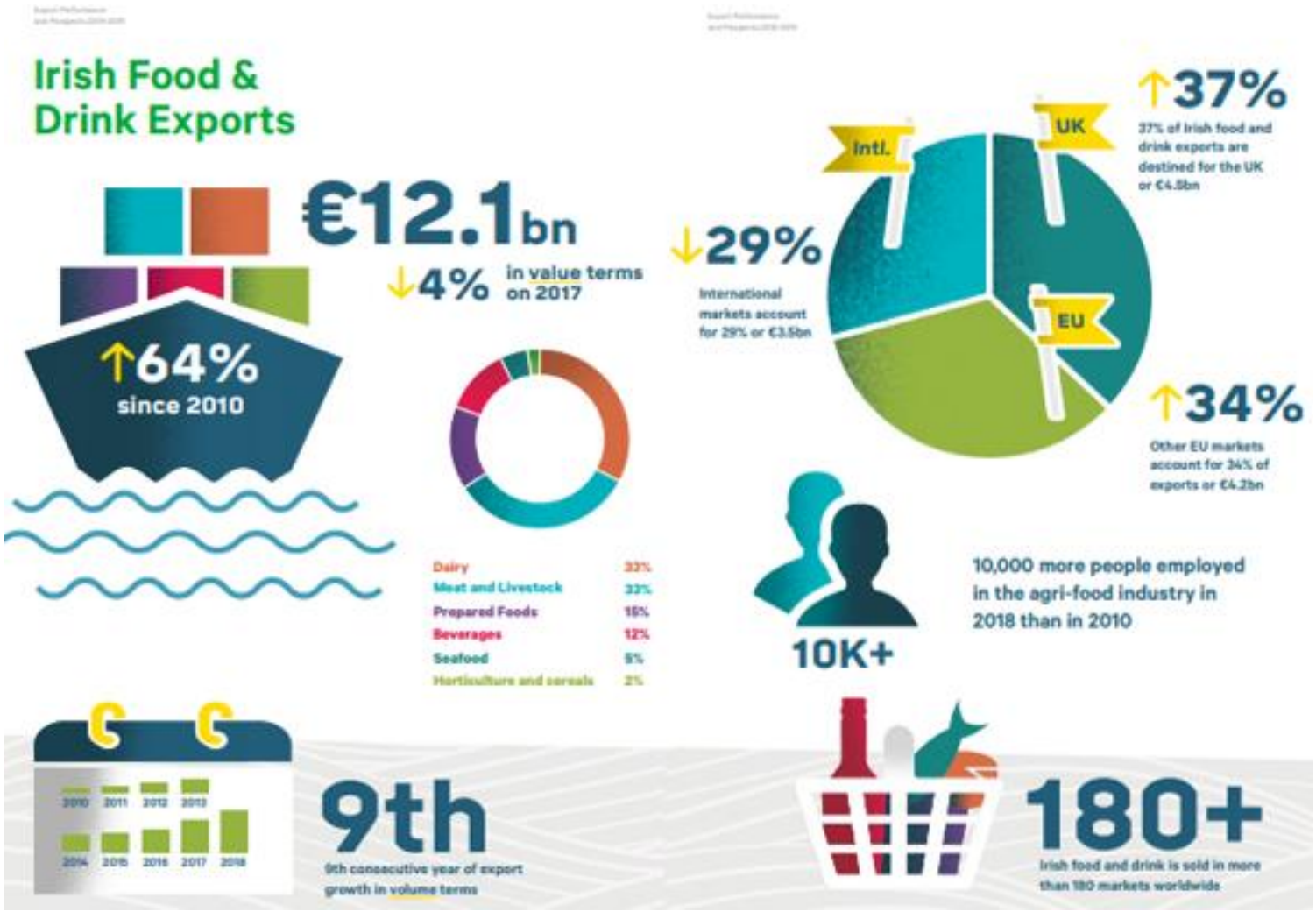


Figure 1.2: Irish food and drink exports (Bord Bia 2018).

1.2 Dairy Processing Wastewater Generation

For every litre of milk processed, 0.2-10 litres of wastewater can be produced (Dhall *et al.* 2012). Different dairy processing products generate characteristic waste streams with varying nutrient compositions, as outlined in Table 1.1. The most significant nutrients in terms of wastewater treatment are chemical oxygen demand (COD), nitrogen (N) and phosphorus (P). The concentrations of these nutrients in dairy wastewater can range from; 1,150 – 68,814 mg/L COD, 14 – 1,462 mg/L N, and 7.2 – 650 mg/L P. EPA regulations allow for no more than 50mg/L COD, 10mg/L N and 5mg/L P in effluent discharged to water bodies. Strict regulatory limits are necessary to prevent eutrophication in receiving water bodies, which are particularly relevant in the case of dairy processing streams which carry high COD, N and P loads. The strength and volume of wastewater produced by the dairy processing industry is not suitable for remediation in municipal treatment facilities. As a result, large dairy processing sites in Ireland incorporate on-site wastewater remediation facilities comprising varying configurations of chemical and biological treatments.

Table 1.1: Organic and inorganic characteristics of various dairy processing wastewater streams (Minescu *et al.* 2016).

Origin	COD (mg/L)	Fats (mg/L)	TKN (mg/L)	P_t (mg/L)	pH	TSS (mg/L)	VSS (mg/L)	C:N	Reference
DP	4000	400	55	45	8–11	675	635	73	Kasapgil *et al.* (1994)
DP	4000	–	200	60	5–9	–	500	20	van den Berg (1984)
DP	2926	294	36	21	6.7	–	–	81	Gutierrez (1991)
Whey	6600	–	650	650	4–6	–	2000	10	van den Berg (1984)
DP	2125		70	100	9.8	280	250	30	Monroy *et al.* (1985)
DP	4500	350	60	50	–	800	–	75	Craggs *et al.* (2000)
DP	1750	–	75	9.1	–	400	355	23	Koyuncu *et al.* (2000)
CP	4430	754	18	14	7.32	1100	–	246	Koyuncu *et al.* (2000)
YB	1500	–	63	7.2	–	191	–	24	Koyuncu *et al.* (2000)
DP	1150–9200	–	14–272	8–68	6–11	340–1730	255–830	33–82	Demirel *et al.* (2005)
Whey	68,814		1462	378	–	–	–	47	Demirel *et al.* (2005)
DP	3380	260	51	22	7.9	830	750	66	Latif *et al.* (2011)

DP, dairy processing; CP, cheese production; YB, yoghurt and buttermilk processing; TKN, total Kjeldahl nitrogen; P_t, total phosphorus; TSS, total suspended solids; VSS, volatile suspended solids.

1.3 Treatment of Dairy Processing Wastewater.

1.3.1 Chemical treatment options.

Chemical treatment of wastewater includes the use of precipitation, oxidation, neutralization and stabilization reactions to remove nutrients and organic matter from wastewater. There are a number of chemical treatments available for phosphate removal, all of which use metal salts or calcium, usually as $Ca(OH)_2$ (lime) at various stages in the treatment scheme. The purpose of adding metal salts is to facilitate adsorption of phosphate into flocs, while the addition of calcium facilitates the precipitation of hydroxyapatite (Metcalf & Eddy 2014).

$$10Ca_2^{+} + 6PO_4^{3-} + 2OH^{-} \longleftrightarrow Ca_{10}(PO_4)_6(OH)_2$$

Figure 1.3: Reaction for chemical removal of phosphate using lime.

Table 1.2: Applications of chemical unit processes in wastewater treatment (adapted from Metcalf & Eddy 2014).

Process	**Application**
Advanced Oxidation Process	Removal of refractory compounds
Chemical coagulation	The chemical destabilization of particles in wastewater to bring about their aggregation during flocculation
Chemical disinfection	Control of biofilm growth, control of odours
Chemical neutralization	pH control
Chemical oxidation	Removal of BOD, grease, ammonium, destruction of microorganisms, control of odours, removal of resistant organic compounds.
Chemical precipitation	Enhanced removal of solids and BOD in primary sedimentation facilities, removal of phosphate, ammonium, and heavy metals
Chemical scale control	Control of scaling due to calcium carbonate and related compounds
Chemical stabilization	Stabilization of treated effluents
Ion exchange	Removal of organic compounds

Both phosphate and NH_4^+ can be removed during the formation of magnesium ammonium phosphate hexahydrate, or struvite. This process is dependent on the ionic strength, pH, alkalinity and temperature of the waste stream.

$$Mg_2^+ + NH_4^+ + PO_4^{3-} + 6H_2O \longleftrightarrow MgNH_4PO_4 \bullet 6H_2O$$

Figure 1.4: Reaction for struvite formation.

Careful control of struvite formation is necessary, as uncontrolled build-up of struvite crystals can cause blockages in pipelines (Metcalf & Eddy 2014). Chemical treatment methods, particularly for phosphate removal, are still used to treat dairy processing wastewater. The ACTIFLO® system from Veolia is one such process. The costs of recovering phosphate during wastewater treatment has been calculated as €2-8 per kg (Molinos-Senante *et al.* 2010). In addition, chemical treatment is an additive process, resulting in an increase in dissolved compounds. The resulting chemical precipitant sludge is often mixed with biological sludge and spread on land. This is in contrast with biological treatment methods, which are subtractive, in that the treatment method does not increase the concentration of dissolved compounds as it removes nutrients from the wastewater.

1.3.2 Biological Nutrient Removal.

Biological nutrient removal (BNR) systems are often used in dairy wastewater treatment for nutrient removal and energy recovery in the form of biogas. Many different BNR configurations have been reported, offering the dairy industry a range of operational system designs depending on the particular wastewater treatment requirements. BNR can be performed as anaerobic digestion; which covers nutrient removal and energy recovery, as an activated sludge process; which removes nutrients, or as a combination of both. Figure 3 below illustrates the activated sludge process.

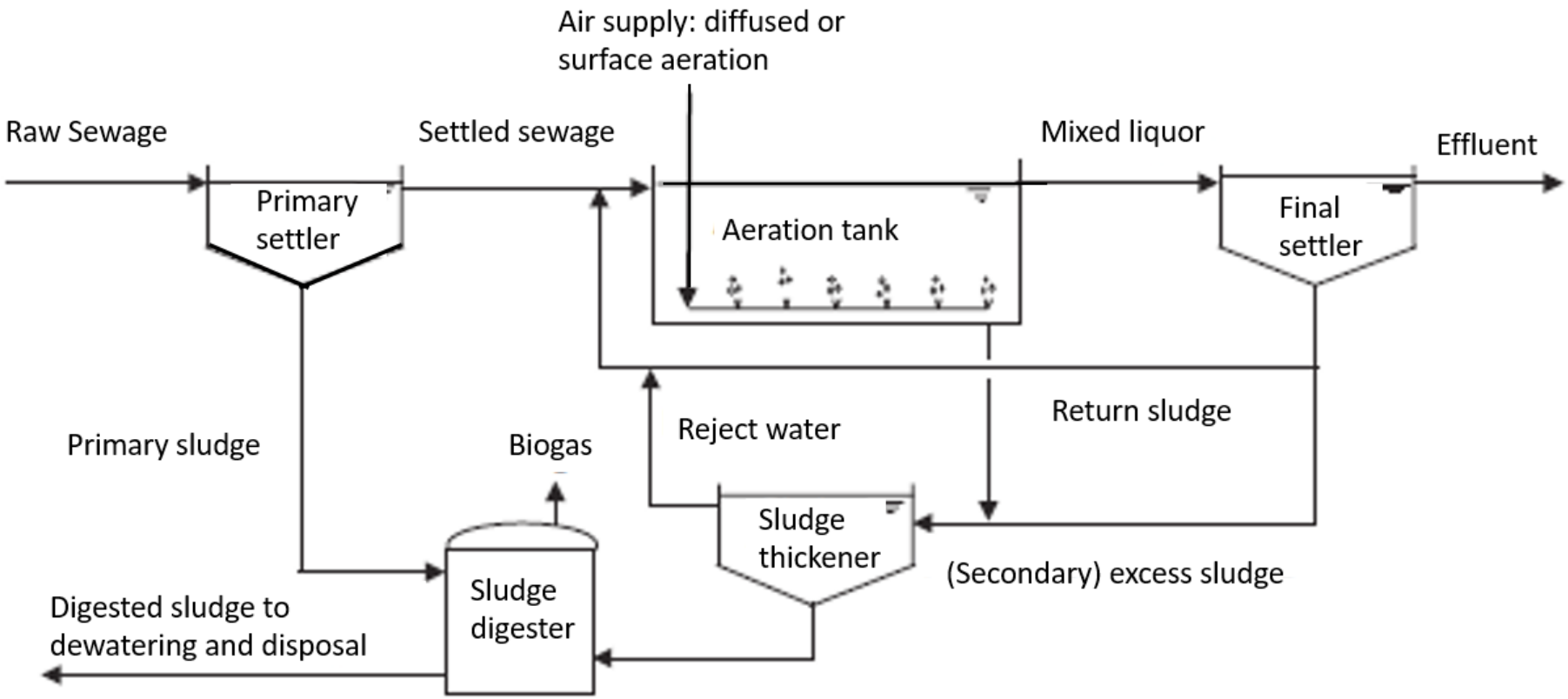


Figure 1.5: Schematic of the basic configuration of the activated sludge system designed for primary and secondary treatment (van Haandel and van der Lubbe 2012).

Primary treatment of wastewater is the initial separation of solid and liquid waste. In the context of dairy processing wastewater, the solids separated are the fats and other large particles that may enter the waste stream from storm water. The activated sludge process comprises part of the secondary treatment step, using aerated microbial biomass to remove COD from the water (van Haandel and van der Lubbe 2012). Tertiary treatment systems focus on removal of N and P from the wastewater and were developed in the latter half of the 20th century. Several system configurations for BNR now exist, all of which are modifications of activated sludge processes used for secondary treatment. Two such configurations are shown in Figures 1.6 and 1.7 below.

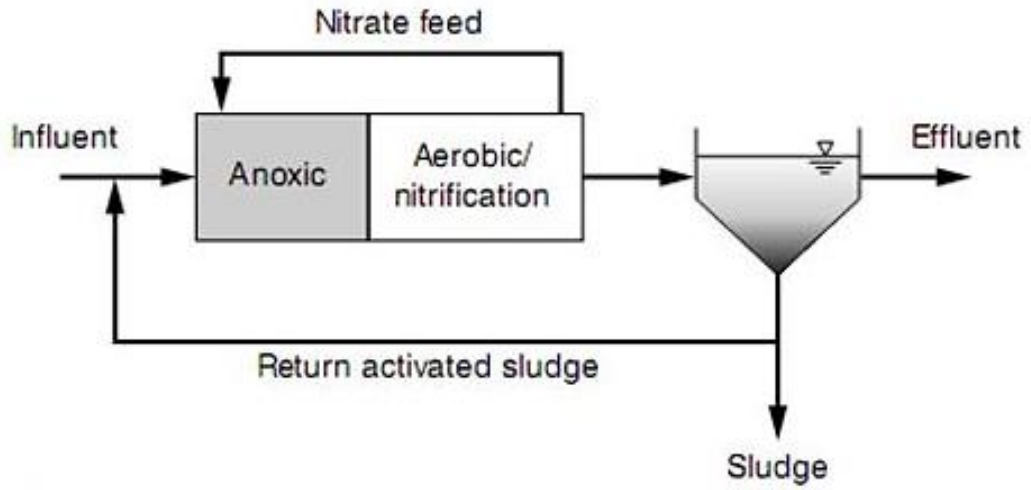


Figure 1.6: Schematic of Modified Ludzak-Ettinger configuration for N removal (adapted from Metcalf & Eddy 2014). The original Ludzak-Ettinger did not contain an internal recycle from aerobic to anoxic zone.

The Ludzack-Ettinger (LE) process was the first system for biological nitrogen removal developed with a pre-anoxic zone for denitrification (Ludzack and Ettinger 1962). The purpose of the aerobic zone is to allow for nitrification (oxidation of NH_3 or NH_4 to NO_3^-). In the modified version, the nitrate is then returned to the anoxic zone where denitrification (reduction of NO_3^- to N_2) occurs, thus removing N from the system. COD removal is carried out in both zones.

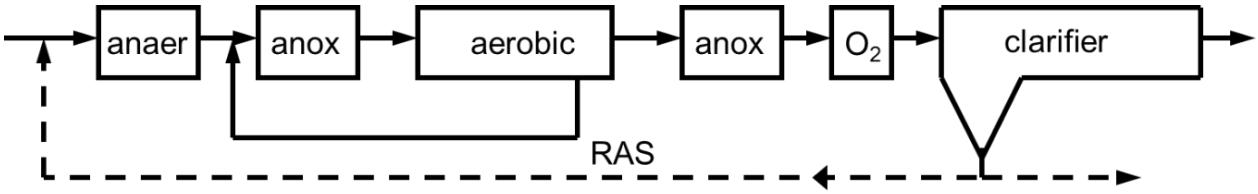


Figure 1.7: Schematic of modified Bardenpho configuration for P removal (Kroiss *et al.* 2011).

The Bardenpho process was developed by James L. Barnard in the early 1970's and was later modified for Enhanced Biological Phosphorus Removal (EBPR) by addition of an anaerobic zone as shown in Figure 1.7 (Kroiss *et al.* 2011, Barnard 1974). During EBPR, mixed liquor from activated sludge is digested anaerobically with influent or other wastewater containing Volatile Fatty Acids (VFAs). This enriches for phosphorus accumulating organisms (PAOs) within the anaerobic zone, which can utilize VFAs and transform them into intracellular carbon storage products.

These configurations were developed for municipal and domestic wastewater treatment but can be applied to industrial wastewater processing. Dairy processing plants often use Anoxic/Oxic configurations (see Figure 1.6) in their activated sludge processes. Another configuration found in dairy processing wastewater treatment is a modification of the A/O process, where an anaerobic zone is added upstream of the anoxic zone. This is known as the A2O process or A/A/O process. The A2O process has a similar function to the modified Bardenpho process. Table 1.3 below compares the use of the A2O and A/O processes from case studies in recent literature. Figure 1.8 shows a typical A2O process configuration.

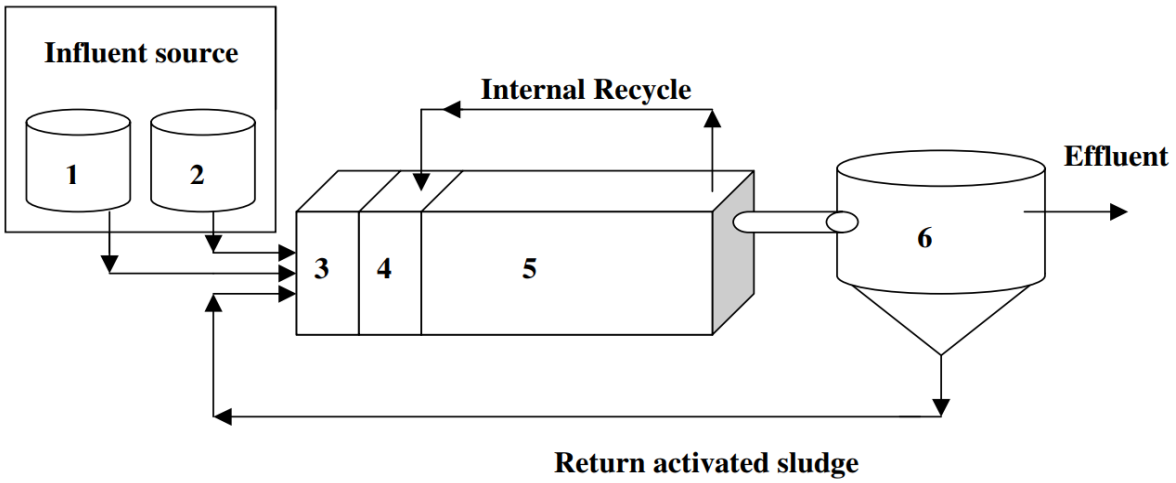


Figure 1.8: Schematic of A/A/O reactor configuration (Mulkerrins *et al.* 2004). Key: 1 = interstage basin, 2 = primary acidogenic fermenter, 3 = anaerobic zone, 4 = anoxic zone, 5 = aerobic zone, 6 = clarifier.

Other options for BNR treatment include but are not limited to; oxidation ditches, membrane filter technology and Sequencing Batch Reactors (SBR). SBR technology operates in a single tank, with 4 phases of operation; fill with a fixed volume of wastewater, treatment by aerating and stirring the mixed liquor, settle the contents by turning off the aeration and mixing, and drawing off a fixed amount of treated effluent from the top. This reactor technology was not widely used in the early days of wastewater treatment due to its labour-intensive operation. However, with the advent of programmable timers and automation, SBRs are now quite easy to use and provide an attractive alternative for BNR at municipal treatment facilities or smaller industrial treatment facilities that may not have the space, funds or wastewater output required for some of the more elaborate processes (Metcalf & Eddy 2014).

The drawback to an SBR is that continuous aeration can be costly, which is particularly problematic for smaller plants or treatment facilities in developing countries. To combat this, a relatively new modification of SBR technology includes intermittent aeration to provide aerobic and anoxic periods throughout the treatment phase. This allows for nitrification and denitrification in a single reactor, as well as COD and P removal. This type of SBR is called Intermittently Aerated Sequencing Batch Reactor (IASBR), and it has been used successfully to treat high strength industrial wastewater at both laboratory and pilot scale, with nutrient removal efficiencies of >95% COD, >90% N and >80% P respectively (Henry *et al.* 2014, Tarpey 2016, Leonard *et al.* 2018(a), Leonard *et al.* 2018(b)).

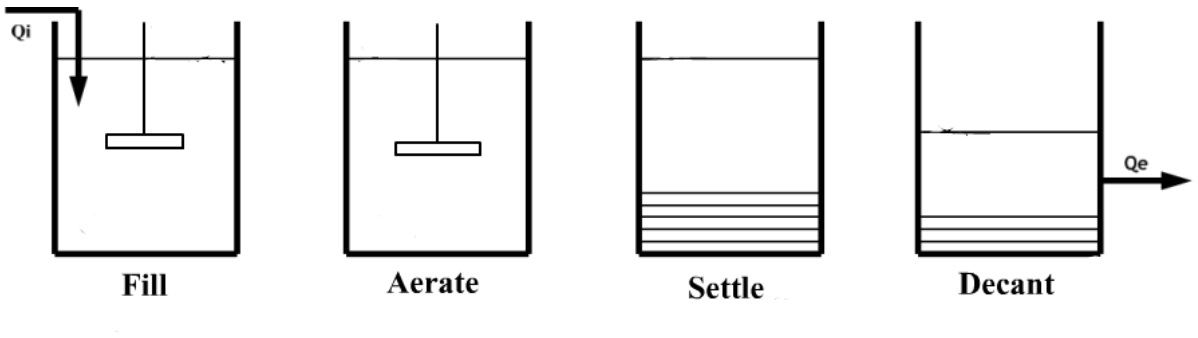


Figure 1.9: Simple schematic of typical SBR operational phases (Lim and Vadivelu 2014).

Table 1.3: Comparison of reactor design configurations based on their performance parameter and effluent characteristics. Question marks represent data that was not provided in the relevant literature, nor could it be extrapolated from data provided.

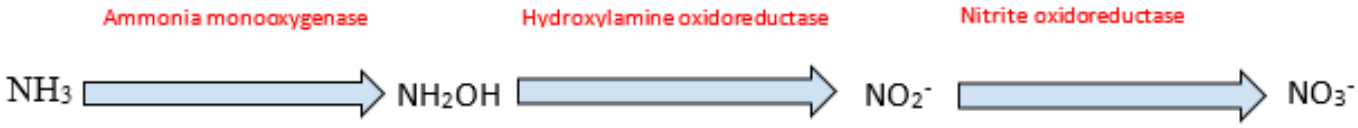
Wastewater type	Process	Wastewater Strength (COD, N, P)	%COD removal	%N removal	%P removal	HRT	SRT(d)	Global Location	Reference
Dairy Processing Wastewater.	A/A/O	555 - 3230 mg/l COD, 5-107 mg/l combined NH_4^+ and NO_3^-, 9.3-93 mg/l - P	94.2	96.7	74.8	28.6 h	10-15	Ireland	(Mulkerrins *et al.* 2004).
Winery effluent	MLE	800 - 3050 mg/l COD (mostly ethanol), 4000-6000 mg/l NH_4^+	98-99	90	n/a	2.2 - 4.0d.	25	Spain	(Carrera *et al.* 2003).
Sewage	A2O/ AO	31.4-102.4 mg/l BOD, 26.3-57.1mg/L TN.	93 BOD removal	72	n/a	12.7h	?	South Korea	(Lim *et al.* 2009).
Domestic wastewater	A2O	105.4-270.6mg/L COD, 59.6-85.6mg/L TN.	n/a	60-85	n/a	9.31-13.96h	15-20	China	(Zeng *et al.* 2010).
Synthetic wastewater	A2O	180-460mg/L COD, 43.3-63.8mg/L TN, 6.18-9.15mg/L PO_4-P,	>90	80-96	>95	8.064h	12	China	(Peng *et al.* 2006).
Dairy Processing Wastewater	IASBR	1210 – 9770mg/L COD, 60 – 220mg/L TN, 44 – 66mg/L TP.	>97	>95	>95	4d	16	Ireland	(Leonard *et al.* 2018(b))
Simulated dairy wastewater	SBR	3900mg/L COD, 113.18mg/L TKN	>90	40-70	n/a	15-30h	20	India	(Kushwaha *et al.* 2013)

For effective N and P removal from wastewaters, certain nutrient/nutrient or nutrient/microorganism ratios have been recommended in literature. According to the EPA (1997) manual on Primary, Secondary and Tertiary Treatment, a COD/N ratio of >10 and a COD/P ratio of >40 is desirable for N and P removal. The food to microorganism ratio is the ratio of COD to microbial biomass in the system. The recommended F/M ratio for 20-30-day SRT is 0.05-0.1g BOD/g VSS.d and for 5-7 day SRT the recommended F/M ratio is 0.3-0.5g BOD/g VSS.d, (Metcalf & Eddy 2014). A ratio of 7:1 for alkalinity (as carbonate or bicarbonate) to ammonia is also promoted. As the data in Table 1.1 suggests, dairy processing wastewater contains sufficient concentrations of COD, N and P to fulfil the COD/N and COD/P ratios recognised as key to effective biological remediation.

1.3.3 Microbiology of Nitrogen Removal for Biological Nutrient Removal.

Nitrogen in wastewater treated by biological means is often carried out by the complementary processes of nitrification and denitrification.

(a)



(b)

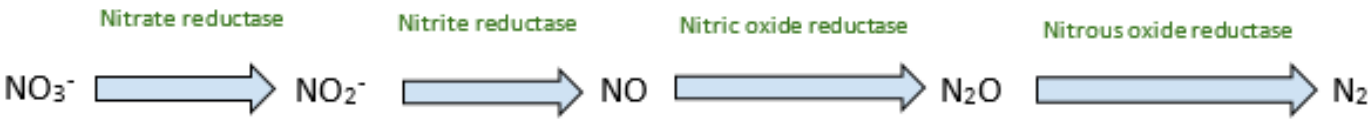
$$NH_3 + 2O_2 \longrightarrow NO_3^- + H^+ + H_2O$$

Figure 1.10: (a) Representation of the nitrification process, including relevant enzymes. (b) Stoichiometry of nitrification.

Microorganisms that carry out nitrification are called nitrifiers. Most nitrifying bacteria are members of the *Proteobacteria*. There is no known single bacterium that can carry out the full process of nitrification, thus nitrifiers are separated into two groups; the ammonia-oxidising bacteria (AOB) and the nitrite-oxidising bacteria (NOB). Both AOB and NOB use carbon dioxide (CO_2) as their carbon source and they use dissolved oxygen (DO) to oxidise NH_4^+ or NO_2^- to obtain energy (Madigan *et al.* 2012). AOB and NOB can therefore be called aerobic chemoautotrophic bacteria. AOB are often given the precursor Nitroso- (eg; *Nitrosomonas*) and NOB are given the precursor Nitro- (eg; *Nitrospira*). Both AOB and NOB operate under aerobic conditions within a reactor setup. This is because AOB require over 3 times as much DO per gram of NH_4^+ in order to carry out the oxidation of NH_4^+ to NO_2^-, and

NOB require approximately equal parts DO to NO_2^- to carry out the oxidation of NO_2^- to NO_3^-. AOB and NOB grow quite slowly when compared to heterotrophs under the same conditions, (Madigan *et al.* 2012) and thus longer sludge retention and hydraulic retention times (SRT and HRT) are required for aerobic zones or phases in a reactor setup.

(a)



(b)

$$2NO_3 + 10H^+ + 10e^- \longrightarrow N_2 + 2OH^- + 4H_2O$$

Figure 1.11: (a) Representation of the denitrification process, including relevant enzymes. (b) Stoichiometry of denitrification.

Microorganisms that carry out denitrification are called denitrifiers. Denitrifying bacteria found in nature include *Pseudomonas* spp, *Bacillus* spp and members of the *Proteobacteria* (Madigan *et al.* 2012). In wastewater treatment systems, denitrification takes place during the anoxic phase of the reactor system, if such a phase is included, as nitrate reductase synthesis is repressed by oxygen (Madigan *et al.* 2012). Denitrifying organisms developing under anoxic conditions grow slowly compared to their growth under aerobic conditions due to the decreased availability of O_2 as a terminal electron acceptor. This leads to more COD consumption in order to free enough energy for biomass synthesis, since the NO_3^-/NO_2^- redox couple releases less energy than O_2/H_2O (Madigan *et al.* 2012). For this reason, the COD requirement for denitrification is higher than the COD required for nitrification, hence the rationale for placing the anoxic zone ahead of the aerobic zone in an A/O or MLE process.

NH_4^+ can also be oxidised using NO_2^- as the electron acceptor to yield N_2 and H_2O. This process is known as Anaerobic Ammonia Oxidation, or ANAMMOX. The Anammox process can be performed in either a single reactor system for high activity ammonium removal over nitrite (SHARON process), or in a completely autotrophic nitrogen removal over nitrite (CANON) process (Xinhong *et al.* 2013). Anammox, SHARON and CANON are relatively modern approaches to N removal, and a pilot-scale annamox system has been established at the Delft University of Technology in The Netherlands.

1.4 Future prospects and challenges.

Sustainable growth, in an environmentally sensitive manner, is one of the challenges faced by the dairy processing industry. At the moment, industries primarily follow a linear economy approach, whereby raw materials are converted to a product for consumption. Once the product is consumed, it is disposed of. Waste materials generated during the process are not recycled or re-purposed. The DAFM published the Food Wise 2025 strategy in 2015 to outline a progressive plan for a sustainable future in the Irish agricultural sector. This strategy states that "Environmental protection and economic competitiveness are equal and complementary: one will not be achieved at the expense of the other". This statement reiterates the principles conveyed in the European Commission legislature on waste disposal, which proposes that Europe move towards circular economy practices in order to sustain economic growth (EU Action plan 2015). Figure 1.12 explains the circular economy approach.

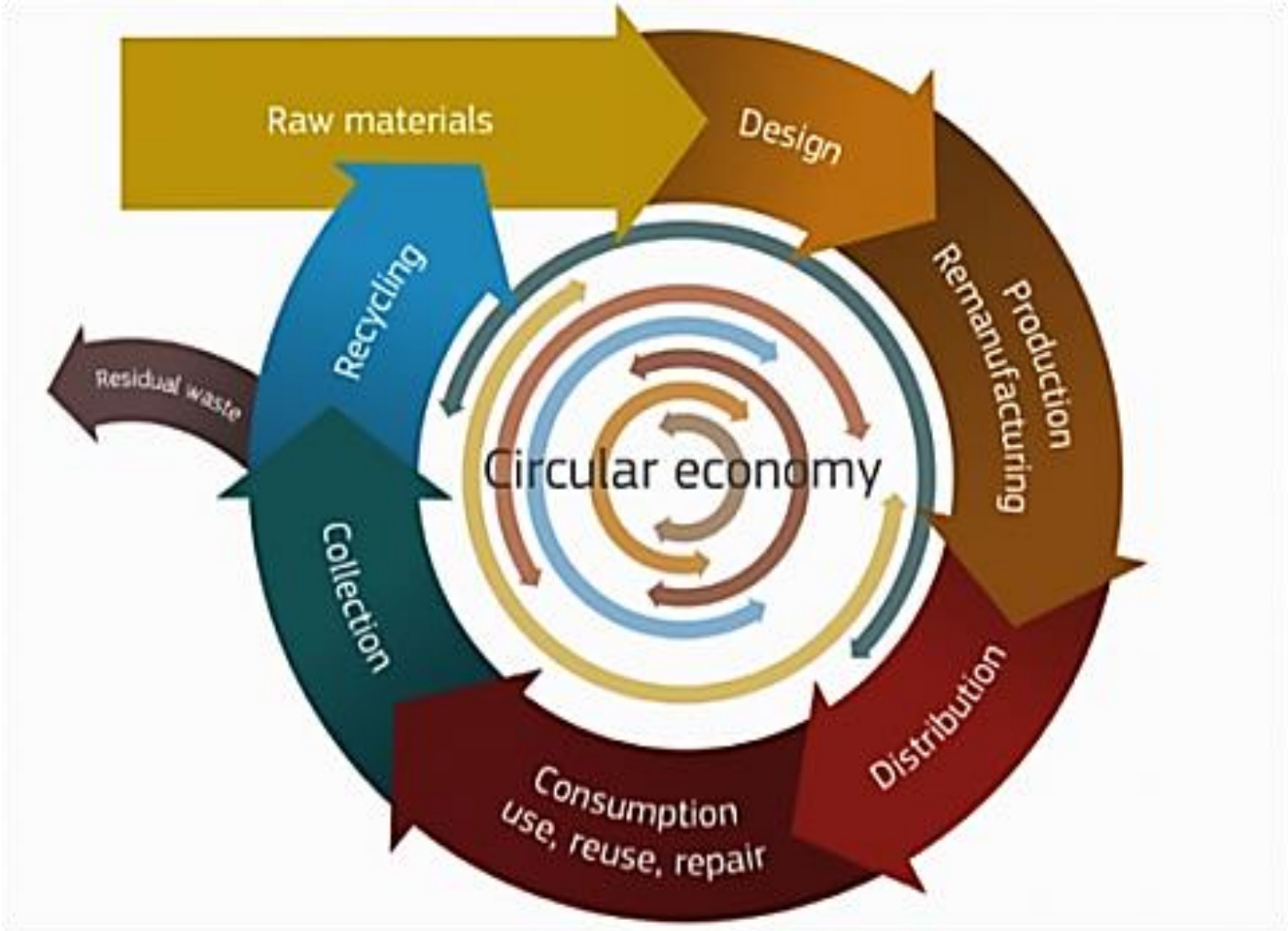


Figure 1.12: The circular economy approach (European Commission 2017).

Opportunities exist therefore to re-examine major agri-sector operators, such as dairy processing, to identify potential for waste capture in value-added products (Raji S., D.D.Sarode 2018). This Thesis examines one such opportunity by refocusing biological treatment strategies to act as conditioners of dairy processing wastewater to generate feedstocks for production of *Lemna minor*, a species of duckweed native to Ireland. Duckweed is a high protein source that could potentially be returned to the dairy herds that produce the milk as a feed during the off-peak season (Leng *et al.* 1995). Such a system would allow for dairy processing companies to secure their milk supply, reduce their effluent output, reduce their water intake from the municipal supply and provide farmers with a competitive alternative to Soya protein.

Key objectives of this study are:

- Determination of optimal aerobic reactor parameters for conditioning of wastewater as *Lemna* feedstock.
- Development of coupled reactor system module for wastewater treatment and *Lemna* propagation.
- Characterization of coupled system resilience to nutrient challenges.

Chapter 2 – AO reactor set-up and performance.

2.1 Introduction

The Dairy Industry in Ireland is one of the most important, generating almost €2 billion in revenue in 2018. Many different products are processed from milk, resulting in large volumes of wastewater with varying characteristics (table 1.1). The wastewater must be treated to remove excess COD and nutrients, in accordance with EPA discharge limits. The most commonly used treatment technology in Irish dairy industry is A/O, also known as modified Ludzack-Ettinger (MLE) process. As can be seen in Figure 1.6, this particular process is designed for N removal via nitrification/denitrification, as well as COD and some P removal. The purpose of placing the anoxic zone first is to ensure that there is sufficient COD available for denitrification without the need to add external carbon sources such as methanol. Denitrification also produces alkalinity, helping to balance the overall pH and avoiding the need to add external buffers. COD remaining after denitrification is utilized for nitrification in the oxic zone, with the internal recycle providing NO_3^- back to the anoxic zone. The clarifier allows the flocs in MLSS to settle, producing a clear effluent which is removed and, in the context of dairy processing effluent, discharged to receiving water bodies. The settled sludge is either returned to the anoxic zone or wasted, depending on the system requirements and the amount of sludge generated.

The A/O process has been previously employed to treat high-strength winery effluent at pilot scale (Carrera *et al.* 2004). The winery selected for this study produced two waste streams; one high in NH_4^+-N and low in COD, the other high in COD and devoid of NH_4^+-N. Figure 2.1 below shows the set-up used in this study, and table 2.1 outlines the composition of the wastewater streams.

Table 2.1: Composition of wastewater streams from Carrera *et al.* 2004.

Component	N-wastewater ($mg\,l^{-1}$)	C-wastewater ($mg\,l^{-1}$)
COD	50	800–3000
NH_4^+–N	4000–6000	0
Cl^-	500–600	700–1000
SO_4^{2-}	15000–20000	300–800

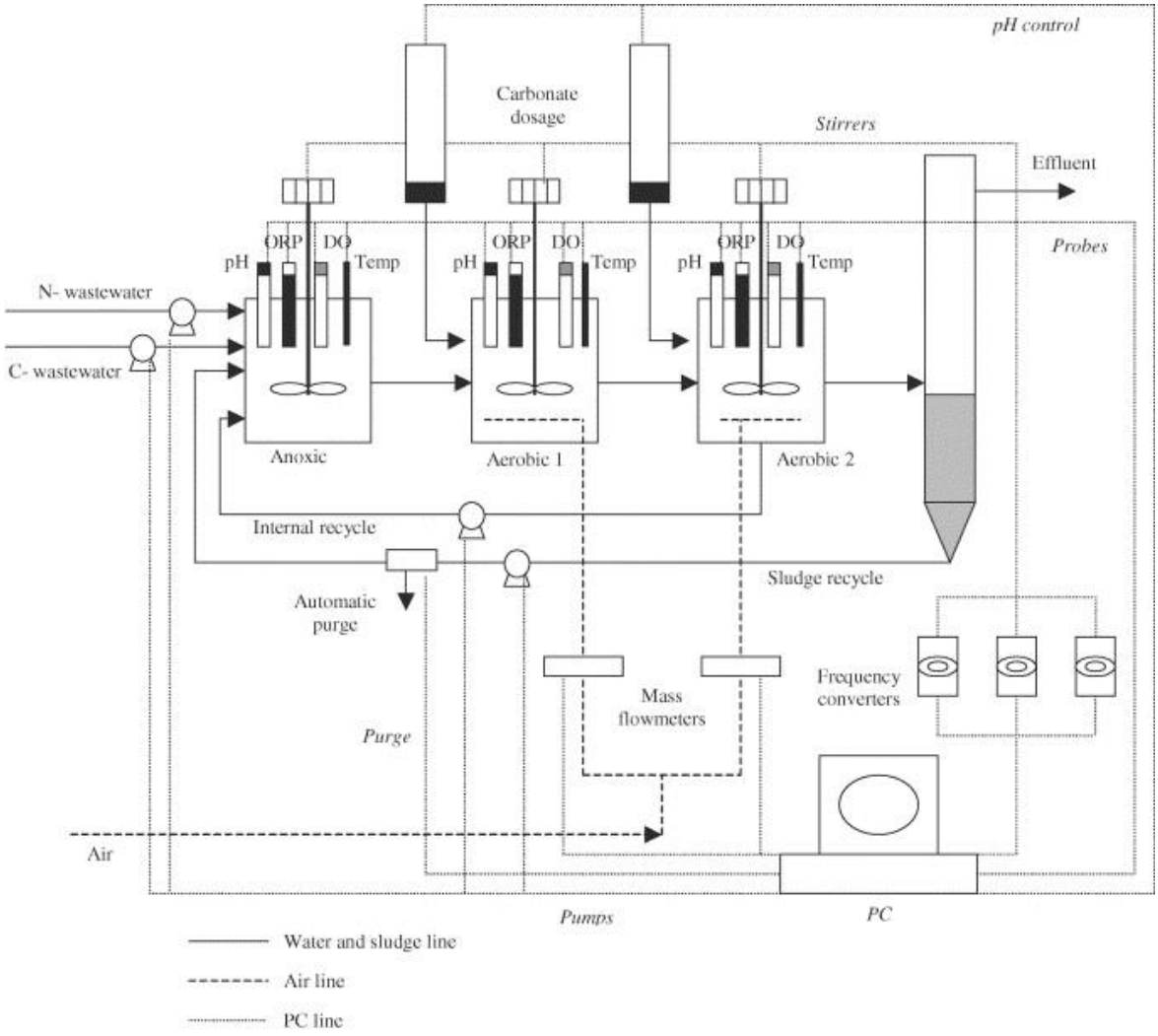


Figure 2.1: Schematic of pilot-scale A/O system from Carrera *et al.* 2004.

Both waste streams were combined in order to provide the required amount of COD for N removal. The study was run for 365 days, achieving efficiencies of up to 99% COD removal and 90% N removal. P removal was not measured. Literature regarding the use of A/O technology to treat dairy processing wastewater is sparse, with many studies focusing on A_2O, SBR or anaerobic treatment technologies instead.

The purpose of the research outlined in this chapter was to condition dairy processing wastewater for downstream *Lemna minor* production. The A/O process was selected for this research as it has been used successfully to treat high-strength wastewater, and because it is a common configuration in existing dairy processing wastewater treatment plants (Foglar *et al.* 2005, G.Z. Breisha and J. Winter 2010).

2.2 Materials and methods.

A laboratory scale A/O wastewater treatment system, shown in Figure 2.2 below, was used to conduct the experiments outlined in this chapter. The system was designed to mimic a typical industrial BNR system at laboratory scale.

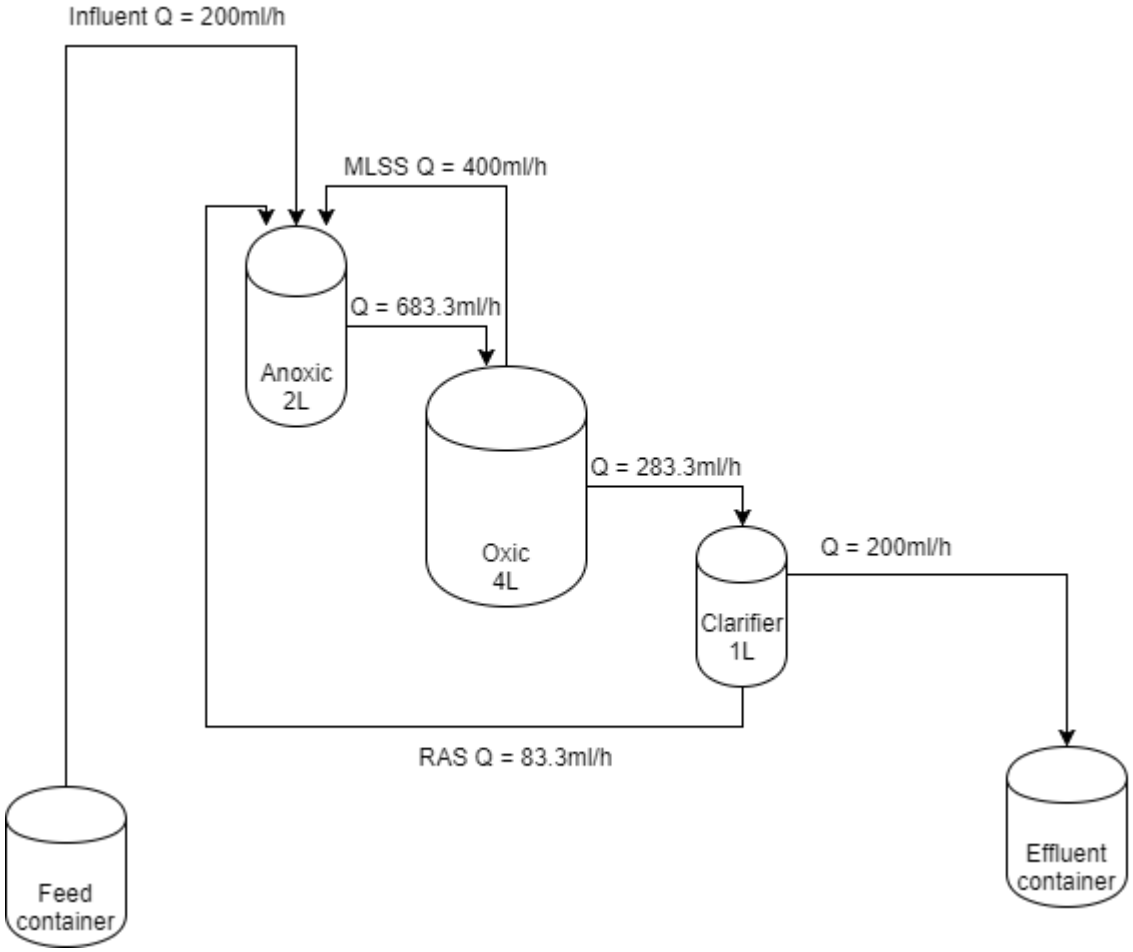


Figure 2.2: Schematic of A/O reactor design, including flow rates and volumes.

The seed sludge for the system was obtained from the anaerobic digestor in Dairygold, Mitchelstown, Co. Cork. Both the anoxic and oxic zones were seeded with a starting MLSS concentration of 4 g/L for synthetic operation and 7 g/L for real-time operation. The system was initially fed with synthetic wastewater, the composition of which is outlined in Table 2.2. Synthetic wastewater was made as necessary using tap water. The purpose of using synthetic wastewater was to investigate key operational parameters with a stable influent before using real wastewater, as composition of dairy processing wastewater varies greatly (table 1.1). Once these key operational parameters were established, real-time dairy processing effluent was fed into the system. Real-time dairy processing wastewater was

supplied by Dairygold, Mitchelstown, Co. Cork. Dairygold produces a variety of products including cheese and butter, among other products. The waste streams from each product are collected and mixed in a balancing tank before entering the anaerobic digestor on site. Therefore, the composition of the wastewater obtained varied greatly depending on the day-to-day operation of the plant. COD ranged from 2500 – 7000 mg/L, PO_4 from 150 – 300 mg/L, NH_4 from 5 – 20 mg/L and pH from 2 – 12. Wastewater was collected in 25L drums approximately every 2 – 4 weeks and stored at 4°C until needed.

The A/O system consisted of two vessels; anoxic, oxic and a clarifier, with working volumes of two, four and one litres respectively (Figure 2.2). The system employed gravity-flow from one vessel to the next, with influent flow rate (Q) controlled by an EW-07520-60 MasterFlex Peristaltic Pump with Motor Drive 6-300 RPM. The attached pump head was a Masterflex L/S Multichannel Pump Head for L/S Tubing; 8-Channel pump head (Cole-Parmer®, USA).

Table 2.2: Synthetic wastewater composition, adapted from Henry (2014).

Sodium acetate	3 g
Yeast Extract	218 mg
Skimmed milk powder	872 mg
NH_4Cl	167.3 mg
Urea	129.9 mg
K_2HPO_4	61.7 mg
$NaHCO_3$	172 mg
$MgSO_4 \cdot 7H_2O$	50 mg
$FeSO_4 \cdot 7H_2O$	10 mg
$MnSO_4 \cdot H_2O$	2 mg
$CaCl \cdot 6H_2O$	3 mg

Mixed liquor recycle from the oxic zone to the anoxic zone was set to be 2Q and was thus controlled using the same pump as influent Q and two sets of tubing. Effluent flowed through a port at the top of the clarifier and was collected in a container for further use as influent for *Lemna* growth studies. The Hydraulic Retention Time for the whole system (anoxic zone, oxic zone and clarifier) was 1.46 days initially, with changes being made as

necessary. A return activated sludge (RAS) recycle was also implemented to control Solids Retention Time (SRT). SRT was initially set to 30 days for synthetic wastewater and was adjusted as necessary throughout the reactor operational period on synthetic wastewater. SRT was much longer on real-time wastewater, as no sludge wastage was implemented, only sludge return. RAS was controlled for both synthetic and real-time experiments using a Watson-Marlow 323u Peristaltic Pump (Watson-Marlow Pumps Group, USA). Aeration was provided using a Welch® 2511 dry vacuum pump/compressor (Gardner Denver, USA) which supplied air at $\geq$ 5 LPM. Mixing was provided by magnetic stirring plates with adjustable RPM to control the mixing speed.

The reactor system was run for 169 days on synthetic wastewater and 210 days on real-time wastewater. Samples of 50ml were taken 3 times per week from Influent, Anoxic and Oxic zones. COD, PO_4^{3-}, NH_3, NO_2^- and NO_3^- were determined with a HACH® DR 2800 spectrophotometer (Hach, USA). The procedures for each chemical test are outlined below. All chemical tests were carried out according to the HACH® DR 2400 procedures manual (Hach company 2004). Reagents used were also as per the HACH® DR 2400 procedures manual unless otherwise specified.

Chemical Oxygen Demand (COD)

Method 8000 for High Range (HR) 20 – 1500 mg/L COD was used to determine the COD of all samples with both synthetic and real-time wastewater. Steps 1 and 2 of the procedure were replaced with centrifugation of the samples. Samples were diluted prior to addition to reagent vials in order to ensure the test volume remained within the measuring range. Synthetic wastewater was diluted to 1:3, with 1:5 sometimes being necessary if settling was poor and suspended solids remained in the sample after centrifugation. Real-time wastewater dilutions ranged from 1:2 – 1:6, depending on the strength of each batch of wastewater. Reagent vials used for this test were COD cuvettes 0 – 1500 mg/L, supplied by Reagecon Diagnostics LTD, Ireland. Digestion of samples was carried out using a Hach COD reactor, model 45600.

Orthophosphate (PO_4^{3-})

Method 8114, Molybdovanadate method for 0.3 – 45.0 mg/L was used to determine the orthophosphate levels of all samples with both synthetic and real-time wastewater. Protocol for reagent solution was followed, not the protocol for AccuVac® Ampules, which is also outlined under method 8114. Samples were centrifuged and diluted prior

to testing in order to ensure the test volume remained within the measuring range. Synthetic wastewater was diluted to 1:3, with 1:5 sometimes being necessary if settling was poor and suspended solids remained in the sample after centrifugation. Real-time wastewater dilutions ranged from 1:3 – 1:10, depending on the strength of each batch of wastewater.

Ammonia (NH_3)

Method 8036, Nessler method for 0.02 – 2.50 mg/L was used to determine the ammonia levels of all samples with both synthetic and real-time wastewater. Samples were centrifuged and diluted prior to testing in order to ensure the test volume remained within the measuring range. Synthetic wastewater dilutions ranged from 1:2 - 1:25, as NH_3 levels fluctuated with natural decomposition of Urea and ammonification of organic nitrogen present in skimmed milk powder and yeast extract. Real-time wastewater dilutions ranged from 1:3 – 1:10, depending on the strength of each batch of wastewater.

Nitrite (NO_2^-)

Method 8153, High Range (HR) for 2 – 250 mg/L was used to determine the nitrite levels of all samples with both synthetic and real-time wastewater. Nitrite levels in synthetic wastewater were measured from day 122 onwards, with real-time levels measured for the duration of the reactor operational period on real-time effluent. Samples were centrifuged prior to testing. Dilutions were not performed on samples for nitrite testing.

Nitrate ($NO3^-$)

Method 8171, Medium Range (MR) for 0.1 – 10.0 mg/L was used to determine the nitrate levels in all samples with both synthetic and real-time wastewater. Nitrate levels in synthetic wastewater were measured from day 45 onwards, with real-time levels measured for the duration of the reactor operational period on real-time effluent. Samples were centrifuged prior to testing. Dilutions were not performed on samples for nitrate testing. Method 8039, High Range (HR) for 0.3 – 30.0 mg/l was also used for real-time wastewater depending on the strength of each batch.

Dissolved oxygen (DO) and Temperature were monitored using a HI 98186 DO meter (Hanna Instruments, USA). Mixed Liquor Suspended Solids (MLSS) and Mixed Liquor Volatile Suspended Solids (MLVSS) were measured in accordance with APHA standard methods (2015). pH was measured using pH strips (Sigma-Aldrich, USA) for synthetic operation and with a Mettler-Toledo® seven-easy pH meter (Mettler Toledo, USA) for real-time operation. Floc size/density was monitored using a Leica DM3000 Type DFC490 light microscope with attached camera for image capture (Leica Microsystems, Germany). Gram staining, Neisser staining and DAPI staining techniques were used to monitor and identify microbial changes within floc structures. Food to microorganism ratio (F/M) was calculated using the following formula adapted from Metcalf & Eddy 2014:

$$F/M = \frac{[\text{g COD}] \times [\text{Q L/d}]}{[\text{g VSS}] \times [V_{reactor}\ \text{L}]}$$

A sample calculation is provided below using data from day 1 of A/O operation on synthetic wastewater. The F/M calculation is for the anoxic zone of the reactor.

$$F/M = \frac{[2.116\ \text{g COD}] \times [4.8\ \text{L/d}]}{[4.04\ \text{g VSS}] \times [2\ \text{L}]} = 1.26\ \text{g COD / g VSS} \bullet \text{d}$$

2.3 Results and Discussion.

The purpose of this project was to condition dairy processing wastewater for downstream application as growth media for *Lemna minor*. An AO (also known as MLE) reactor system was selected for this experiment as it is commonly used in dairy processing wastewater treatment, and it has been shown to remove COD and N with high efficiency from high – strength industrial wastewater (Carrera *et al.* 2004). The system was initially run with synthetic wastewater (Henry 2014) in order to establish key operational parameters for conditioning, prior to feeding with real dairy processing wastewater. Results of both synthetic and real-time wastewater trials will be presented and discussed in this section.

2.3.1 Floc formation and MLSS with synthetic wastewater.

MLSS levels fluctuated between 2000 and 6000 mg/L for the first 30 days of operation on synthetic media. Between days 30 and 75 MLSS dropped to between 1000 and 2500 mg/L, after which MLSS levels stabilized around 2000 – 3000 mg/L. The peak seen in the anoxic measurement at day 117 (Figure 2.3) happened because the magnetic stirrer lost power for 48 hours, creating an anaerobic environment, which caused the anoxic zone to become septic. Once the issue with the stirrer was rectified, the MLSS returned to previous levels. However, the anoxic sludge remained septic. As a result, sludge had to be wasted instead of recycled, causing the drop in MLSS seen between days 150 and 162.

Initial floc size in the seed sludge was measured as 100 – 200µm in diameter. Each individual floc was rather compact, with sludge settling occurring quite rapidly. Floc density in the seed sludge was also quite high, as can be seen from Figure 2.4. However, floc size and density had deteriorated by day 30, with flocs becoming less compact and spaced further apart. Filaments could also be seen among the floc structures. This continued for the remainder of the synthetic trial, with some more stable flocs appearing on day 117 in the anoxic zone. However, the flocs quickly returned to their previous state and remained that way until the end of the trial. Average F/M ratio for the anoxic zone across the operational period was 3.13 g COD / g VSS • d. High F/M can be attributed to high influent COD levels and relatively low VSS across the operational period as well as the smaller volume in the anoxic zone. The Oxic zone yielded an average F/M of 0.42 g COD / g VSS • d. This is in line with the reference literature values of 0.3 – 0.5 g BOD / g VSS • d given in chapter one.

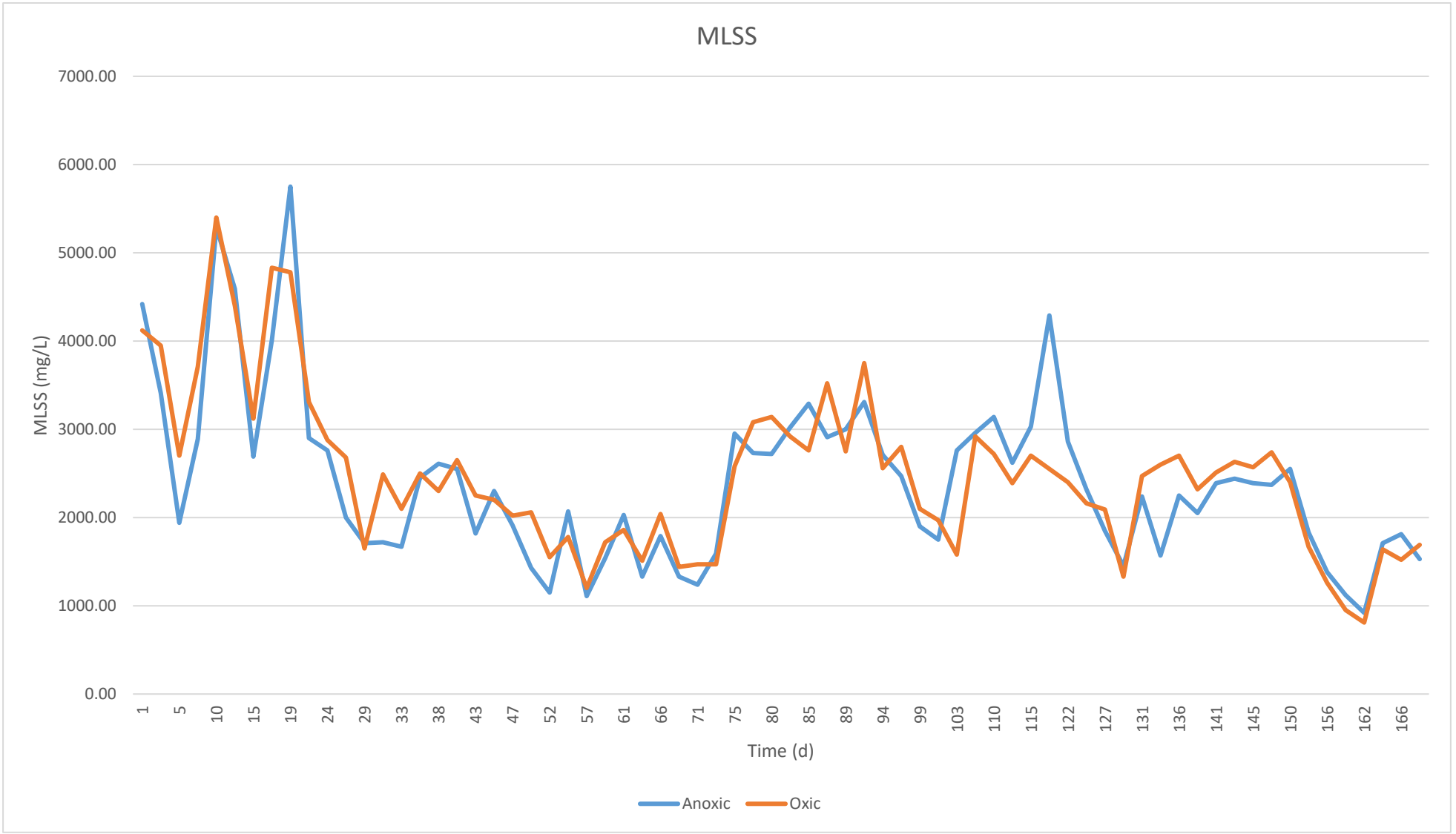


Figure 2.3: MLSS levels with synthetic wastewater.

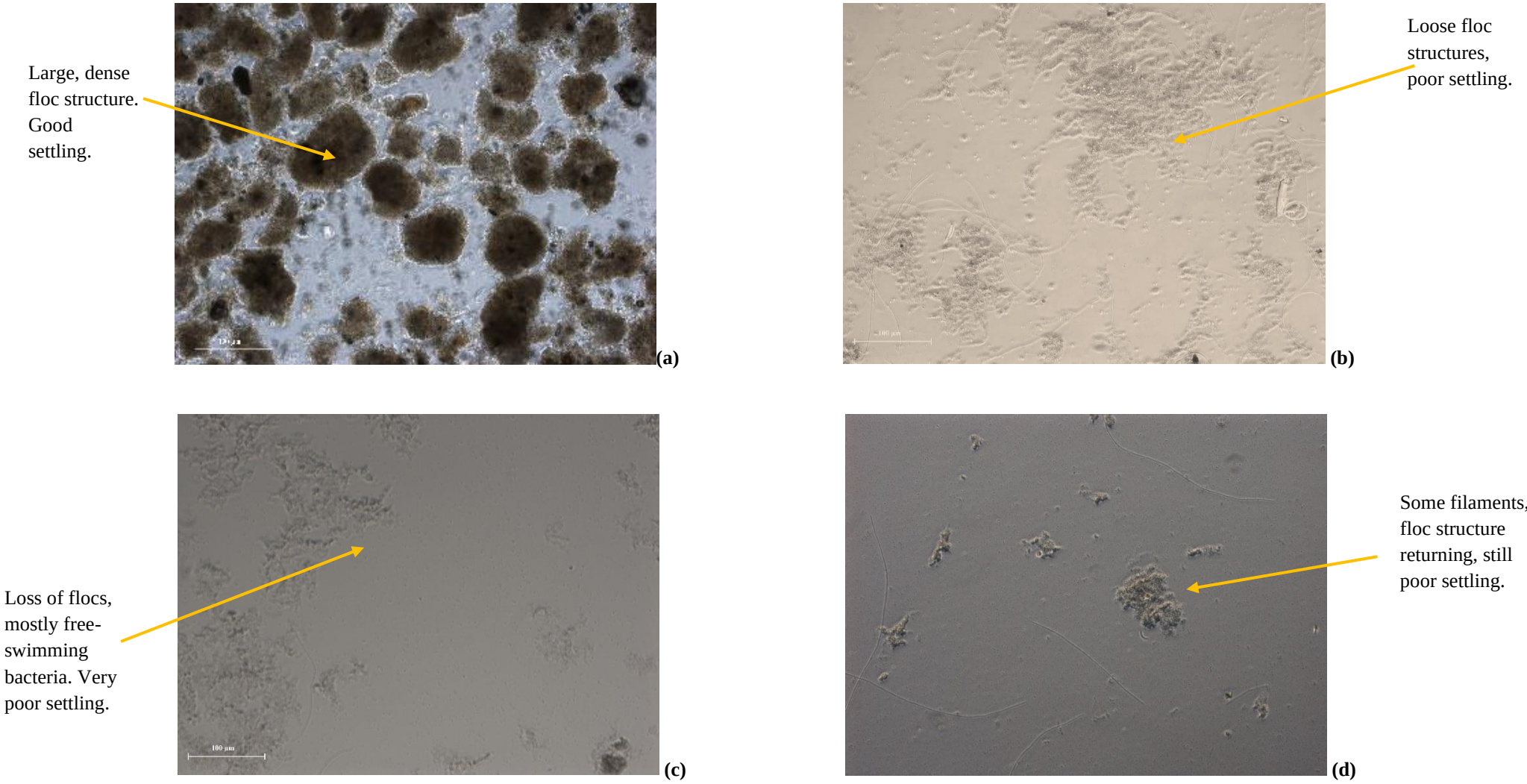


Figure 2.4: (a) Image of seed sludge under phase contrast 10x magnification. (b) Image of sludge flocs from day 75 in oxic zone under phase contrast 10x magnification. (c) Image of sludge flocs from day 117 in oxic zone under phase contrast 10x magnification. (d) Image of sludge flocs from day 169 in oxic zone under phase contrast 10x magnification. Scale bar in all images is 100µm.

2.3.2 Chemical analysis of performance with synthetic wastewater.

Temperature in both the anoxic and oxic zones remained between 20 and 25°C for the full 169 days of operation. Dissolved oxygen (DO) remained at 0 mg/L in the anoxic zone and ranged from 1 – 8.5 mg/L in the oxic zone. Influent pH ranged from 6 – 7, with pH in both the anoxic and oxic zones remaining at pH 8 – 8.5. COD removal ranged from 80 – 99% across the synthetic trial (Figure 2.5), which matches reported values for COD removal in literature. COD removal remained efficient despite the MLSS being lower than reported industry values, which typically range from 4000 – 5000 mg/L MLSS. COD removal was not affected by the sepsis that occurred from day 117 onwards. The majority of COD was removed in the anoxic zone, supporting evidence of denitrification. However, despite the high removal efficiency, effluent COD was rarely below 100 mg/L, and was only once within the EPA discharge limits.

During the course of the synthetic trial, the system performance in terms of COD removal corresponded to reported values of 90 – 99% (table 1.3) for BNR systems. This was despite the MLSS levels being lower than typical industry levels of 4000 – 5000 mg/L MLSS. The fact that the majority of COD removal during both synthetic and real-time operation was observed in the anoxic zone is evidence for the occurrence of denitrification. The process of denitrification (Figure 1.11) consumes COD in order to convert nitrate or nitrite to nitrogen gas through a series of enzymatic steps. The purpose for placing the anoxic zone first in this type of reactor is to ensure enough COD for denitrification, as otherwise COD would be consumed by heterotrophic microbes in the oxic zone and an external carbon source would need to be added to provide the COD for denitrification.

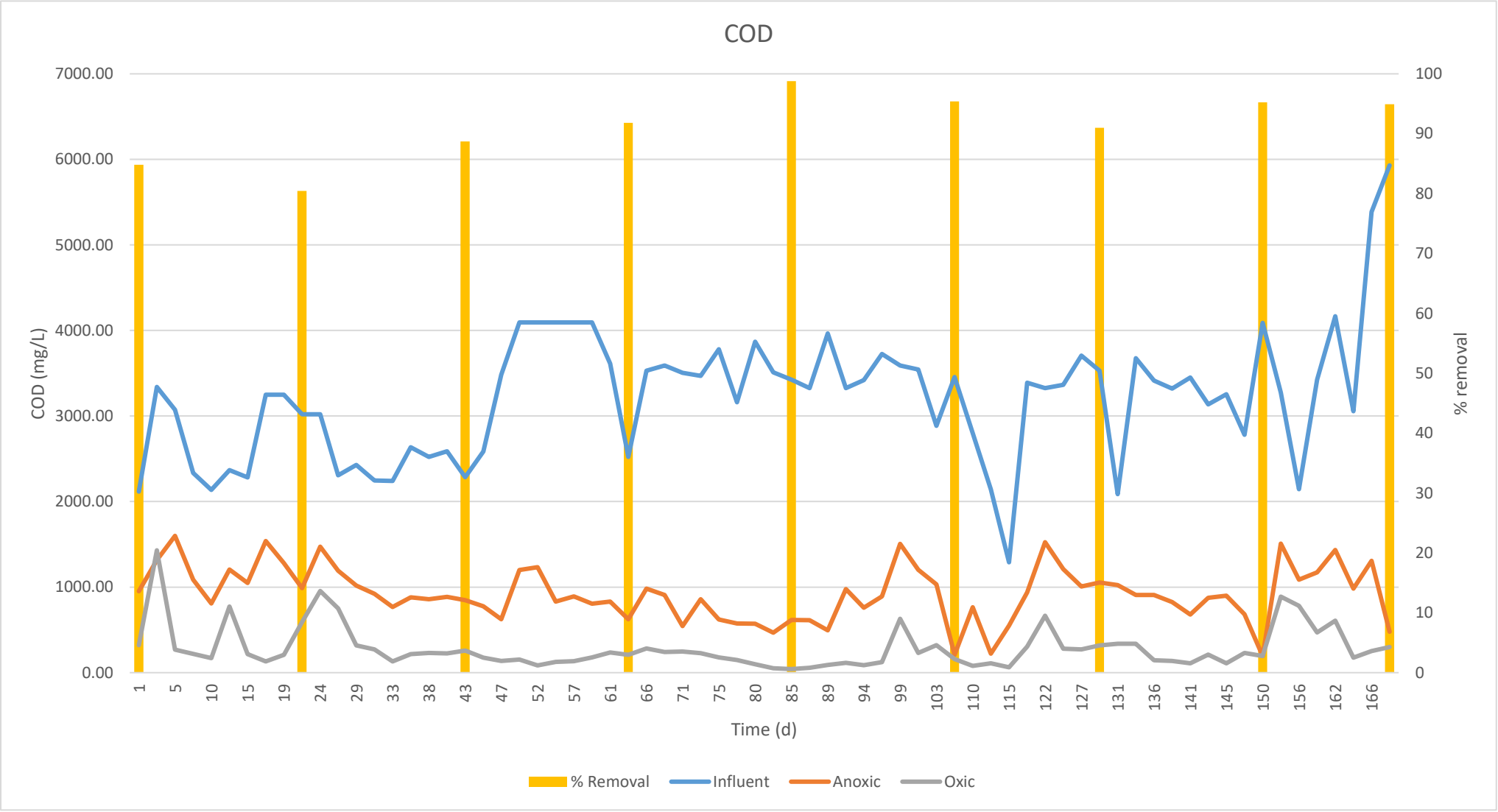


Figure 2.5: COD levels with synthetic wastewater, showing % removal every 21 days (rolling average).

Phosphate removal was measured as Orthophosphate (PO_4^{3-}). Removal ranged from 15 – 55% (Figure 2.6), which is lower than values for P removal reported in literature. The spike in influent PO_4^{3-} on day 64 was due to turbidity in the sample, which affected the spectrophotometry reading. Turbidity was caused by poor settling of MLSS. As a result, suspended solids remained in the test sample after centrifugation. Filtering the sample and diluting it prior to testing did not remove sufficient solids to prevent an inaccurate measurement. P removal did not appear to be affected by the period of sepsis from days 117 to 162. Effluent phosphate levels ranged from 21 – 155 mg/L, which does not fall within EPA discharge limits. However, this was desirable, as Orthophosphate is needed in the effluent at a range of 0.03 – 300 mg/L to provide the optimal levels for *Lemna* growth (table 2.4).

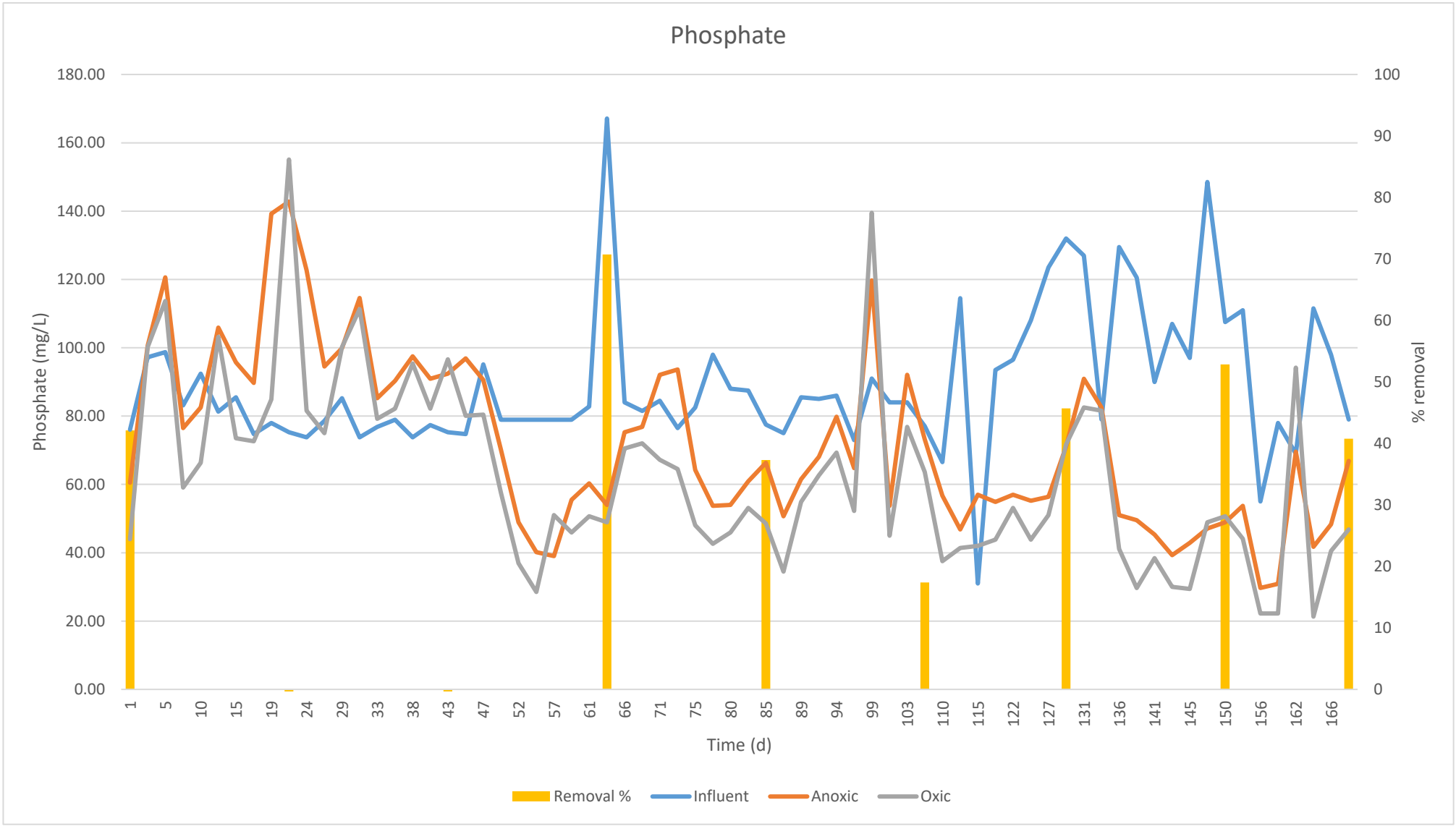


Figure 2.6: Phosphate levels with synthetic wastewater, showing % removal every 21 days (rolling average).

Nitrogen removal was measured primarily by NH_4^+ removal, with measurements for NO_2^- and NO_3^- providing data on nitrification. NH_4^+ removal ranged between 55 and 99% up to day 109, after which efficiency declined to below 20% (Figure 2.7). This was due to the period of sepsis that occurred in the anoxic zone from days 117 to 162. Removal efficiency began to improve towards the end of the trial. For the most part, removal efficiencies matched those reported in literature. Partial nitrification can be seen on day 162, with NO_2^- reaching 133 mg/L. What is interesting about the nitrogen removal observed with synthetic is that nitrite and nitrate levels did not correlate with ammonia levels, indicating that nitrification did not contribute much to overall removal. To try and explain the observed nitrogen removal, an investigation was launched into alternative nitrification processes such as SHARON, ANAMMOX and CANON to see if they were responsible.

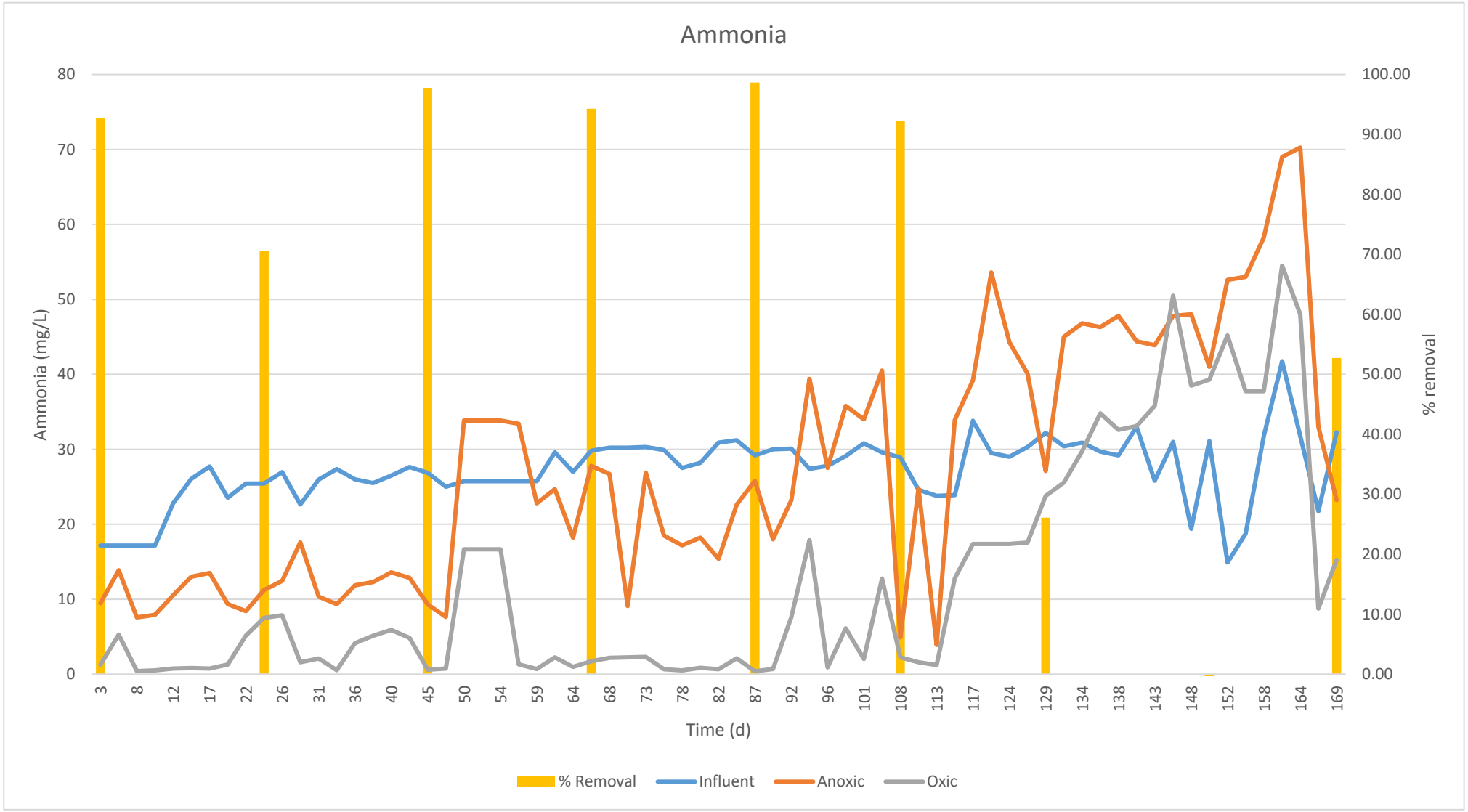


Figure 2.7: Ammonia levels with synthetic wastewater, showing % removal every 21 days (rolling average).

SHARON stands for Single-reactor High temperature Ammonia Removal Over Nitrite. This process uses temperatures of 30 – 40°C to exploit the difference in growth rate between AOB and NOB, allowing AOB to dominate within the system. SHARON does not employ sludge retention like A/O or BNR systems employing a more traditional nitrification approach. This prevents build-up of NOB, and therefore nitrite is not converted to nitrate. The lack of NOB within the system allows for the use of a lower aeration rate (< 1LPM), as AOB have a higher affinity for DO than NOB (Madigan *et al.* 2012). SHARON has been used successfully at full scale in the Netherlands since the late 1990's (Hellinga *et al.* 1998, Schmidt *et al.* 2003).

ANAMMOX stands for Anaerobic Ammonia Oxidation. This process uses anaerobic bacteria such as *Candidatus Brocadia anammoxidans* and *Candidatus Kuenenia stuttgartiensis*, which utilize ammonia as the electron acceptor for denitrification of nitrite. It is operated at high temperatures of around 35°C with long SRT due to the slow growth rate of anammox bacteria. Lower temperatures result in a less efficient nitrogen removal process, making this type of process ideal for warmer climates. Since it is an anaerobic process, aeration rate is not a factor. ANAMMOX has been used successfully in the Netherlands, in tandem with SHARON processes, to treat municipal wastewater in full-scale plants since early 2000's (Schmidt *et al.* 2003). CANON (Completely Autotrophic Nitrogen removal Over Nitrite), is a combination of ANAMMOX and partial nitrification. CANON takes place in a single reactor using high temperature (30 – 40°C), low aeration rate (< 1LPM) and long SRT. AOB oxidize ammonia to nitrite, while anammox bacteria perform denitrification as described above.

Table 2.3: Comparison of operational parameters of different nitrogen removal processes (Schmidt *et al.* 2003).

Technology or process	Conventional	SND	Short-cut (SHARON)	ANAMMOX	Aerobic deammonitrification	CANON	OLAND
Number of reactors	2	1	2	1	1	1	1
Feed	Wastewater	Wastewater	Wastewater	Ammonia + Nitrite	Wastewater	Wastewater	Wastewater
Discharge	NO_3^-, NO_2^-, N_2	N_2	NO_2^-, N_2	NO_3^-, N_2	N_2	NO_3^-, N_2	N_2
Operating conditions	Aerobic, anoxic	Aerobic	Aerobic, anoxic	Anaerobic	Aerobic	Oxygen limited	Oxygen limited
Oxygen requirements	High	Low	Low	None	Low	Low	Low
Biomass retention	None	None	None	Yes	Yes	Yes	Yes
COD requirements	Yes	No	No	No	No	No	No
Sludge production	High	Low	Low	Low	Low	Low	Low
Bacteria	Nitrifiers + heterotrophs	Heterotrophic Nitrifiers + aerobic denitrifier	Aerobic Ammonium Oxidizer	Planctomycetes	Aerobic nitrifiers + aerobic Denitrifier	Aerobic ammonium Oxidizer + planctomycetes	Aerobic ammonium Oxidizer + anaerobic Ammonium oxidizer
Max N loading (kg N m^{-3} reactor d^{-1})	2–8	1–3.5	0.5–1.5	10–20	1–2	2–3	0.1
Total-N removal efficiency	95%	100%	90%	87%	60%	75%	85%
Optimum temperature (°C)	12–35	20–30	Above 25	30–40	Unknown	30–40	30–40
Common reactor configuration	Activated sludge and biofilm	Oxidation ditch, SBR	Activated sludge and biofilm	Fixed and fluidized-bed reactor, gas-lift reactor, SBR	Biological rotating contactor, gas-lift reactor, fixed and fluidized-bed reactor	Fixed and fluidized-bed reactor, SBR	Fixed and fluidized-bed reactor, SBR
Application status	Established	Laboratory	Full-scale plants	Full-scale initiated	Laboratory	Laboratory	Laboratory
Electron donor	COD	Unknown	COD	Ammonium	Ammonium	Nitrite	Ammonia
Biofilms or suspension	Biofilms/ suspension	Biofilms/ suspension	Suspension	Biofilms/ suspension	Biofilms/suspension	Biofilms/suspension	Biofilms/suspension

SND, simultaneous nitrification and denitrification; COD, Chemical oxygen demand; SBR, sequencing batch reactor.

Temperature and DO profiles in the A/O system were not compatible with any of the alternative process parameters outlined in table 2.3. All three of the above-mentioned systems require temperatures above 25°C and little if any DO. Temperature ranged from 20 – 25°C in both anoxic and oxic zones, with average DO concentration in the oxic zone at 2.43 mg/L, which surpassed the upper limits for the alternative models. Both temperature and DO range in the A/O system were suited to the growth of both AOB and NOB, so it is unlikely that alternative nitrification processes were taking place at any significant level.

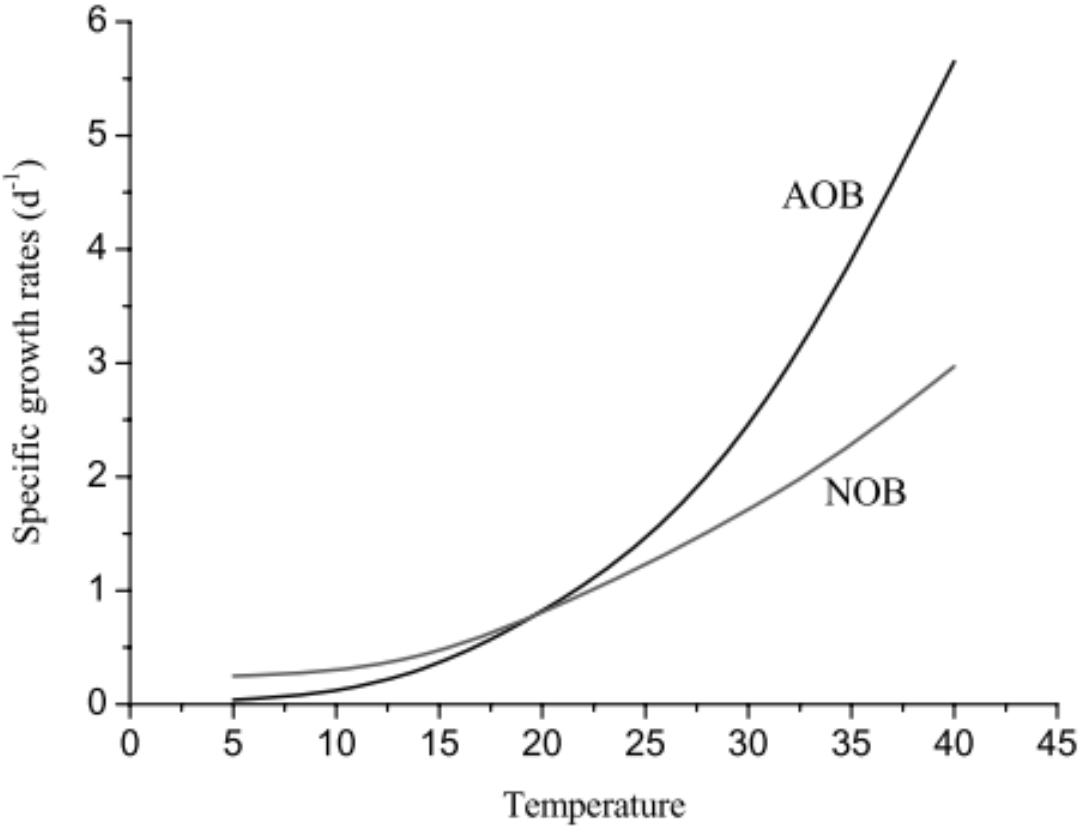


Figure 2.8: Growth rates of AOB and NOB at different temperatures (Zhu *et al.* 2008).

Figure 1.10 shows the currently accepted process of nitrification; ammonia oxidation to hydroxylamine by ammonia monooxygenase (AMO) followed by hydroxylamine oxidation to nitrite by hydroxylamine oxidoreductase (HAO). However, a recent study into the nitrification process by Caranto and Lancaster (2017) contradicts the current model of nitrification. It is posed in this study that NO_2^- formation is a result of spontaneous reaction with dissolved oxygen (DO) rather than the result of HAO activity. The study tested HAO activity under both aerobic and anaerobic conditions, and it was found that HAO does not produce NO_2^- in the absence of oxygen. Under aerobic conditions, NO_3^- was much more

abundant than NO_2^-, suggesting that NO_2^- is not the end-product of HAO activity, which contradicts the currently accepted model. Under low oxygen conditions, N_2O and NO were also found to be produced by HAO. This provides new insights into the specifics of nitrification and provides an explanation as to why NH_4^+ removal rates (Figure 2.7) were relatively high considering the low levels of NO_2^- and NO_3^-. Future work with synthetic wastewater should include measurement of gaseous outputs to investigate the possibility of Nitrogen removal through this type of enzymatic activity. Figure 2.9 below contrasts the new model of nitrification with the currently accepted model.

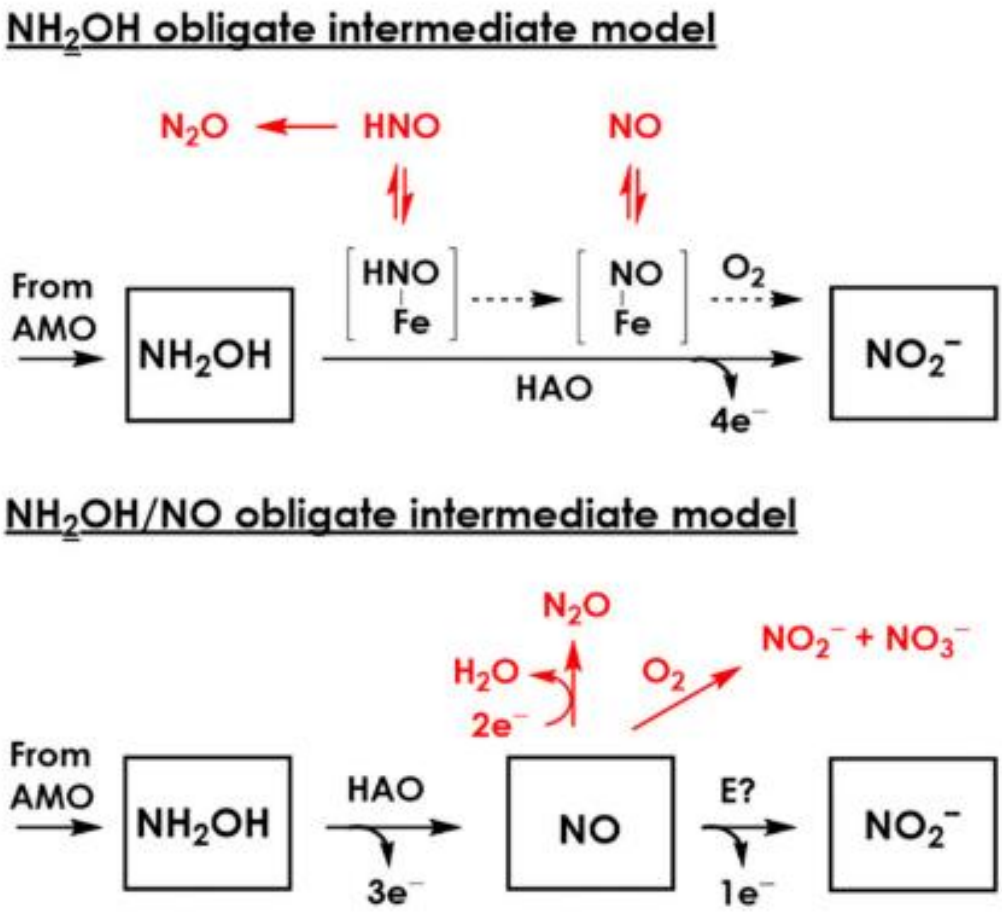


Figure 2.9: Two models for the obligate intermediates of NH_3 oxidation to NO_2^- by AOB. The HAO NH_2OH oxidation products in each model are those proposed in previous literature (NH_2OH obligate intermediate model) or this work (NH_2OH /NO obligate intermediate model). Solid boxes surround obligate intermediates and products. Square brackets enclose proposed enzyme-bound intermediates. Pathways in red are proposed non-enzymatic pathways leading to N_2O, HNO, NO, NO_3^-, and non-enzymatic NO_2^- production. E is a proposed NO oxidoreductase necessary to oxidize NO to NO_2^- (Caranto and Lancaster 2017).

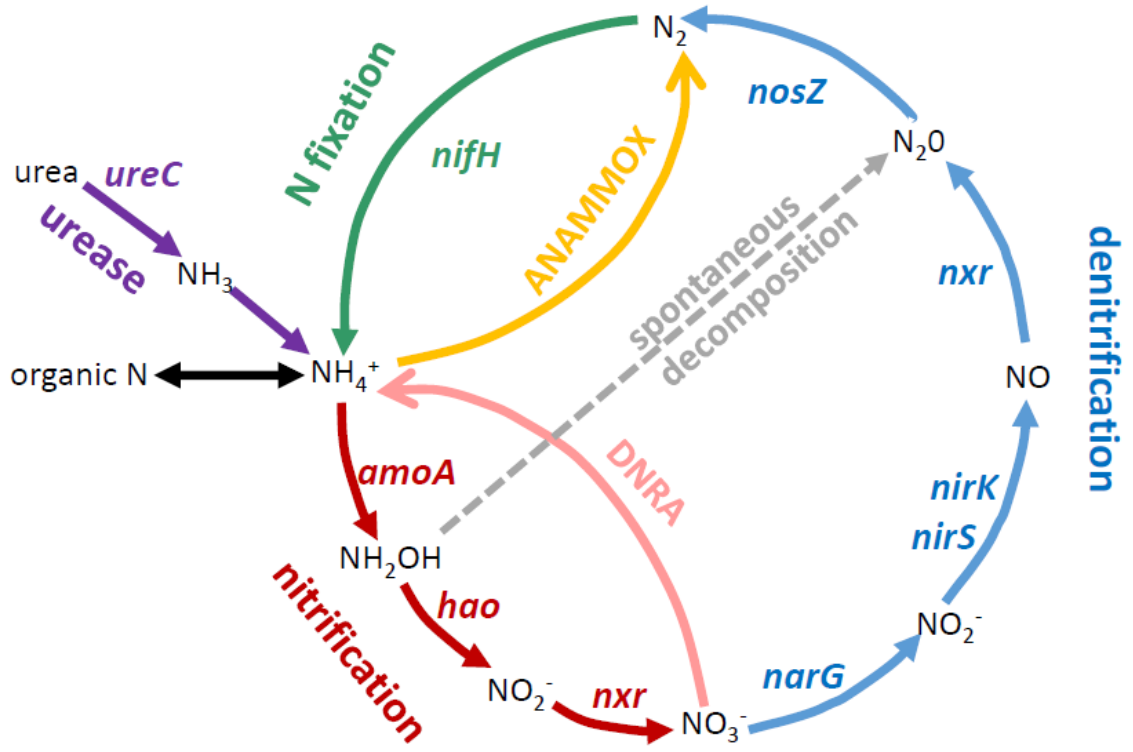


Figure 2.10: Diagram of Nitrogen Cycle genes and the enzymes they produce (Hirsch 2017). Enzymatic activity is also shown, and it can be seen from the diagram that hydroxylamine spontaneously decomposes to Nitrous Oxide.

During the course of evaluating reactor performance, the composition of synthetic wastewater (table 2.2) was also investigated in relation to floc formation and MLSS settling. The evaluation of synthetic wastewater composition highlighted the need for trace elements Copper (Cu) and Molybdenum (Mb) in addition to the elements already in supply. Two enzymes involved in nitrification, ammonia monooxygenase and nitrite oxidoreductase, require Cu and Mb (Ge *et al.* 2015, Metcalf & Eddy 2014). It was therefore decided that synthetic wastewater should be supplemented with a trace element solution to improve flocculation and increase nitrification efficiency for future synthetic work.

2.3.3 Floc formation and MLSS with real-time wastewater

MLSS levels in the anoxic zone rose to 11000 mg/L by day 2 of operation on real-time wastewater, but MLSS in both anoxic and oxic zones dropped to around 3000 mg/L by day 7. MLSS remained relatively stable between 3000 and 6000 mg/L from days 7 - 125. The decrease in MLSS seen between days 60 and 70 (Figure 2.11) was partially due to low COD in the wastewater for that particular 10-day period. The wastewater used from days 37 – 51 was pH 11 – 12; the highest pH across the entire operational period. This particular wastewater likely contained detergent from cleaning-in-place (CIP) processes, as the biomass within each zone was bleached within 48 hours of feeding, and large amounts of foam could be seen. As a result, sludge wastage was implemented without RAS to remove the affected biomass. Colour returned to the biomass after feeding with a new batch of wastewater with a neutral pH. Once the biomass recovered, RAS was re-implemented and MLSS began to increase. The drop in MLSS after day 126 was a result of lower COD levels in the wastewater from that period onwards. MLSS continued to fluctuate for the remainder of the real-time wastewater trial.

Initial floc size and density in the seed sludge was the same as for operation on synthetic wastewater (Figure 2.4), as the seed sludge was from the same source. Floc size and density did not change very much until day 28 (Figure 2.12) when flocs became sparse and less compact. Floc structures remained loose for the remainder of the experiment, even when individual flocs were quite large (> 500µm across). Filaments were present in both zones from day 28 onwards, with growth of filaments becoming more prominent towards the end of the operational period. By day 210, floc structures in the anoxic zone appeared more compact. Floc structures did not deteriorate as much with real-time wastewater as with synthetic wastewater. This was because real wastewater contained trace elements such as Cu and Mb that were absent from synthetic wastewater.

Average F/M ratio for the anoxic zone across the operational period was 3.79 mg COD / mg VSS • d. Once again, this can be attributed to high influent COD and relatively low VSS across the operational period as well as the smaller volume in the anoxic zone. The Oxic zone yielded an average F/M of 0.92 mg COD / mg VSS • d. This is slightly higher than the reference literature values of 0.3 – 0.5 g BOD / g VSS • d given in chapter one. This is most likely due to the higher levels of influent COD coupled with lower concentrations of biomass within the 4L volume of the oxic zone.

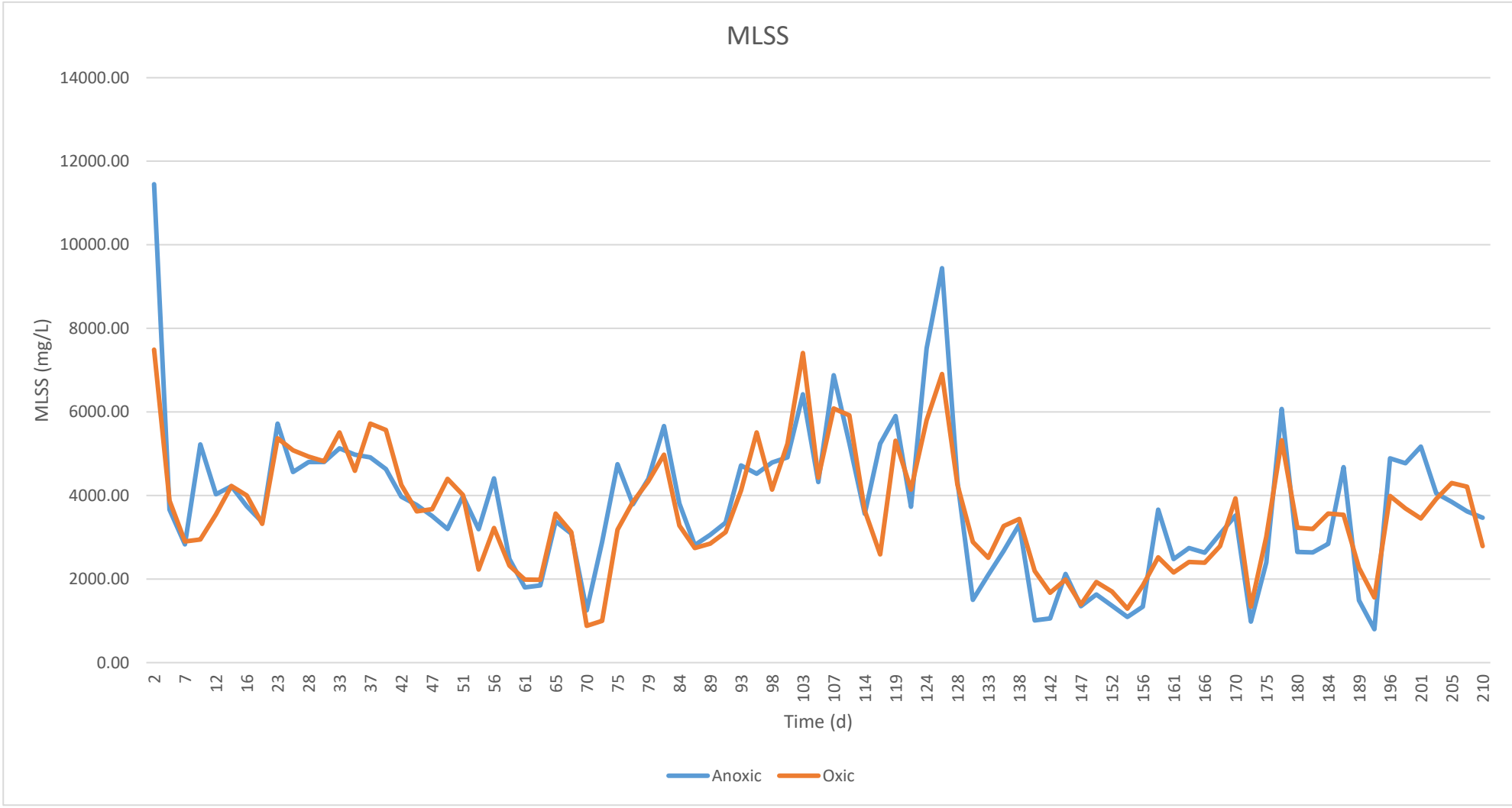


Figure 2.11: MLSS levels with real-time wastewater.

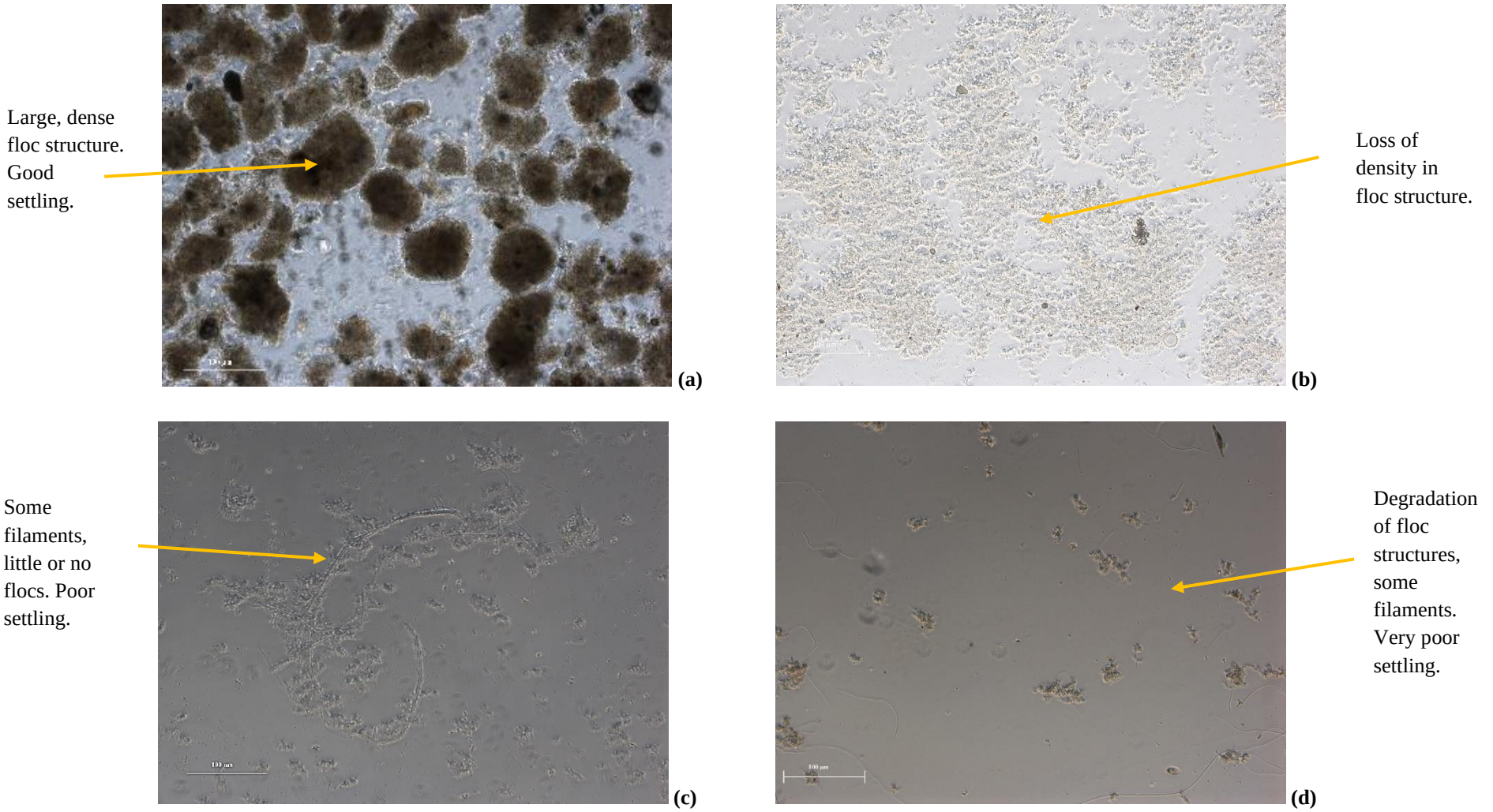


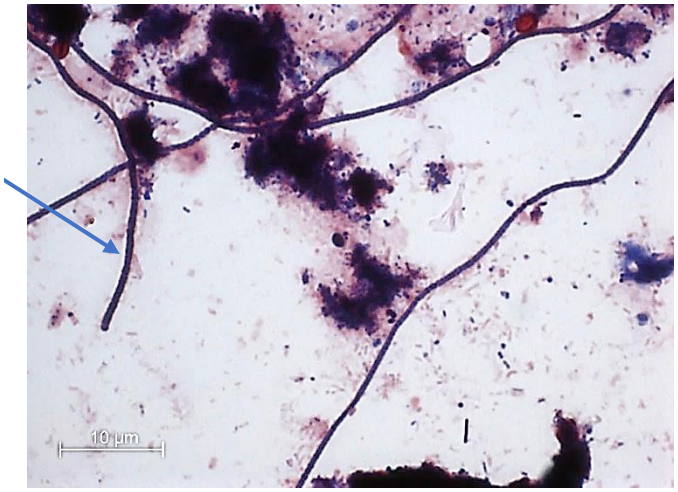
Figure 2.12: (a) Image of seed sludge under phase contrast 10x magnification. (b) Image of sludge flocs from day 28 in oxic zone under phase contrast 10x magnification. (c) Image of sludge flocs from day 128 in oxic zone under phase contrast 10x magnification. (d) Image of sludge flocs from day 210 in oxic zone under phase contrast 10x magnification. Scale bar for all images is 100µm.

2.3.3 A/O filament identification.

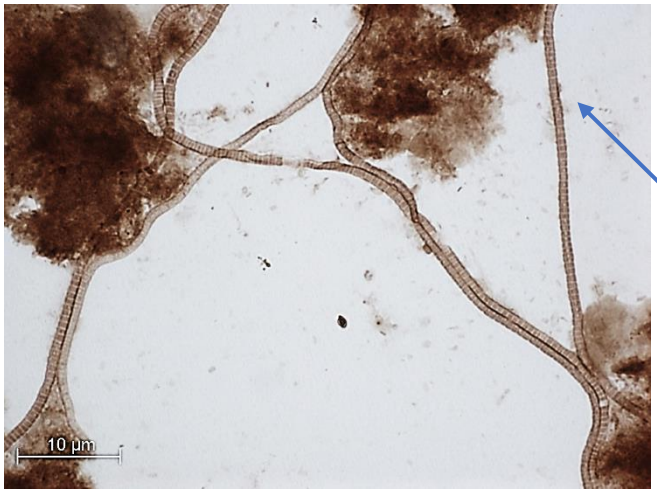
Filamentous organisms are necessary to a certain extent in wastewater treatment systems, as they provide a structural platform upon which flocs can be built. However, under inefficient operational conditions, filamentous growth can become problematic, causing issues with sludge bulking, foaming and poor sludge settling. Different filaments thrive under different operational conditions, and the type of filament observed in the system can aid diagnosis of operational problems. Staining procedures such as; Gram stains, Neisser stains and DAPI stains, among others, can allow for identification of different filaments, therefore helping to diagnose problems with reactor operational conditions (van Loostrecht *et al.* 2016, Metcalf & Eddy 2014, Eikelboom 2000, Jenkins *et al.* 2004).

The above-listed stains were carried out on A/O and IASBR sludge samples across their respective operational periods in order to identify filaments that caused sludge bulking and poor settling. The main filament types identified in the oxic zone of the A/O (Figures 2.13 – 2.15) were Type 0041 and Type 0675. These filaments are Gram positive, Neisser negative and do not accumulate poly-phosphate. Cells are round/disc shaped with indentation in cell septa. Cells are also encased in a sheath, to which other microbes attach to form flocs. These filaments are often the backbone of floc structures. There is little difference between Type 0041 and Type 0675 except for Type 0675 have a smaller cell diameter and appear narrower under the microscope. Both of these filament types have been phylogenetically identified as members of the candidate phylum TM7, although some filaments of this type have also been identified as part of the phylum *Chloroflexi* (Nielson *et al.* 2009).

Rounded cells with indentation at cell septa. No sheath visible in Gram stain

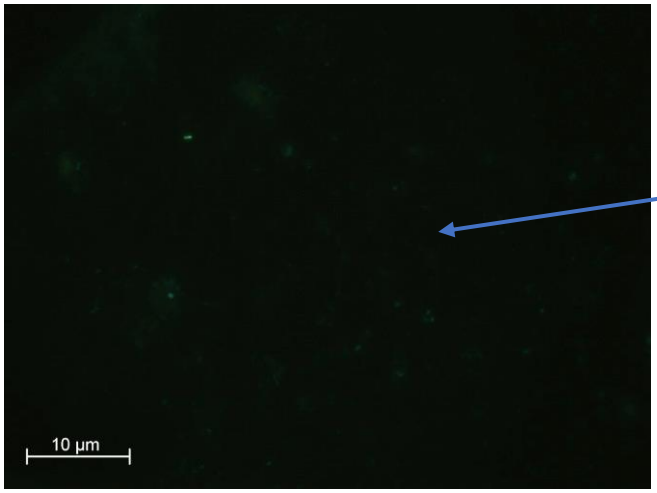


(a)



(b)

Rounded cells with indentation at cell septa. Sheath visible in Neisser stain



(c)

No filaments can be seen in the image. Cells appear green under DAPI fluorescent stain. Poly-P inclusions would fluoresce yellow if present.

Figure 2.13: (a) Oxic zone Gram stain day 68 of real-time operation. (b) Oxic Neisser stain day 68 of real-time opearation. (c) Oxic DAPI stain day 68 of real-time operation. Scale bar for all images is 10µm.

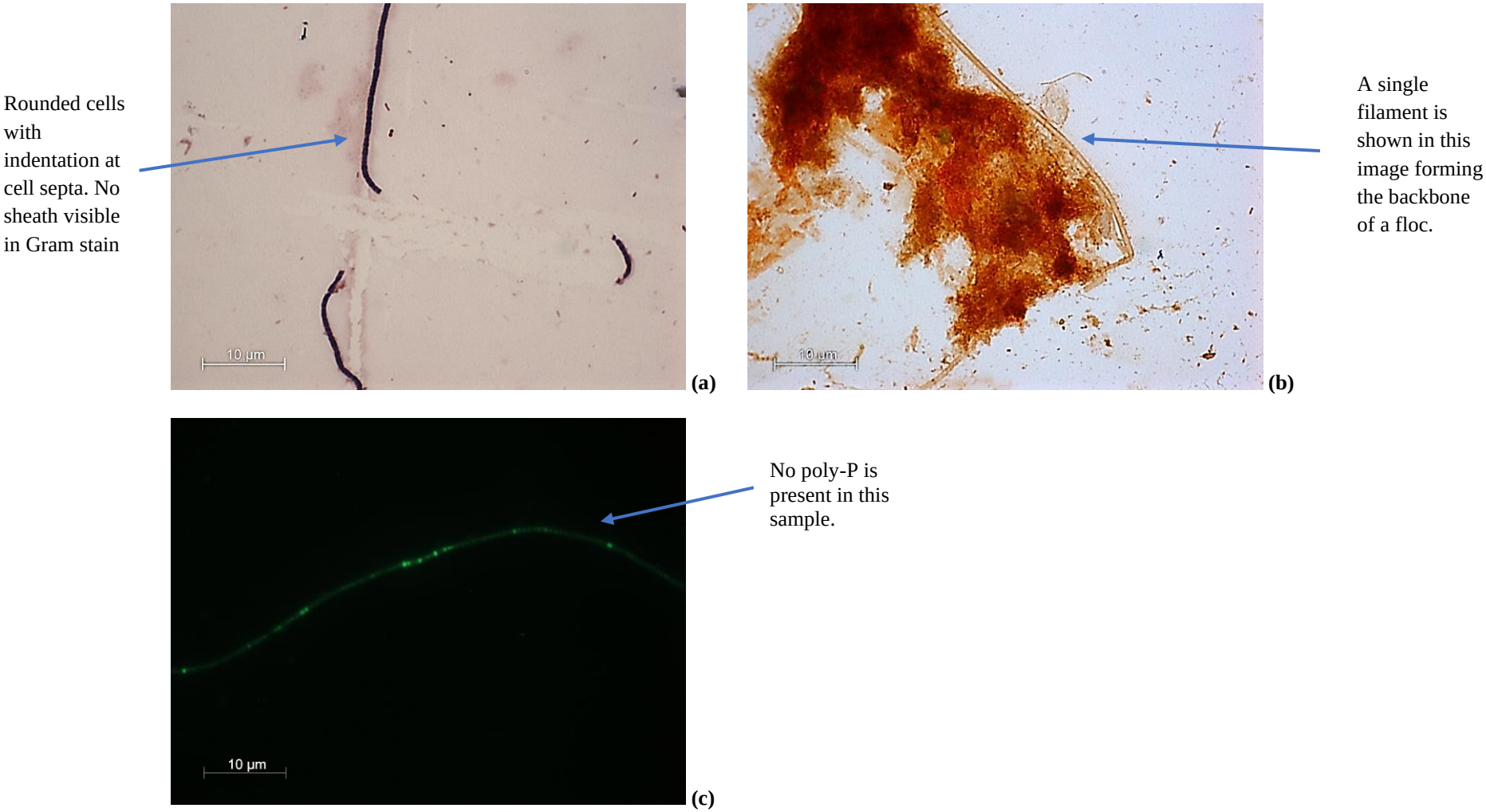


Figure 2.14: (a) Oxic Gram stain day 152 of real-time operation. (b) Oxic Neisser stain day 152 of real-time operation. (c) Oxic DAPI stain day 152 of real-time operation. Scale bar for all images is 10µm.

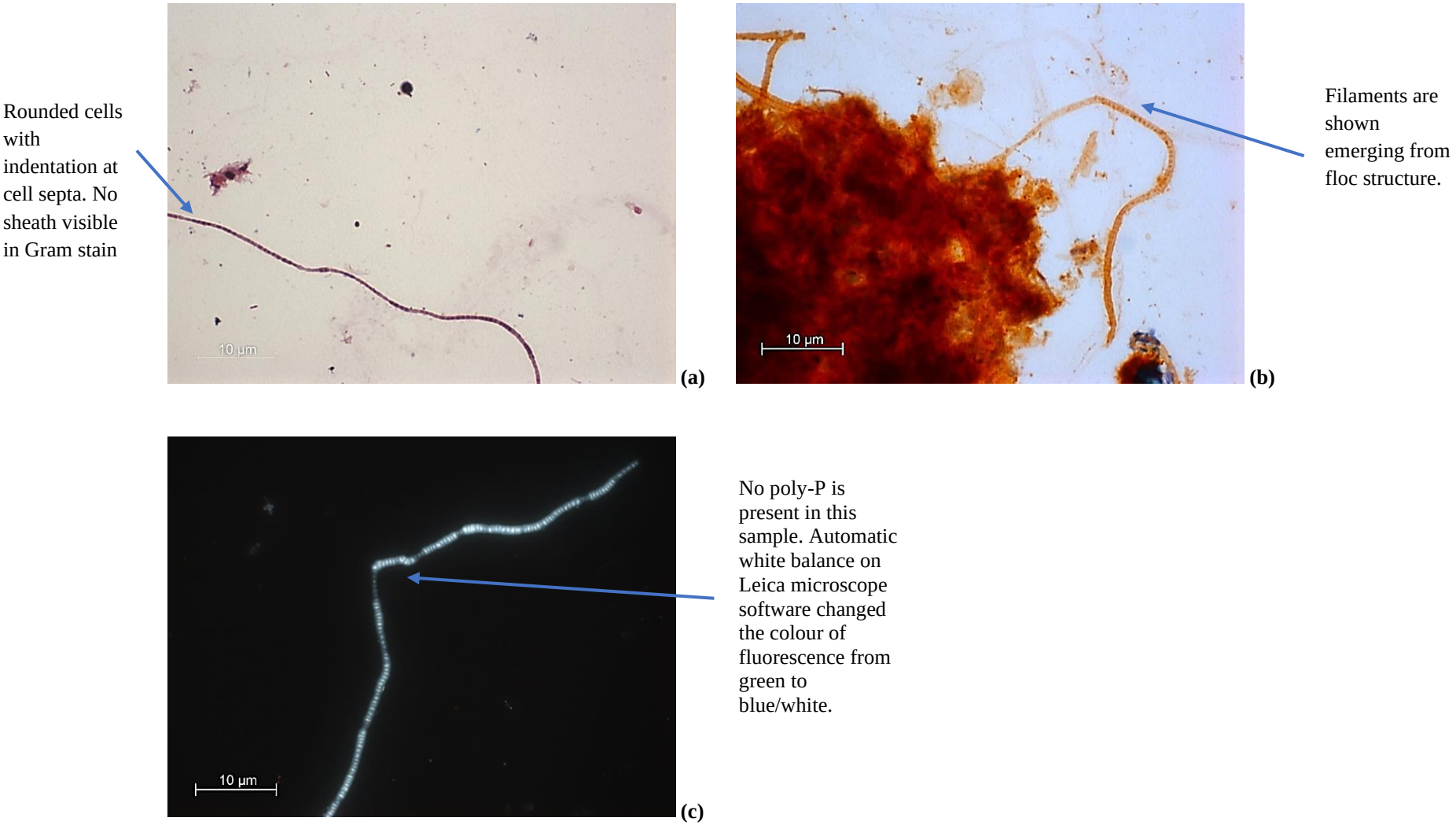


Figure 2.15: (a) Oxic Gram stain day 210 of real-time operation. (b) Oxic Neisser stain day 210 of real-time operation. (c) Oxic DAPI stain day 210 of real-time operation.

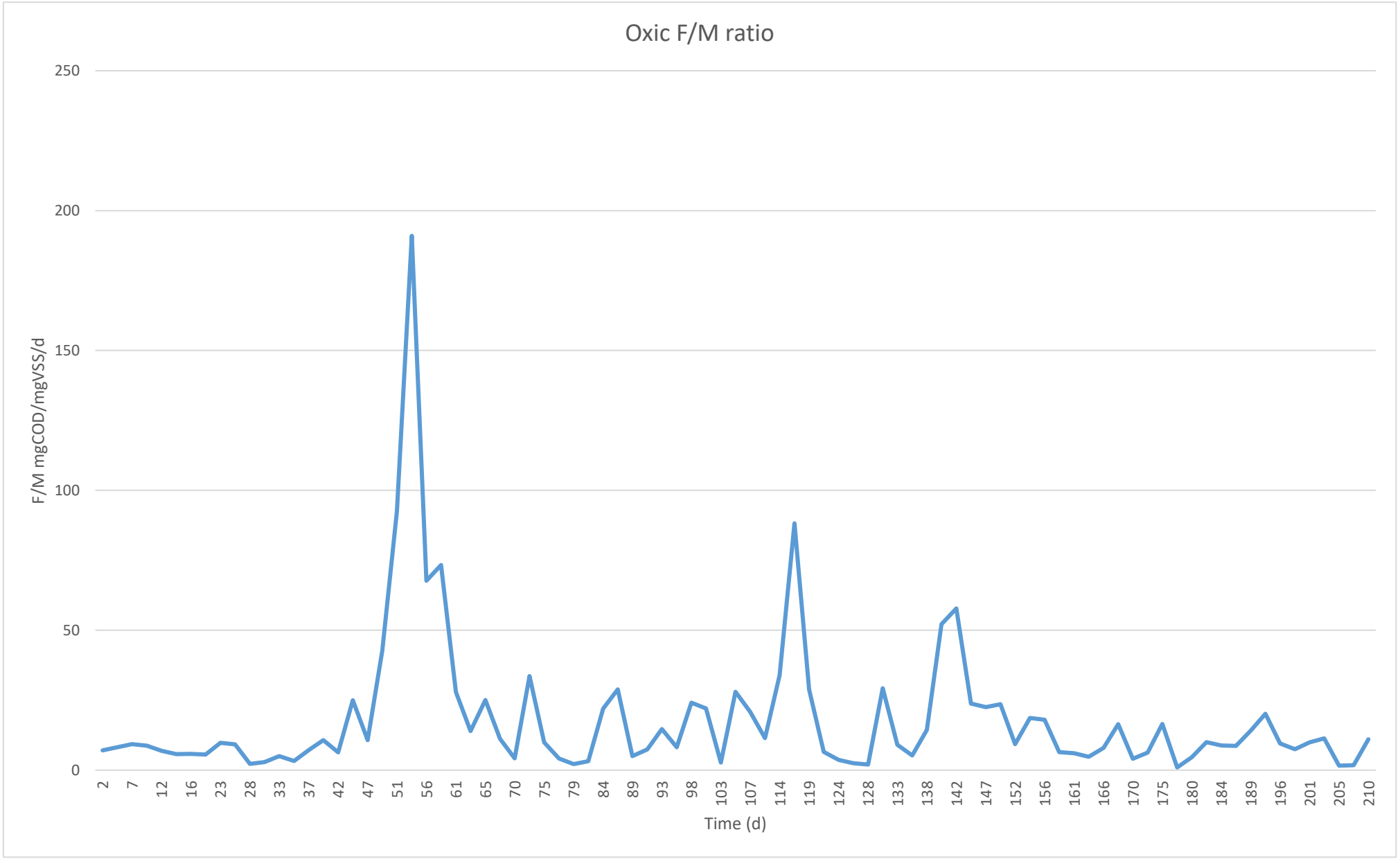


Figure 2.16: Oxic F/M ratios for the operational period on real-time wastewater.

Little data is currently available in the literature about these filaments or the operational conditions under which they thrive, except that they are aerobic and are normally problematic under low F/M conditions and, to a lesser extent, long SRTs (Eikelboom 2000). F/M ratios for the oxic zone of A/O varied with each batch of wastewater, ranging from 0.97 - 190.99 mg COD/mg VSS/d (Figure 2.16). These F/M ratios are generally lower than the literature recommended range of 50-100mg BOD/mg VSS.d for 20 – 30d SRT. The SRT adopted for A/O operation was almost 210d due to lack of sludge wastage and probably contributed to the abundance of these filament types, particularly towards the end of operation.

2.3.4 Chemical analysis of performance with real-time wastewater.

Temperature in both the anoxic and oxic zones remained between 20 and 25°C for the full 210 days of operation. Dissolved oxygen (DO) remained at 0 mg/L in the anoxic zone and ranged from 0 – 12 mg/L in the oxic zone. Influent pH ranged from 4 – 12, with pH in both the anoxic and oxic zones ranging from 4.5 – 9. COD removal ranged from 70 – 97% (Figure 2.17), which matches reported values for COD removal in literature, as well as COD removal with synthetic wastewater. The majority of COD was removed in the anoxic zone, likely used for denitrification. However, despite the high removal efficiency, effluent COD was only occasionally below 100 mg/L, and never went below 50 mg/L.

COD removal efficiencies with real-time wastewater were objectively similar to COD removal efficiencies with synthetic wastewater, with both trials maintaining >90% removal for most of the respective operational periods. However, the fluctuation in COD levels in real-time wastewater meant that % removal also fluctuated. Since synthetic wastewater COD was relatively constant, fluctuations in % removal were more subtle.

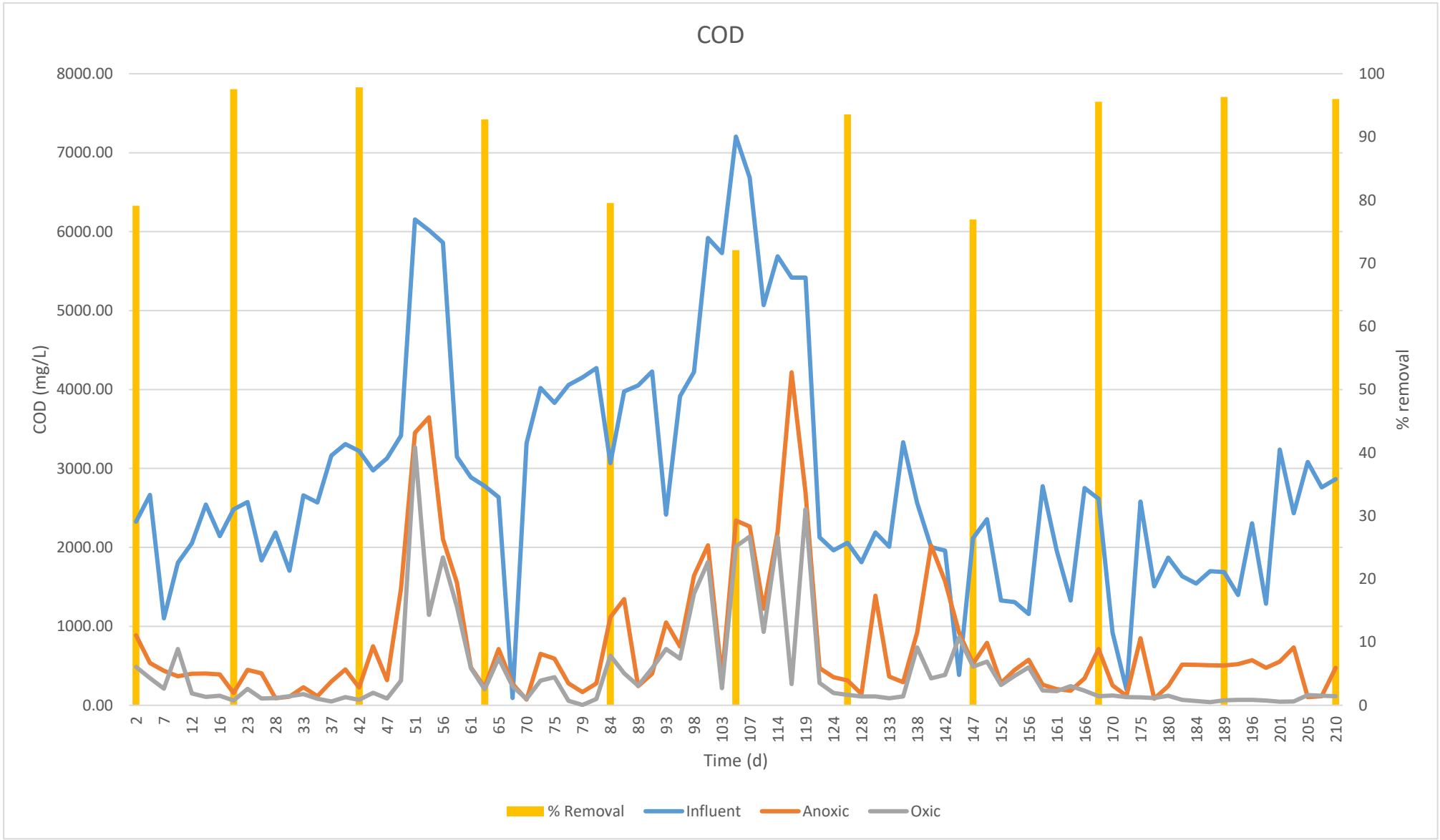


Figure 2.17: COD levels with real-time wastewater, showing % removal every 21 days (rolling average).

Phosphate removal was measured as Orthophosphate (PO_4^{3-}). Removal ranged from 50 – 95% (Figure 2.18) for most of the trial, with short periods of poor removal. P removal, for the most part, matched values reported in literature and was higher than P removal achieved during operation with synthetic wastewater (< 60%). Phosphate levels in real-time wastewater were quite high for the first 120 days, ranging from 100 – 500mg/L. In contrast, P levels in synthetic wastewater rarely measured higher than 100mg/L. P removal increased steadily up to day 63, reaching 80% removal. The decrease in efficiency at day 84 is likely due to the sludge wastage that was carried out after days 37 – 51. Biomass that had been successfully removing P would have been washed out during the wastage period and needed time to recover, which is evidenced by the fact that P removal began to increase again afterwards. The second drop in P removal was likely caused by insufficient MLSS. Efficiency quickly recovered as MLSS increased and was reaching 95% removal by the end of the trial. Effluent phosphate levels ranged from 4 – 190 mg/L, which does not fall within EPA discharge limits, but does fall within the optimal range for *Lemna* growth of 0.03 – 300 mg/L (table 2.4).

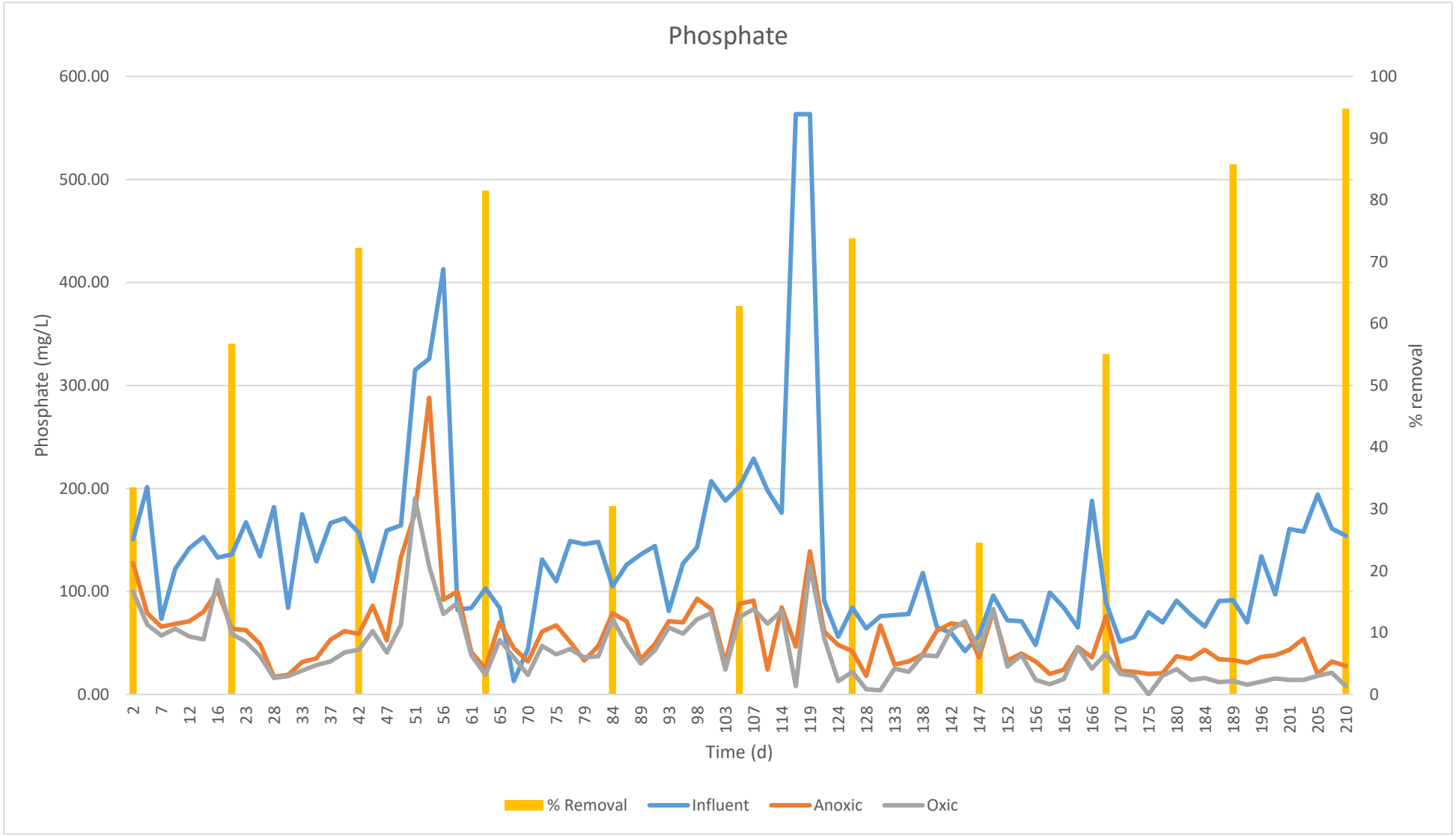


Figure 2.18: Phosphate levels with real-time wastewater, showing % removal every 21 days (rolling average).

Nitrogen removal was measured primarily by NH_4^+ removal, with measurements for NO_2^- and NO_3^- providing data on nitrification. NH_4^+ removal ranged between 20 and 99% (Figure 2.19), with NH_4^+ levels in constant flux with each batch of wastewater. NH_4^+ removal with synthetic wastewater was more consistent, with % removal remaining above 50% for the majority of the 169 days of operation. Quite often, NH_4^+ levels with real-time wastewater were higher in the anoxic zone than in the influent, particularly from days 61 – 68, days 120 – 147, and then again at day 168. This was most likely due to ammonification of influent organic nitrogen. Nitrate and nitrite were observed at high levels in real-time wastewater when new batches were obtained and tested. However, they dropped quite rapidly during storage. This fluctuation would most likely not happen in a treatment plant, as the wastewater would be continuously fed to the reactor, not stored for long periods of time.

NO_2^- and NO_3^- levels, shown in Figures 2.20 and 2.21, indicate that partial nitrification was achieved to a degree. This is quite different from NO_2^- and NO_3^- levels observed with synthetic wastewater. Once again, however, it is unlikely that SHARON, ANAMMOX or CANON were responsible for nitrogen removal, as temperatures in both the anoxic and oxic zones were sub-optimal (below 30°), and the DO profile in the oxic was above 1mg/L, making any of the above-mentioned processes unlikely (table 2.3). For most of the A/O operation on real-time wastewater, effluent NH_4^+ levels were within EPA discharge limits, but overall nitrogen levels were not, due to the contribution of NO_2^- and NO_3^-. This was somewhat desirable, although higher NH_4^+ levels would have been better for *Lemna* cultivation.

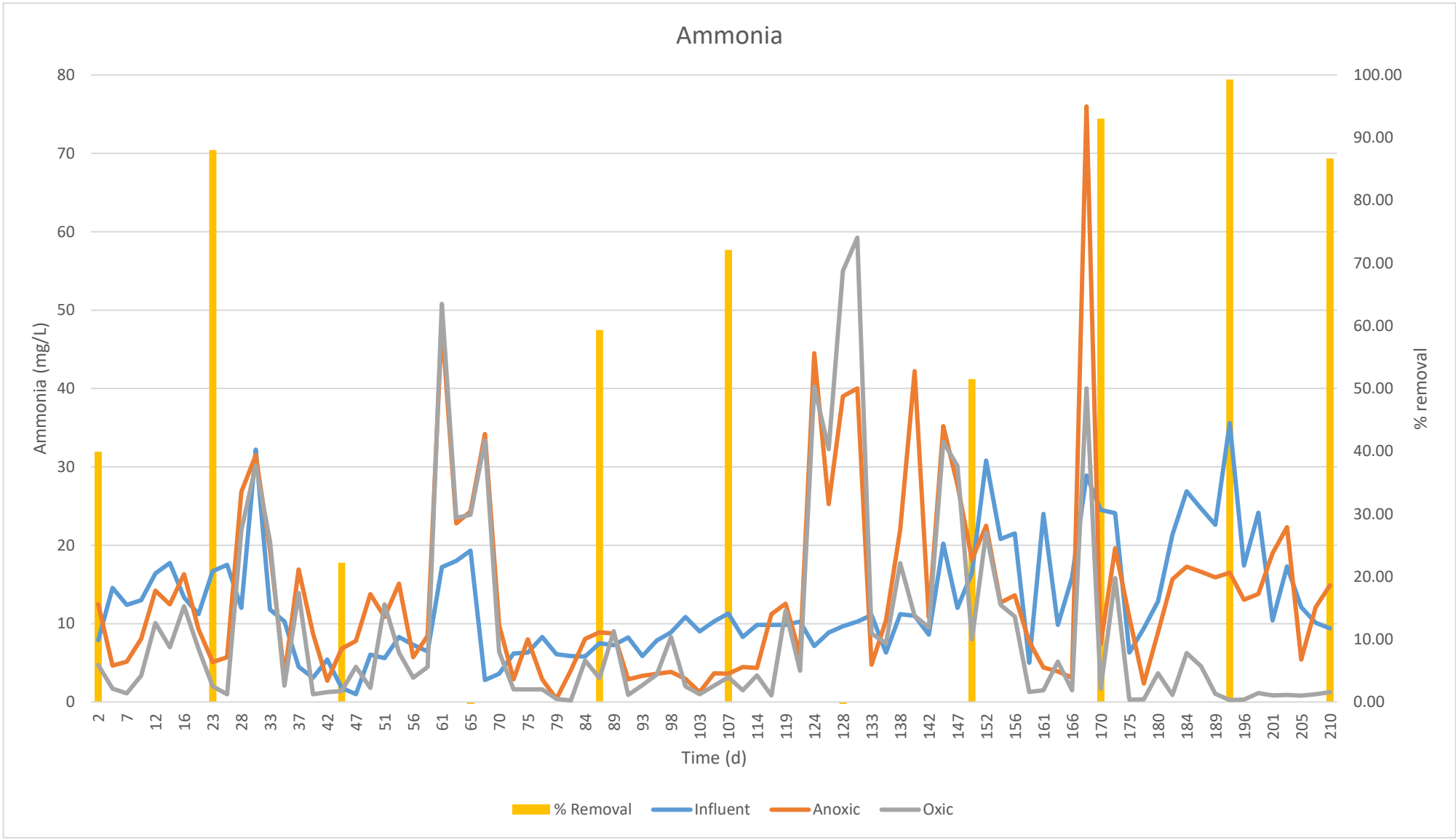


Figure 2.19: Ammonia levels with real-time wastewater, showing % removal every 21 days (rolling average).

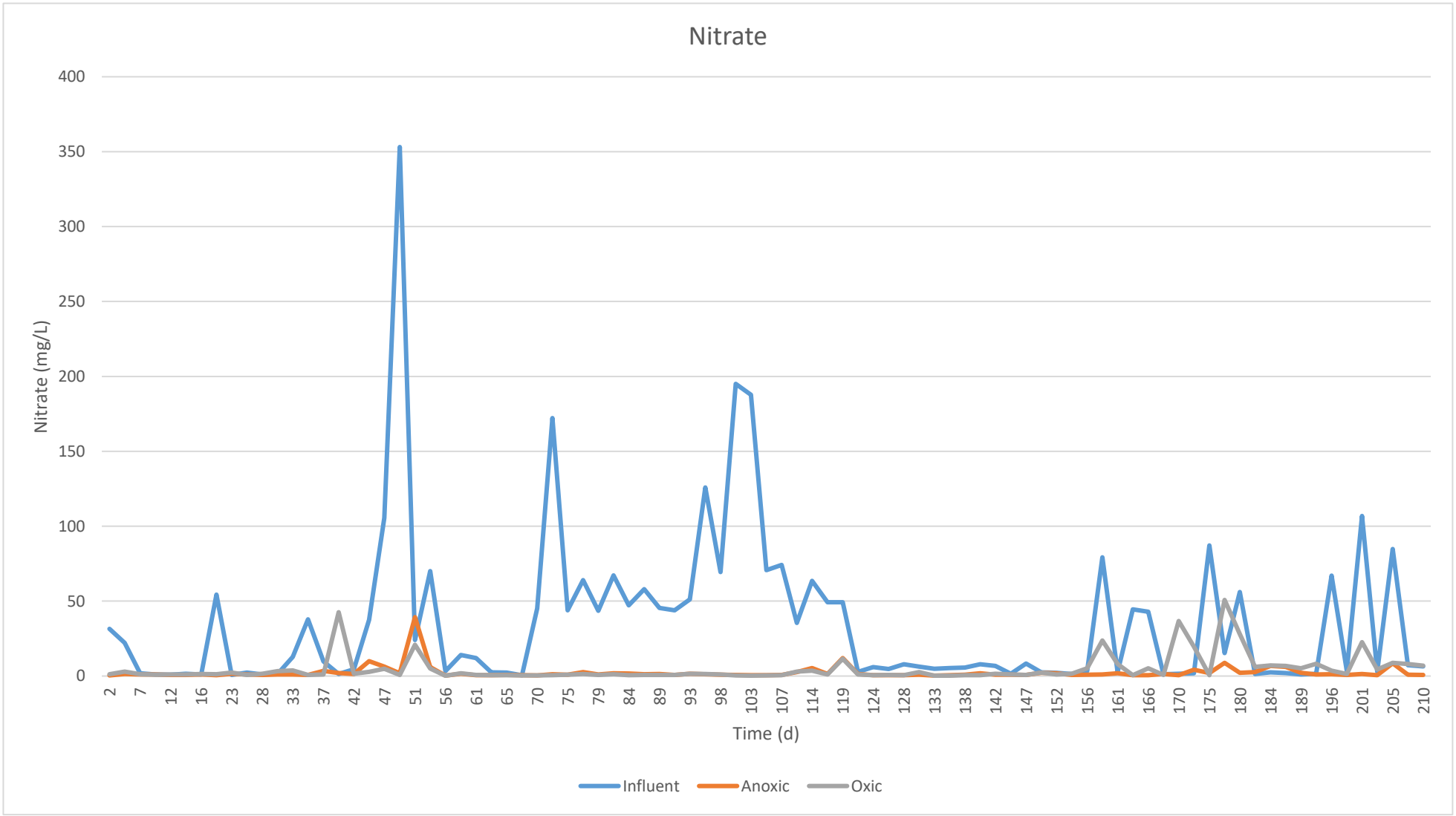


Figure 2.20: Nitrate levels with real-time wastewater.

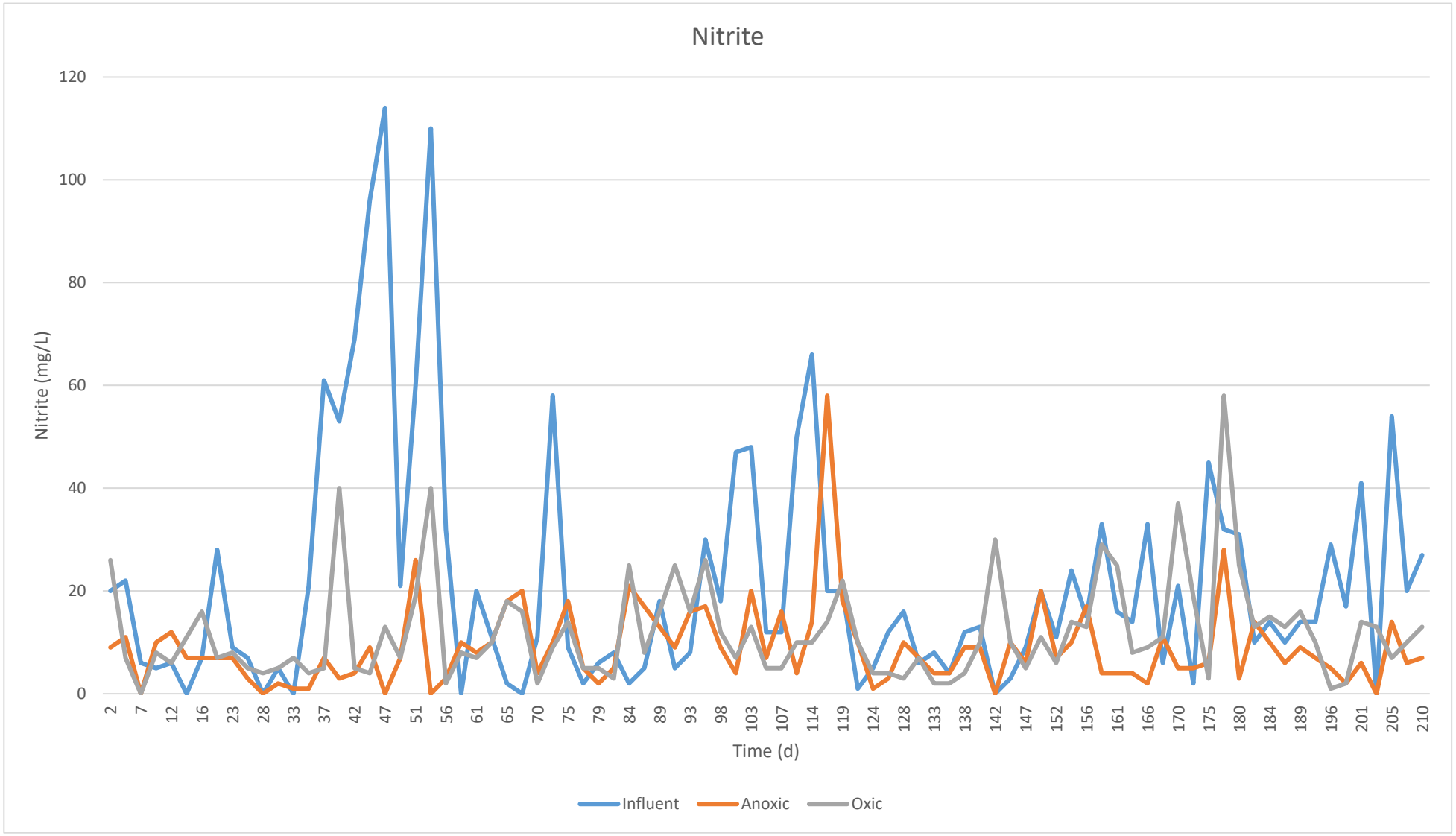


Figure 2.21: Nitrite levels with real-time wastewater.

2.4 Conclusion

Once again, the purpose of the project was to condition dairy processing wastewater for *Lemna minor* production, but first, key reactor parameters such as HRT, SRT and flow rate (Q) needed to be established before conditioning could take place. Effluent COD needed to be as low as possible, while maintaining as much N and P as possible, preferably in the form of NH_4^+, as this is the preferred form of N for *Lemna minor*. This was particularly challenging as BNR systems are designed to achieve complete nutrient removal by utilizing the competing metabolisms present in mixed microbial populations. Reactor parameters that provided the most suitable effluent with synthetic wastewater were as follows; HRT = 1.8d and Q = 200ml/h. SRT was essentially equal to the length of the operational period, since sludge wastage was only employed when problems occurred and was not constant.

These operational parameters were applied for real-time dairy processing wastewater over 210 days with limited success. Table 2.4 below details the N/P requirements for *Lemna* growth. Effluent NH_4^+ levels ranged from 0.2 – 59.25 mg/L throughout operation, averaging at 9.93 mg/L. Despite this falling within the optimal range for *Lemna*, figure 2.19 shows effluent NH_4^+ levels were too unstable to support *Lemna* growth, and did not stabilise, no matter what parameters were changed. Effluent P values averaged at 44.52 mg/L, which falls within the lower end of optimal range for *Lemna*. Figure 2.18 shows that P levels were relatively stable within the system compared to NH_4^+ levels and are therefore less likely to be a limiting factor in *Lemna* growth. Changing HRT or SRT to stabilise NH4+ levels introduced other problems such as sludge bulking and insufficient COD removal. Therefore, wastewater conditioning for *Lemna* growth was not achieved using an A/O system.

Table 2.4: Nutrient requirements for *Lemna* growth obtained from literature.

	Ammonium – NH_4 (mg/L)	**Total [N] (mg/L)**	**Phosphate – PO_4 (mg/L)**	**Total [P] (mg/L)**
Minimum required	0.1	0.07	0.1	0.01
Maximum tolerated	Depends on pH	1400 – 2100	950	310
Optimal Range	5 – 50	3 – 350	0.03 – 300	0.01 – 100

In order to address the issues arising with lack of available nitrogen, an IASBR reactor was established to limit nitrification and denitrification and to increase available nitrogen. Design and operation of the IASBR will be discussed in chapter 3

Chapter 3 – IASBR set-up and performance.

3.1 Introduction

Intermittently aerated Sequencing Batch Reactors (IASBRs) are an adaptation of conventional SBRs (cSBR). cSBRs have been a popular technology in many different areas of wastewater treatment, particularly since the advent of programmable timers and automation, making SBR operation easier than ever. The main difference between a cSBR and an IASBR is the frequency of aeration, which is constant in cSBRs. The purpose of intermittent aeration is to create opportunity for improved N and P removal by allowing for both aerobic and anoxic periods within the same reactor. Intermittent aeration also helps to lower operational costs, making IASBRs a more attractive option than cSBRs.

IASBR technology has been used successfully to treat a number of different wastewater types; slaughterhouse wastewater (Li *et al.* 2011, Zhan *et al.* 2009) and dairy processing wastewater (Tarpey 2016, Leonard *et al.* 2018(a), Leonard *et al.* 2018(b)). Removal efficiencies for COD, N and P were reported as >90% in all cases. This is more efficient than cSBRs and other traditional BNR approaches, especially in terms of P removal. This is of particular significance to industrial wastewater treatment, where P removal is normally carried out by chemical precipitation. If P removal can be carried out efficiently by biological processes, it would reduce overall operational costs and create a more sustainable treatment process.

Tarpey 2016 investigated the efficiency of IASBR treating dairy processing wastewater from an Irish processing plant. The lab – scale IASBR was operated over 211 days with a 12h cycle for most of the experiment. Aeration schedule as outlined in Figure 3.1 below. HRT was maintained at 4d. Aeration rate varied from 0.4 – 0.8 LPM, and SRT ranged from 16 – 20d.



Figure 3.1: Aeration schedule for IASBR over 12h (Tarpey 2016).

The wastewater used in this study had an average strength of 3186mg/L COD, 44 – 66mg/L Total P and 60 – 220mg/L Total N. Removal efficiencies reached >95% for COD, N and P over the course of the experiment. SRT and aeration rate were found to have the most impact on IASBR performance, with highest removal efficiencies occurring during 20d SRT with 0.6LPM aeration and 16d SRT with 0.8LPM aeration.

Leonard *et al.* 2018(b) used similar operational parameters in a pilot-scale IASBR treating dairy processing wastewater from the same treatment plant as wastewater used by Tarpey 2016. Cycle length was 12h, HRT was 4d, and SRT was 16d. Removal efficiencies for COD, N and P during this study were >95%, with effluent COD and nutrient levels measuring within EPA discharge limits. These studies at both laboratory and pilot scale demonstrate the capability of IASBR technology to perform BNR and EBPR to a high degree of efficiency, even when treating high-strength industrial wastewater. IASBRs are therefore an attractive option for BNR as they lower operational costs associated with wastewater treatment and they have a lower carbon footprint, making operation more sustainable and environmentally friendly.

Chapter 3 of this thesis outlines the set-up and performance of a lab-scale IASBR system for conditioning of dairy processing wastewater for *Lemna minor* production. The aim of using an IASBR for this purpose is to limit nitrification and denitrification, thus increasing effluent NH_4^+ levels, as A/O treatment was found to be ineffective for this purpose.

3.2 Materials and methods

3.2.1 IASBR set-up and operation

An IASBR reactor (shown in Figure 3.2) was established in parallel with the A/O system outlined in chapter 2. It was operated for 265 days with the same real-time dairy processing effluent that was fed to the A/O system. Seed sludge was also from the same source as for A/O. The IASBR consisted of a single vessel with a working volume of two litres and was operated on a 12h cycle, details of which are provided in table 3.1. Influent flow rate was controlled using a MasterFlex L/S 4-channel peristaltic pump with digital display (Cole-Parmer, USA). Effluent removal was controlled using a Watson-Marlow 323u Peristaltic Pump (Watson-Marlow Pumps Group, USA). Aeration was provided using a Welch® 2511 dry vacuum pump/compressor (Gardner Denver, USA) which supplied air at ≥ 5 LPM. An Influx air meter (Caché, UK) was used to control aeration at 0.4LPM. Mixing was provided by magnetic stirring plates with adjustable RPM to control the mixing speed. Both pumps, air supply and magnetic stirrer were connected to Theben TR 610 top programmable timers (Theben, Germany).

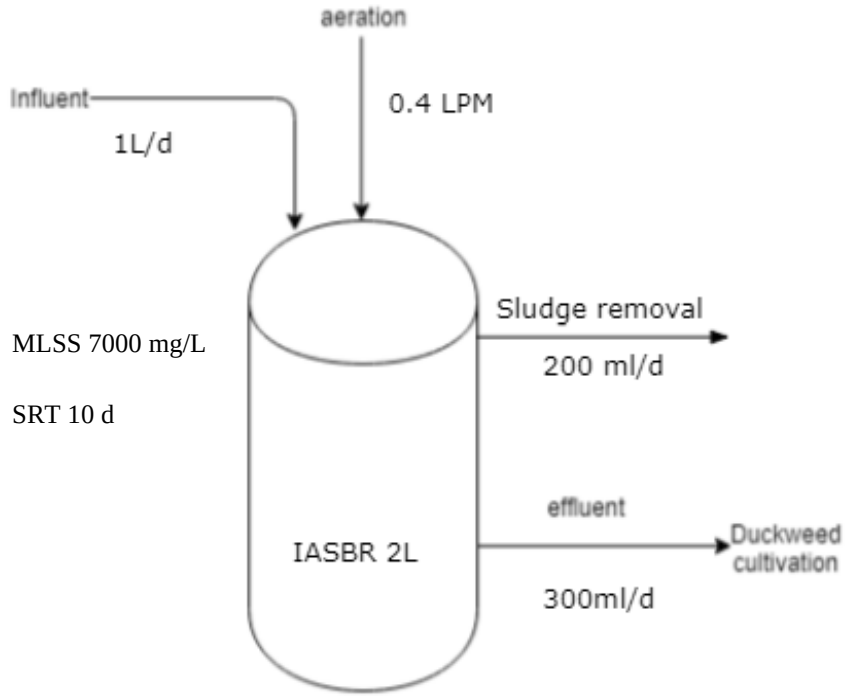


Figure 3.2: Schematic of IASBR.

Table 3.1: IASBR operational parameters for 12-hour cycle.

Fill	120 mins	20 mins	120 mins	20 mins	120 mins	20 mins	120 mins	20 mins	120 mins	20 mins	Settle & Draw

Aeration	Non-aeration

At the beginning of the cycle 500ml of wastewater was pumped into the reactor. For the following 11 hours, the reactors were stirred continuously to ensure complete mixing. The aeration pump operated intermittently with 2-hour aeration periods followed by 20 min non-aeration periods. The settle phase lasted for 40 minutes with no mixing or aeration. In the last 20 minutes of the cycle, 300ml of treated wastewater (supernatant) was pumped out of the reactors into the effluent collection basin. 200 ml of mixed liquor was removed once daily as sludge waste, giving the system an initial SRT of 10 days. Reactor was seeded to an MLSS concentration of 7000 mg/L.

3.2.2 Chemical analysis and other tests.

Samples of 50ml were taken 3 times per week from influent and IASBR MLSS. COD, PO_4^{3-}, NH_3, NO_2^- , NO_3^- and later Total Nitrogen (day 160 onwards) were determined with a HACH® DR 2800 spectrophotometer (Hach, USA). The procedures for each chemical test are outlined in chapter 2 with the exception of Total Nitrogen, which is described below. All chemical tests were carried out according to the HACH® DR 2400 procedures manual (Hach company 2004). Reagents used were also as per the HACH® DR 2400 procedures manual unless otherwise specified.

Total Nitrogen (TN)

Method 10072 Persulfate digestion method for High Range (HR) 10 – 150mg/L TN was used to determine the total nitrogen levels in both influent and IASBR samples. Samples were centrifuged prior to testing in order to remove suspended solids. Influent samples were sometimes diluted 1:2 to ensure the test volume remained within measuring range. Dilution was performed depending on the strength of each batch of wastewater.

Dissolved oxygen (DO) and Temperature were monitored using a HI 98186 DO meter (Hanna Instruments, USA). Mixed Liquor Suspended Solids (MLSS) and Mixed Liquor Volatile Suspended Solids (MLVSS) were measured in accordance with APHA standard methods (2015). pH was measured with a Mettler-Toledo® seven-easy pH meter (Mettler Toledo, USA). Floc size/density was monitored using a Leica DM3000 Type DFC490 light microscope with attached camera for image capture (Leica Microsystems, Germany). Gram staining, Neisser staining and DAPI staining techniques were used to monitor and identify microbialchanges within floc structures. Food to microorganism ratio (F/M) was calculated as per chapter 2.

3.3 Results and Discussion

The A/O system discussed in chapter 2 was found to be unsuitable for use in conditioning wastewater growth media for *Lemna minor*. An IASBR configuration was selected for conditioning, as it had been successfully used at both laboratory and pilot scale to treat dairy processing wastewater. The system parameters from Tarpey (2016) were adapted to provide an operational regime that would remove COD but limit N and P removal. Therefore, the IASBR in this study was not designed to operate efficiently. Results of IASBR operation for wastewater conditioning will be presented and discussed in this section.

3.3.1 Floc formation and MLSS

MLSS dropped to around 2000mg/L within the first 18 days of operation. From day 20 – 76 SRT was increased from 10d to 40d to improve COD removal and recover MLSS. During this period, MLSS levels steadily increased to 10000mg/L by day 76. HRT and SRT were decreased to 4d from days 78 – 113, during which MLSS levels decreased to around 4000mg/L. On day 116, HRT and SRT were decreased to 2d to decrease P removal, as effluent P levels had become too low to support *Lemna* growth. As a result, MLSS decreased to around 2000mg/L. On day 132 HRT and SRT were increased to 4d with the aim of recovering MLSS. However, this did not have the desired effect and MLSS remained stable at 2000mg/L for the remainder of the operational period.

From days 78 – 223 cycle length was set to 24h to promote greater COD removal and to improve sludge settling, as MLSS settling was very poor from day 20 onwards, with high levels of suspended solids remaining in the effluent. This did not change, despite the increase in cycle length, which was changed back to 12h after day 223 to provide sufficient effluent volumes for *Lemna* growth experiments. From day 78 onwards, MLSS was removed with the effluent at the end of each cycle instead of manual removal. The aim of this was to remove biomass that did not form flocs and to promote biomass that did flocculate. Unfortunately, this did not work, as by then filaments had begun to appear in the floc formations and had started to take over the biomass. Filamentous growth was present for most of the IASBR operational period. Average F/M ratio for across the operational period was 0.12 g COD / g VSS • d. This is lower than the reference literature values of 0.3 – 0.5 g BOD / g VSS • d given in chapter one. This can be attributed to high levels of influent COD coupled with low VSS within the reactor.

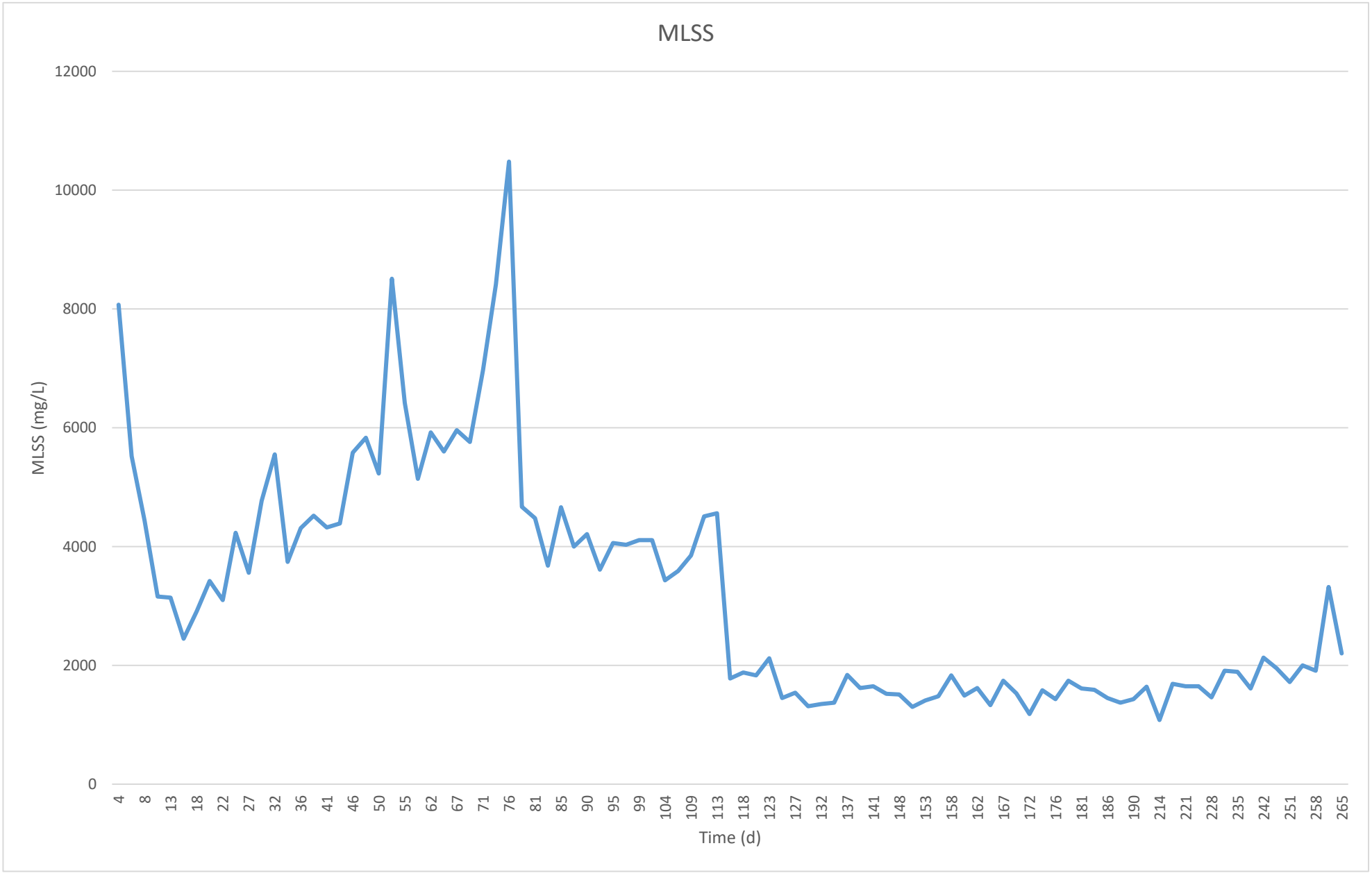


Figure 3.3: IASBR MLSS levels.

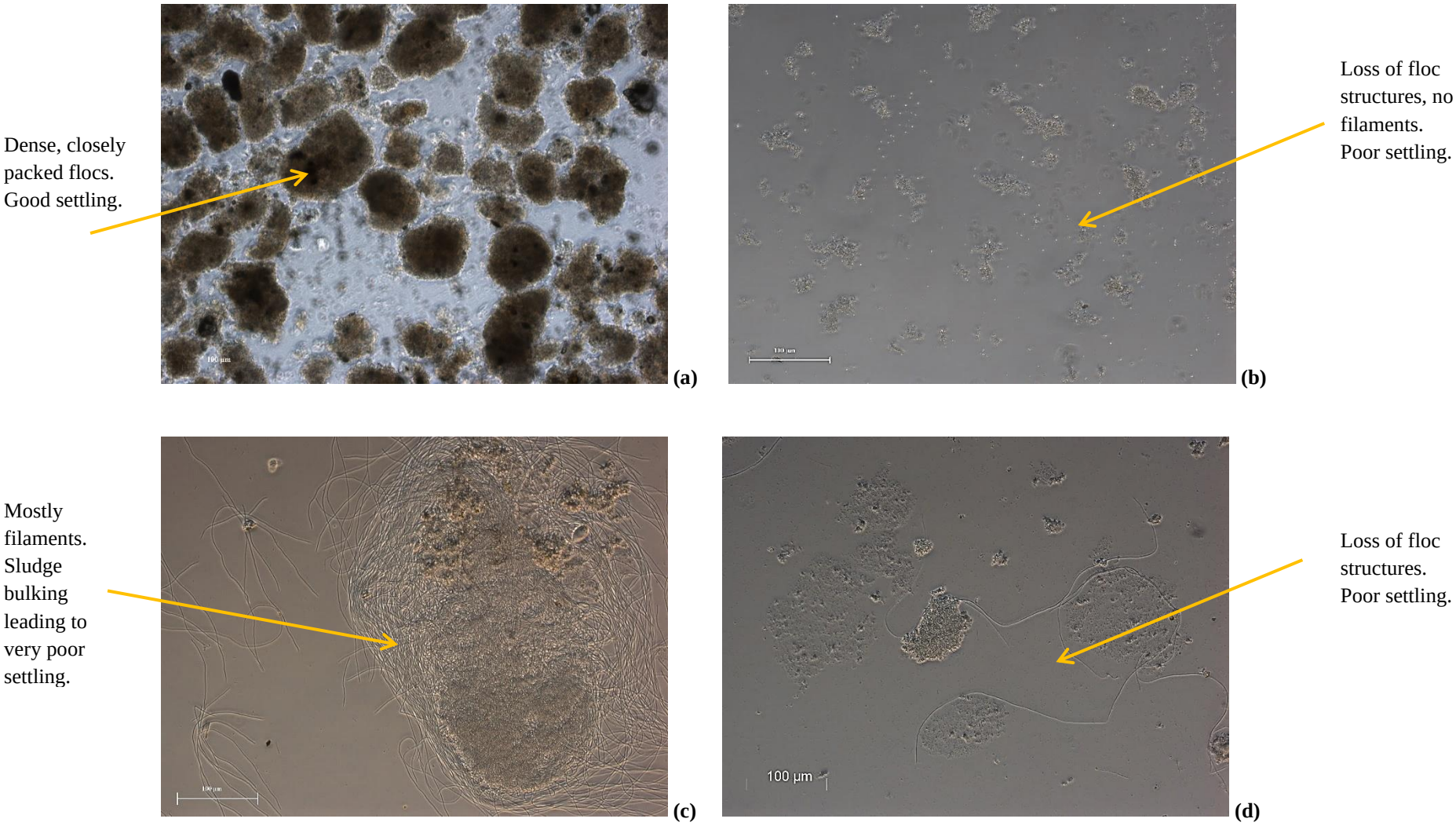


Figure 3.4: (a) Image of seed sludge under phase contrast 10x magnification. (b) Image of sludge flocs from day 78 under phase contrast 10x magnification. (c) Image of sludge flocs from day 162 under phase contrast 10x magnification. (d) Image of sludge flocs from day 265 in IASBR under phase contrast 10x magnification. Scale bar for all images is 100µm

3.3.2 IASBR filament identification.

Gram, Neisser and DAPI stains (as per chapter 2) were carried out on sludge samples across the IASBR operational period in order to identify filaments that caused sludge bulking and poor settling. The main filament types identified were Type 0041 and Type 0675 (Figures 3.5 – 3.7). The criteria for identifying these filament types, as well as their significance for reactor performance, is as discussed previously in chapter two (pages 47 and 52).

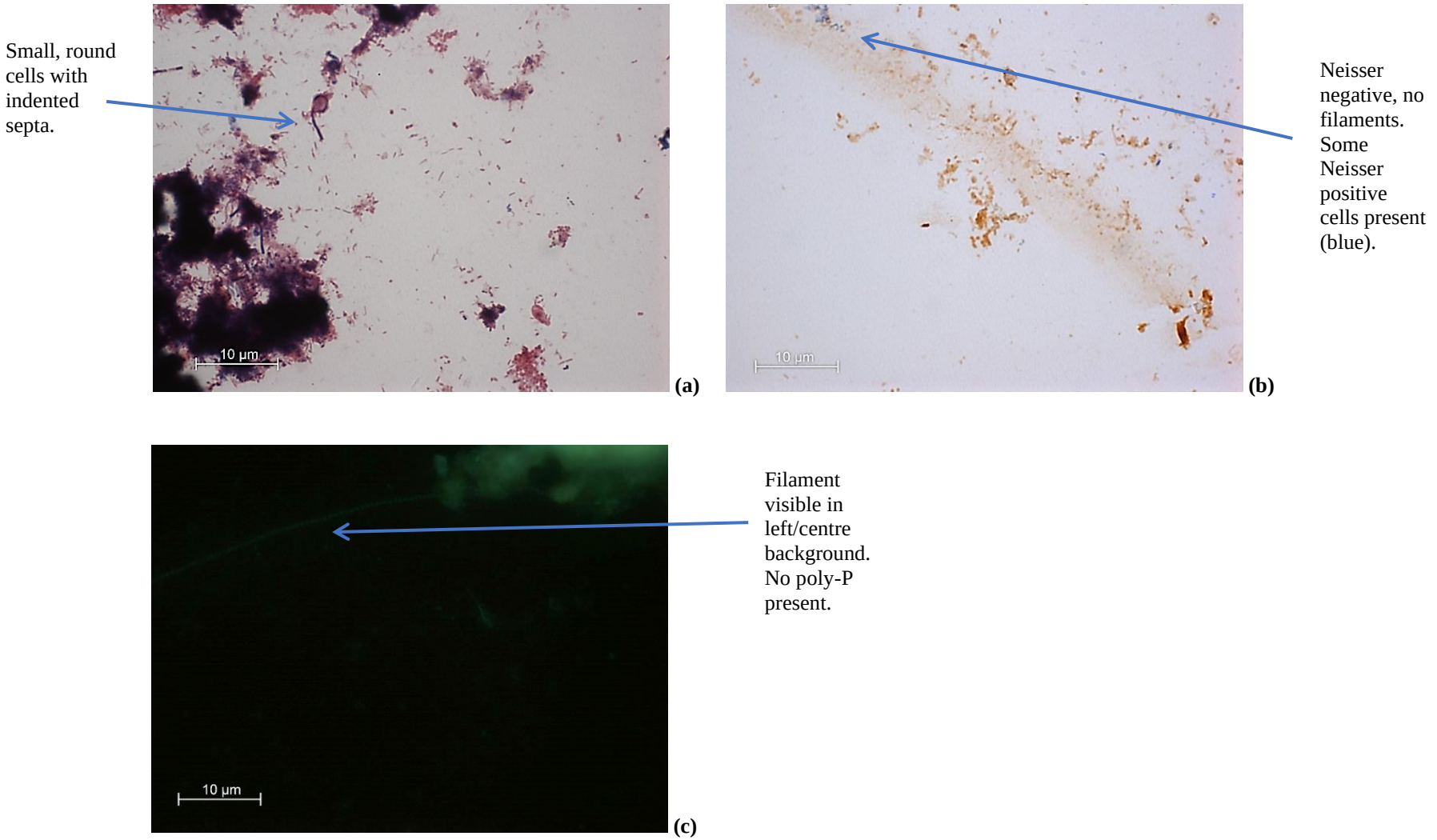
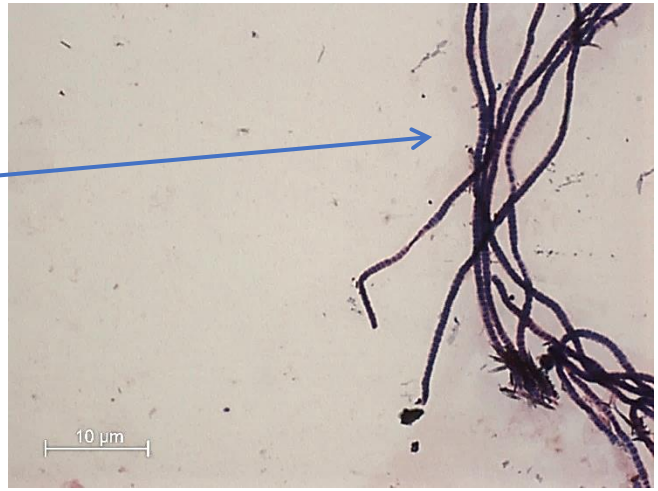
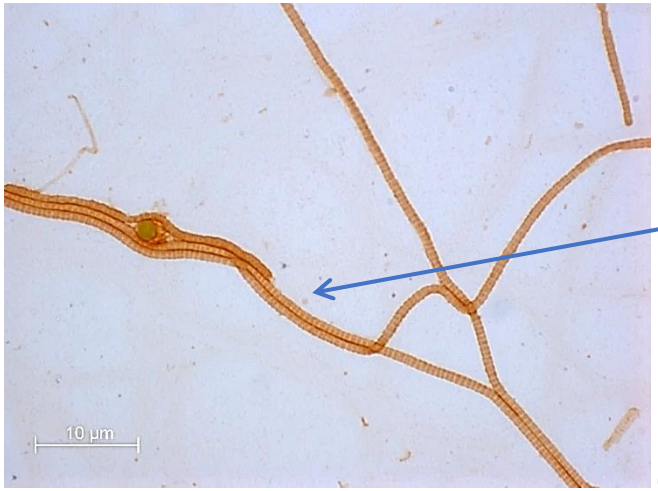


Figure 3.5: (a) IASBR Gram stain day 88. (b) IASBR Neisser stain day 88. (c) IASBR DAPI stain day 88.



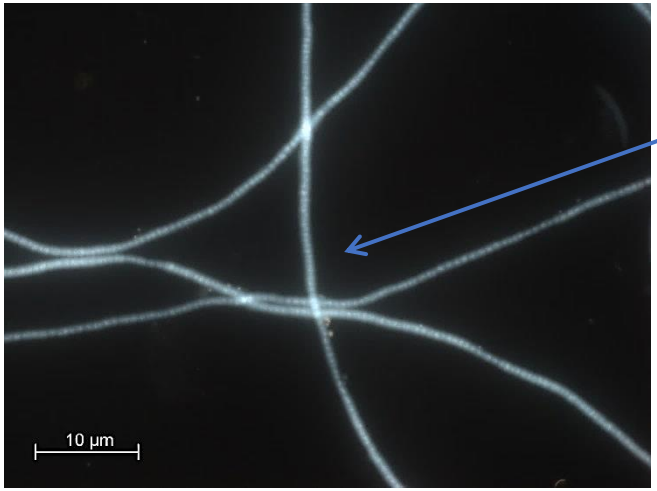
Small, round cells with indented septa.

(a)



Sheath clearly visible on filaments.

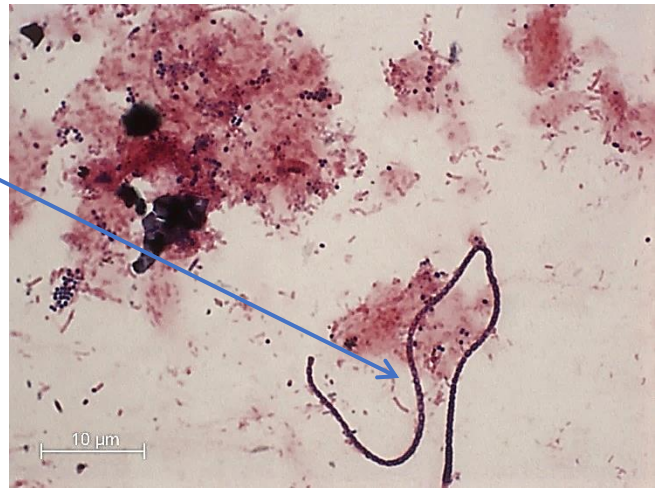
(b)



Automatic white balance on Leica microscope software changed the colour of fluorescence from green to blue/white. No poly-p present.

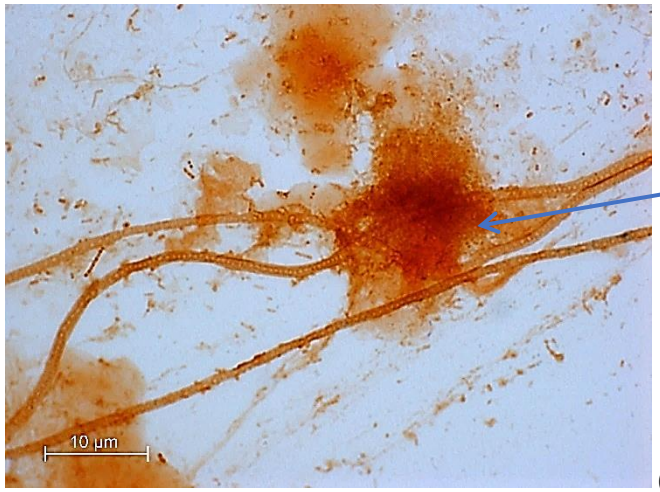
(c)

Figure 3.6: (a) IASBR Gram stain day 173. (b) IASBR Neisser stain day 173. (c) IASBR DAPI stain day 173.



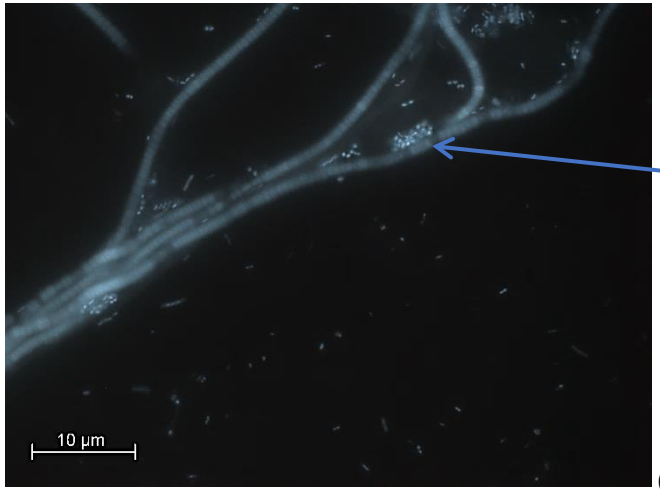
Small, round cells with indented septa.

(a)



(b)

Filaments can be seen as part of floc structure.



(c)

Automatic white balance on Leica microscope software changed the colour of fluorescence from green to blue/white. No poly – P present. Epiphytic (attached) growth is visible.

Figure 3.7: IASBR Gram stain day 265. (b) IASBR Neisser stain day 265. (c) IASBR DAPI stain day 265.

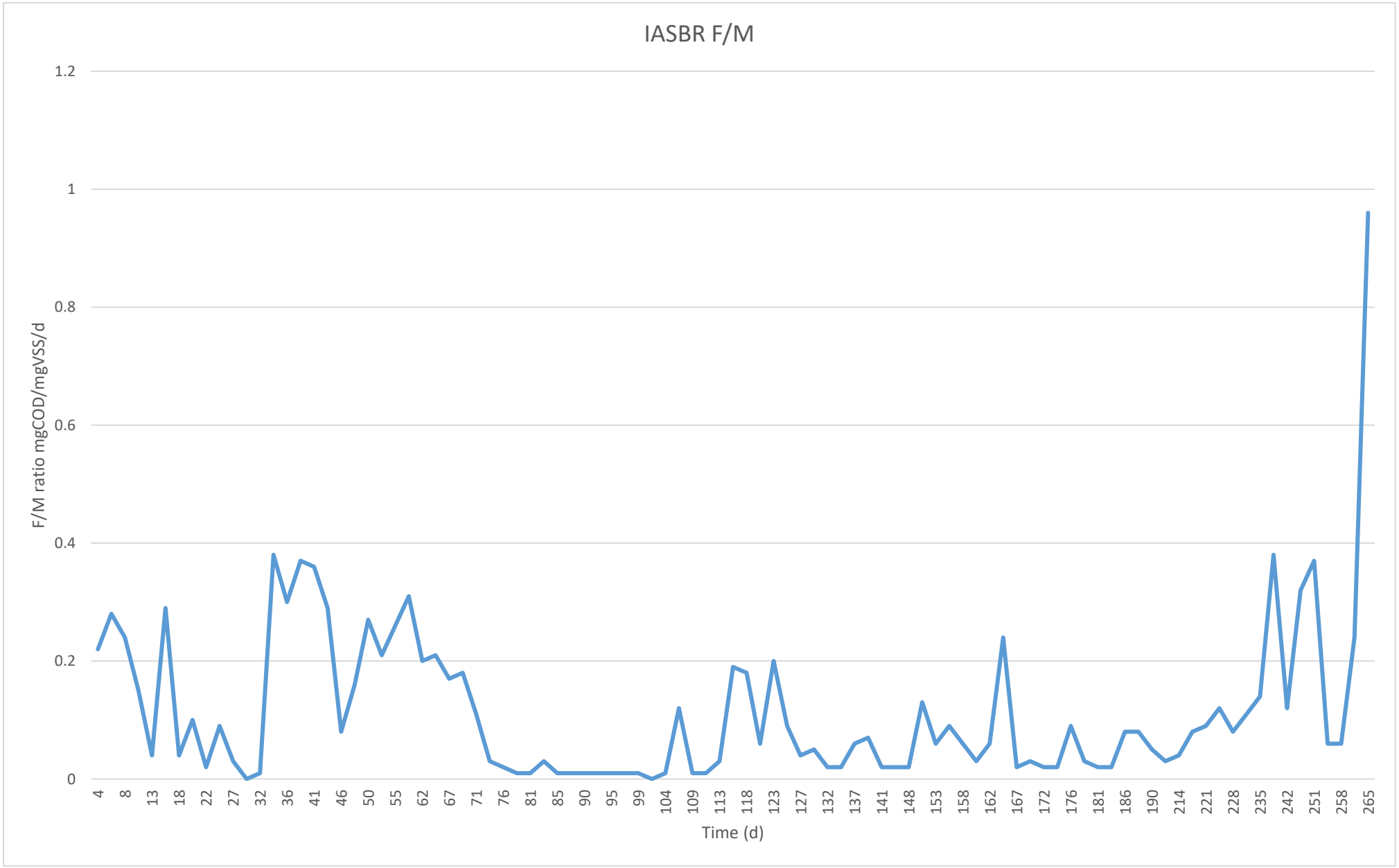


Figure 3.8: IASBR F/M ratios for the operational period.

As stated in chapter 2, little data is currently available in the literature about Type 0041 and Type 0675 filaments, or the operational conditions under which they thrive, except that they are aerobic and are normally problematic under low F/M conditions and, to a lesser extent, long SRTs (Eikelboom 2000). F/M ratios in the IASBR ranged from 0 to 0.96 g COD/g VSS/d (Figure 3.8), averaging at 0.12 g COD/g VSS/d. Calculations are provided below for the highest and lowest data points across the operational period.

$$\text{F/M day 29} = \frac{[0.04\text{g COD}] \times [1\text{ L/d}]}{[4.11\text{g VSS}] \times [2\text{ L}]} = 0.0048 \approx 0.00\text{ g COD/g VSS/d}$$

$$\text{F/M day 265} = \frac{[4.114\text{g COD}] \times [1\text{ L/d}]}{[2.14\text{g VSS}] \times [2\text{ L}]} = 0.9612 \approx 0.96\text{ g COD/g VSS/d}$$

The average F/M ratio was much lower than the literature recommended ratio of 0.3 – 0.5 g BOD / g VSS • d for 5 – 7d SRT and was certainly low enough to provide ideal conditions for the identified filament types, even with the shorter SRT of 2 – 4d (Metcalf & Eddy 2014). The reduced MLSS around days 13-15 of reactor operation (Figure 3.3) prompted a reduction in sludge wastage from 200ml/d to 60ml/d to promote increased MLSS. This was increased again to 500ml/d after MLSS levels reached 10050 mg/L by day 76, after which it stabilised around 2-3 g/L. Influent COD levels fluctuated over the operational period due to a combination of factors including differences in chemical profiles between batches of wastewater, and because influent was fed to the reactor from a 5L glass beaker at room temperature (~25°C), which allowed for microbial growth as influent Q = 1L/d. The combination of high MLSS and relatively low COD levels are the reasons for the low F/M ratios over most of the operational period.

3.3.3 Chemical analysis of performance.

Temperature in the IASBR remained between 20 and 25°C for the full 265 days of operation. Dissolved oxygen (DO) ranged from 0 – 8.31 mg/L. Influent pH ranged from 4 – 12, with pH in the IASBR ranging from 5 – 9. COD removal ranged from 50 – 97% (Figure 3.9), which mostly matches reported values for COD removal in literature, as well as COD removal with the A/O system. However, despite the high removal efficiency, effluent COD

was only occasionally below 100 mg/L, and only once went below 50 mg/L. Therefore, effluent COD did not fall within EPA discharge limits. This, while not entirely desirable, was acceptable.

COD removal efficiencies with IASBR were similar to COD removal efficiencies with A/O, as both systems maintained >90% removal for most of the respective operational periods. However, the fluctuation in COD levels in real-time wastewater meant that % removal also fluctuated. The drop in COD around day 194 occurred around the time that Dairygold shut down the anaerobic digestor for the winter period when milk production is low. As a result, new wastewater was no longer available and the wastewater remaining from the previous batch had to be diluted. After it was observed that COD was too low in the diluted wastewater, it was supplemented with synthetic wastewater as per chapter 2 (table 2.2). A trace element solution was used with the synthetic supplement, as the results of the A/O synthetic trial highlighted the need for such. No change in efficiency was observed with synthetic wastewater.

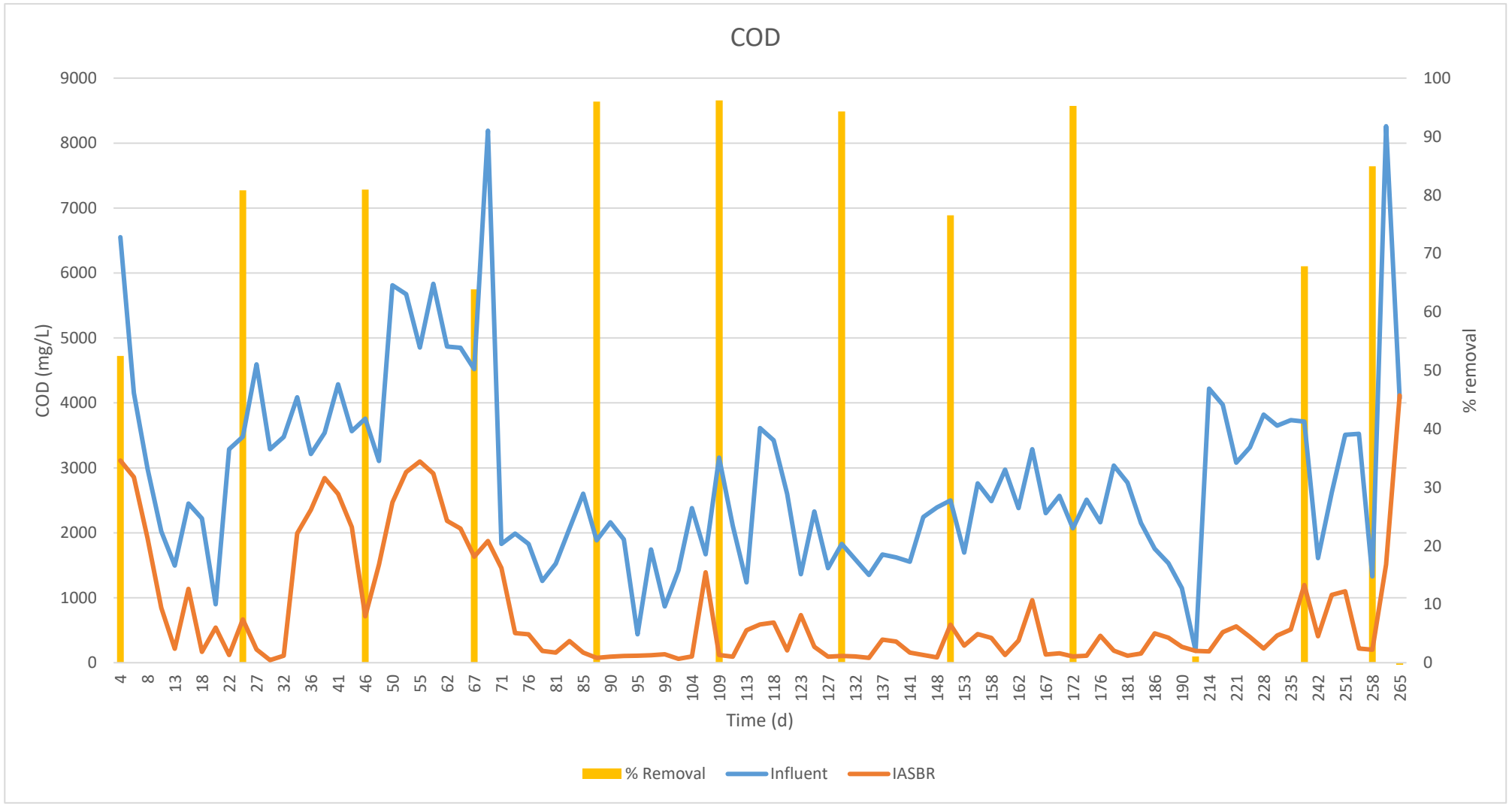


Figure 3.9: IASBR COD levels, showing % removal every 21 days (rolling average).

Phosphate removal was measured as Orthophosphate (PO_4^{3-}). Removal ranged from 30 – 80% (Figure 3.10). P removal for the most part, matched values reported in literature and matched P removal observed with A/O on real-time wastewater. The peak in influent P levels seen at day 67, and subsequently the 94% removal, was due to a high level of suspended solids in the test sample for that particular day, despite centrifugation and filtration of the sample prior to testing. Efficiency decreased towards the end of the trial, likely a result of feeding with synthetic wastewater, as similar low removal efficiencies were seen during A/O operation with synthetic wastewater (20 – 50%). Effluent phosphate levels did not fall within EPA discharge limits despite the observed removal efficiency, which was desirable as the optimal phosphate range for successful *Lemna* cultivation is 0.03 – 300 mg/L (Figure 2.4). The A/O system P levels ranged from 0 – 190.5 mg/L and IASBR system P levels ranged from 97 – 252.5 mg/L across the respective operational periods. This shows that, while both systems produced P within the optimal range for *Lemna*, the IASBR was more closely aligned with *Lemna* needs.

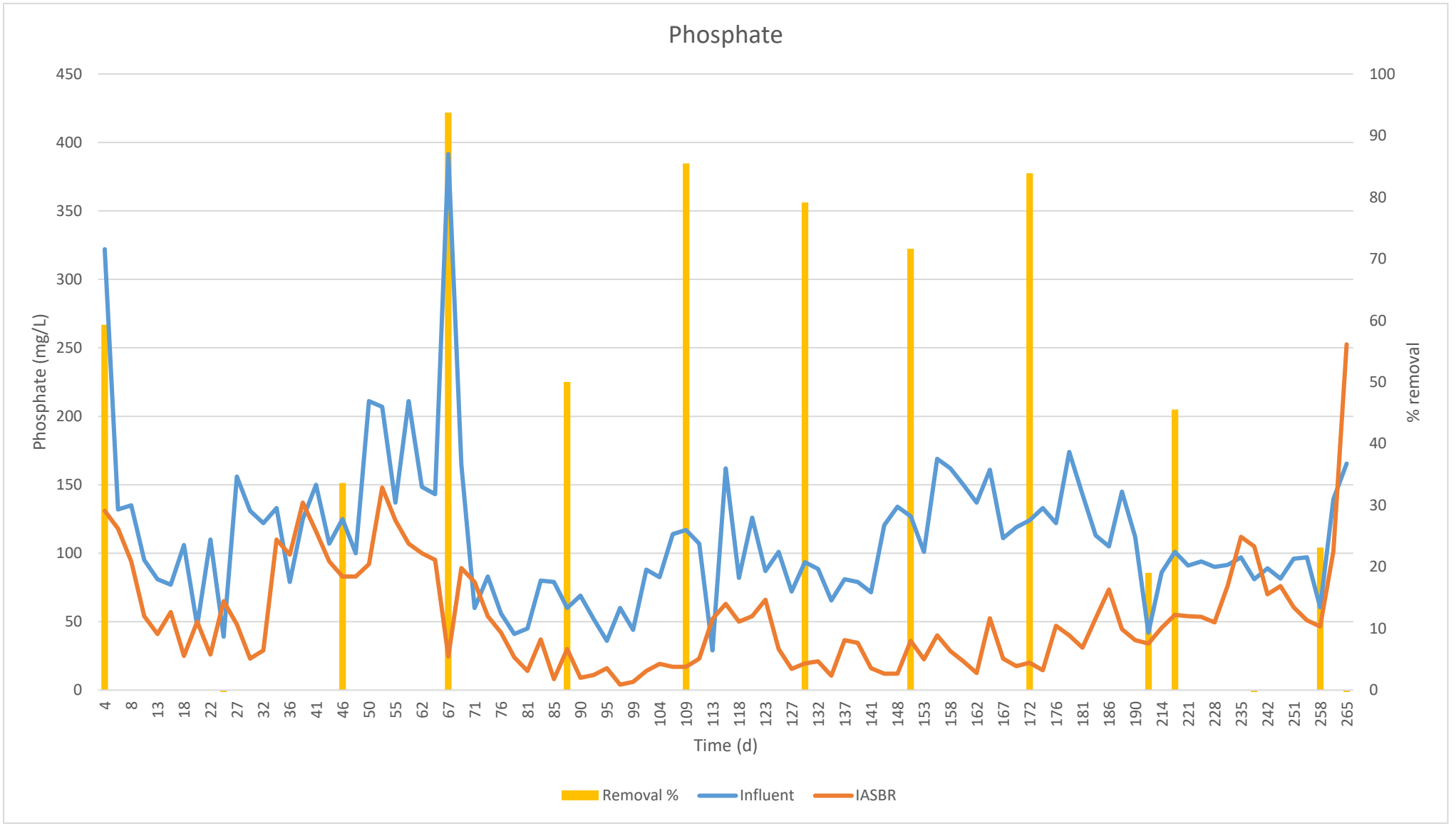


Figure 3.10: IASBR Phosphate levels, showing % removal every 21 days (rolling average).

Nitrogen removal was measured primarily by NH_4^+ removal, with measurements for NO_2^- and NO_3^- providing data on nitrification. Total nitrogen was measured from day 160 onwards to assess the contribution of ammonification to overall NH_4^+ levels within the IASBR. For the first 116 days, NH_4^+ levels were often much higher than NH_4^+ levels in the influent wastewater up to day 116, after which activity shifted towards removal rather than accumulation. NH_4^+ removal ranged between 4 and 94%, with NH_4^+ levels in constant flux with each batch of wastewater (Figure 3.11). The sustained increase in ammonia levels in the IASBR up to day 116 was most likely due to ammonification of high levels of organic nitrogen in the influent, combined with low levels of nitrification, thus allowing NH_4^+ to accumulate. This, however, can only be shown from days 160 onwards (Figure 3.14).

NO_2^- and NO_3^- levels, shown in Figures 3.12 and 3.13, indicate that partial nitrification was achieved for most of the operational period, with low levels of full nitrification achieved after day 130. Peaks seen in IASBR NO_3^- and NO_2^- levels on days 41 and 109 are due to high levels of suspended solids in the test sample that remained after dilution and filtration and are therefore not representative of actual levels on those particular days. Partial nitrification is most likely due to a combination of a short HRT and aeration rate of 1 – 2LPM for most of the IASBR operational period. AOB have a shorter doubling time and a higher affinity for DO than NOB, making them more difficult to wash out of the system. It is also possible that heterotrophic nitrifiers were present. The IASBR operational parameters for most of the trial were compatible with the SHARON process, except for temperature. However, this may have only reduced the rate of nitrogen removal through SHARON rather than preventing it altogether. ANAMMOX or CANON were unlikely because of the aeration rate. For most of the IASBR operation, effluent NH_4^+ levels were not within EPA discharge limits. A desirable result, as the optimal NH_4^+ range for successful *Lemna* cultivation is 5 – 50 mg/L (Figure 2.4). The A/O system NH_4^+ levels ranged from 0.2 – 59.25 mg/L and IASBR system NH_4^+ levels ranged from 0.1 – 70 mg/L across the respective operational periods. While the A/O was more closely aligned with *Lemna* needs in terms of NH_4^+ range, the NH_4^+ levels in the IASBR were more stable across the operational period compared to the A/O, meaning increased likelihood of NH_4^+ availability for *Lemna* growth.

The shift from ammonia addition to ammonia loss could be attributed to changes in microbial community composition within the reactor, or they could be a result of seasonal changes in wastewater composition. If it was the latter rather than the former, it is possible that seasonal changes in wastewater composition caused changes in microbial community

composition. In order to assess this, it would be necessary to repeat the experiment and observe changes in microbial community composition using metagenomics to build a community profile for selected stages of reactor operation.

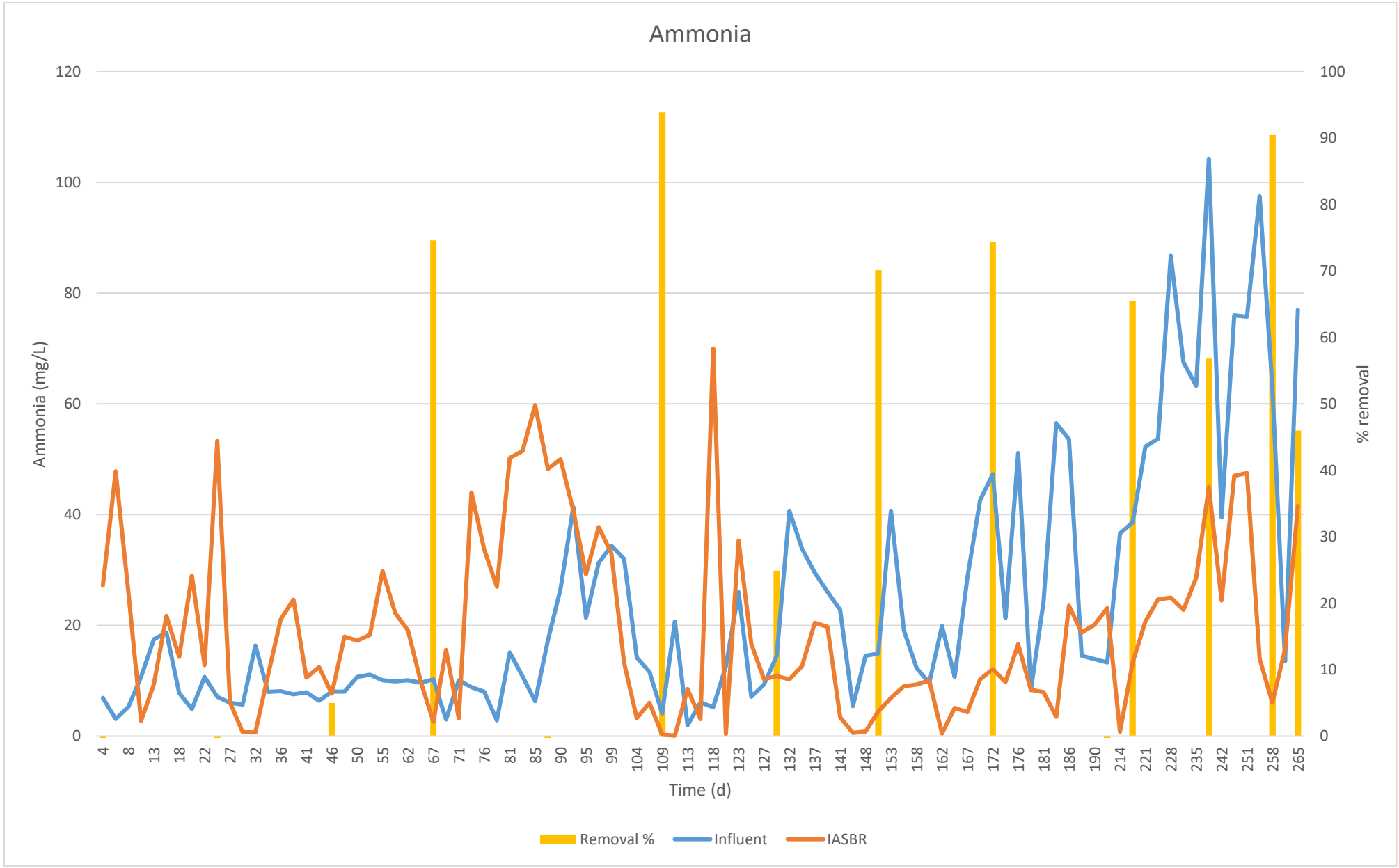


Figure 3.11: IASBR ammonia levels, showing % removal every 21 days (rolling average).

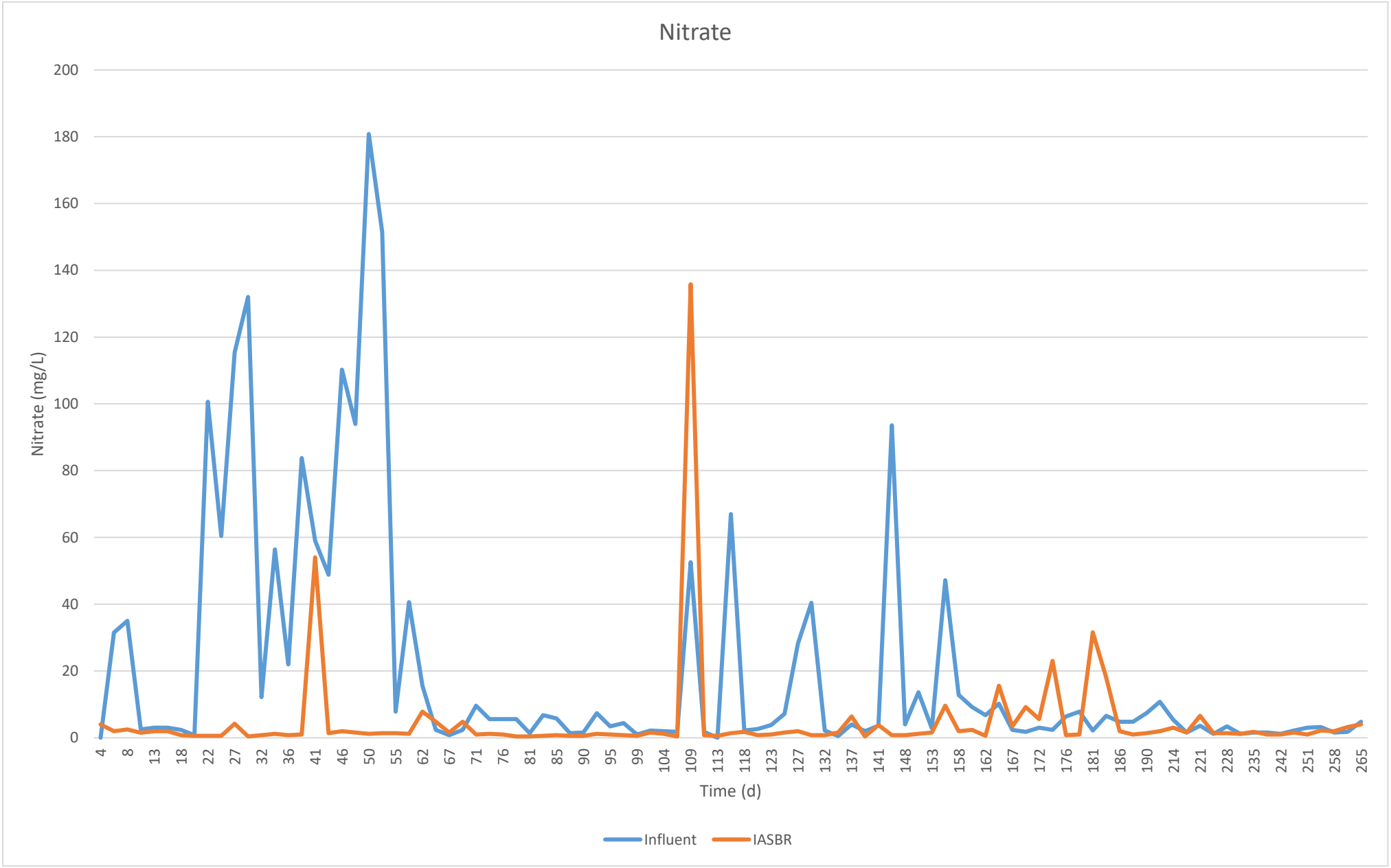


Figure 3.12: IASBR nitrate levels.

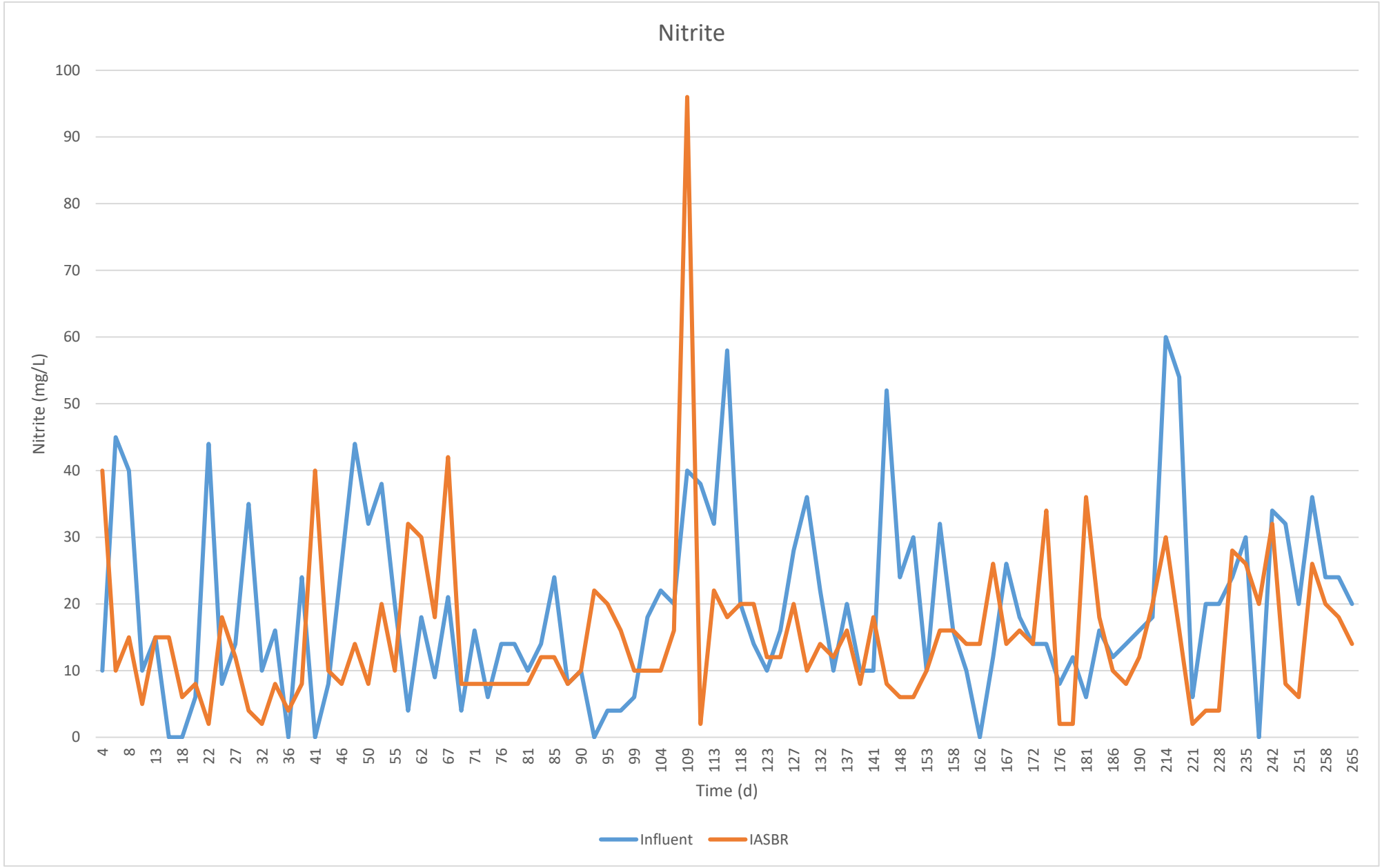


Figure 3.13: IASBR nitrite levels.

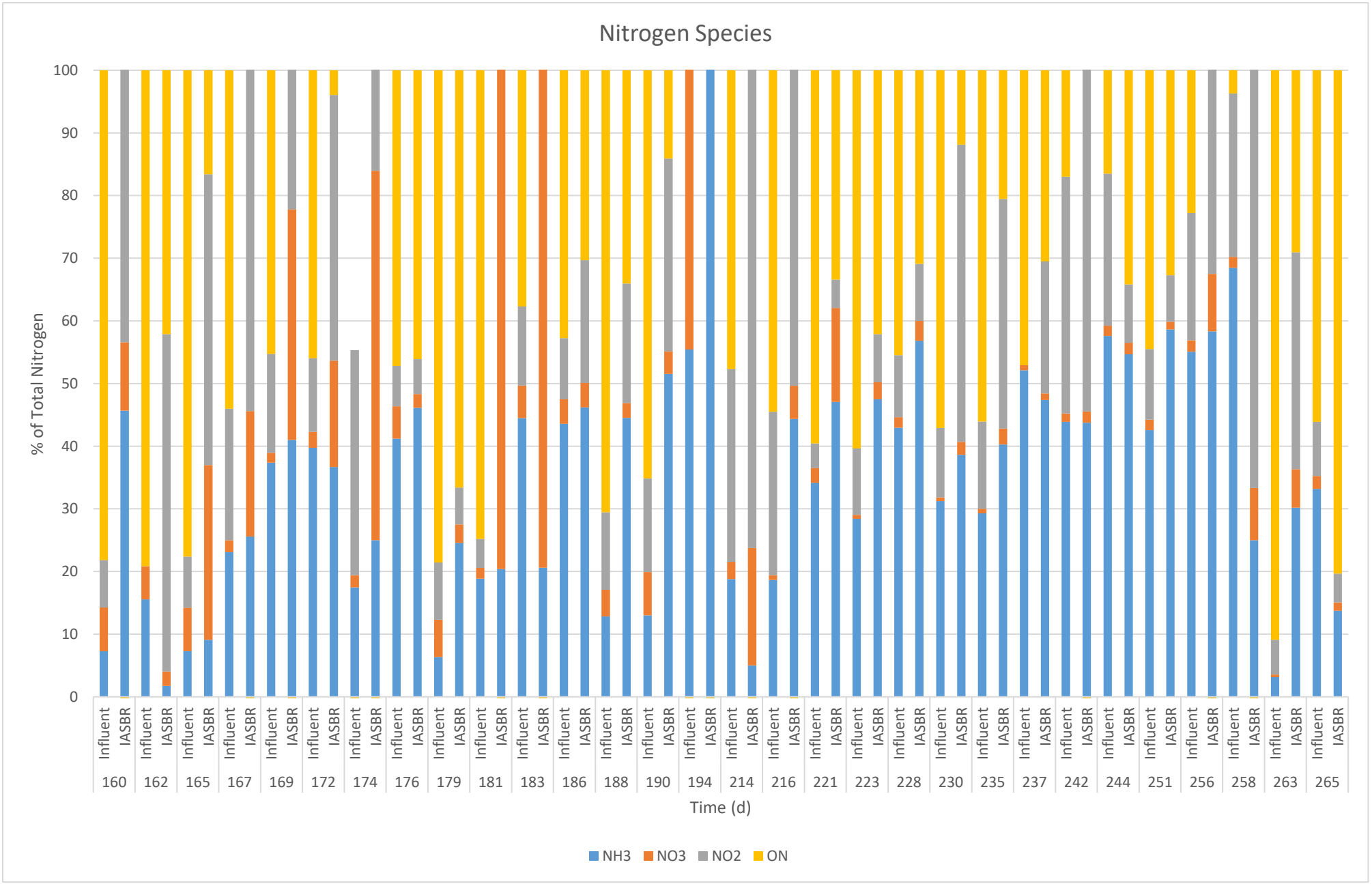


Figure 3.14: IASBR nitrogen species represented as % of total nitrogen.

3.4 Conclusion.

According to the nutrient requirements for *Lemna minor* outlined in table 2.4, the effluent from the AO system was not suitable due to lack of available nitrogen. In order to address the issues arising with lack of available nitrogen, an IASBR reactor was established to limit nitrification and denitrification and to increase available nitrogen.

During operation of the IASBR it became clear that the COD and nutrient removal profiles for both A/O and IASBR systems are similar, despite the differences in their operation. Considering that the IASBR has a smaller carbon footprint and requires less aeration than the A/O, it is a cheaper system to run and is more environmentally friendly than an A/O system. There is also a greater degree of control over the operation of an IASBR system, and therefore the effluent quality, making it a better system to have in an industrial plant than A/O.

The reactor parameters outlined in Tarpey 2016 achieved high nutrient removal efficiencies with dairy processing wastewater, which was the aim of the work carried out therein. For work package 4 of the Newtrients project, these parameters (mainly SRT and aeration rate) were adapted to establish a system that would not achieve the same high levels of nutrient removal, with the aim of producing an effluent that would be suitable for *Lemna* cultivation. Unfortunately, this did not occur, as relatively high removal efficiencies were achieved regardless of inadequate operation, and sludge bulking became a significant issue due to the altered operational parameters.

According to table 2.4, the IASBR reactor effluent was preferable for *Lemna* production. In order to ascertain which effluent was most suitable for *Lemna* growth, test batches of 250 ml effluent from both A/O reactor zones and the IASBR were used to cultivate *Lemna* over the course of 2 weeks. These initial tests showed that effluent from the anoxic zone of the A/O reactor resulted in the highest yields. This was likely due to the fact that COD was removed but N and P remained relatively close to influent levels, as is the case with anaerobic digestor effluent. For this reason, it was decided to utilize an anoxic reactor going forward to *Lemna* growth trials.

Chapter 4: Integration of *Lemna minor* cultivation system with IASBR wastewater treatment.

4.1 Introduction

Lemna minor is a species of duckweed native to Ireland. It is a small monocotyledonous aquatic plant that grows quite quickly (doubling time 1 – 3 days) and absorbs nutrients through all of its exposed surfaces. Due to duckweed's unique characteristics, its nutrient composition is mostly sugar and protein (Kutschera & Niklas 2015, Skillicorn *et al.* 1993).

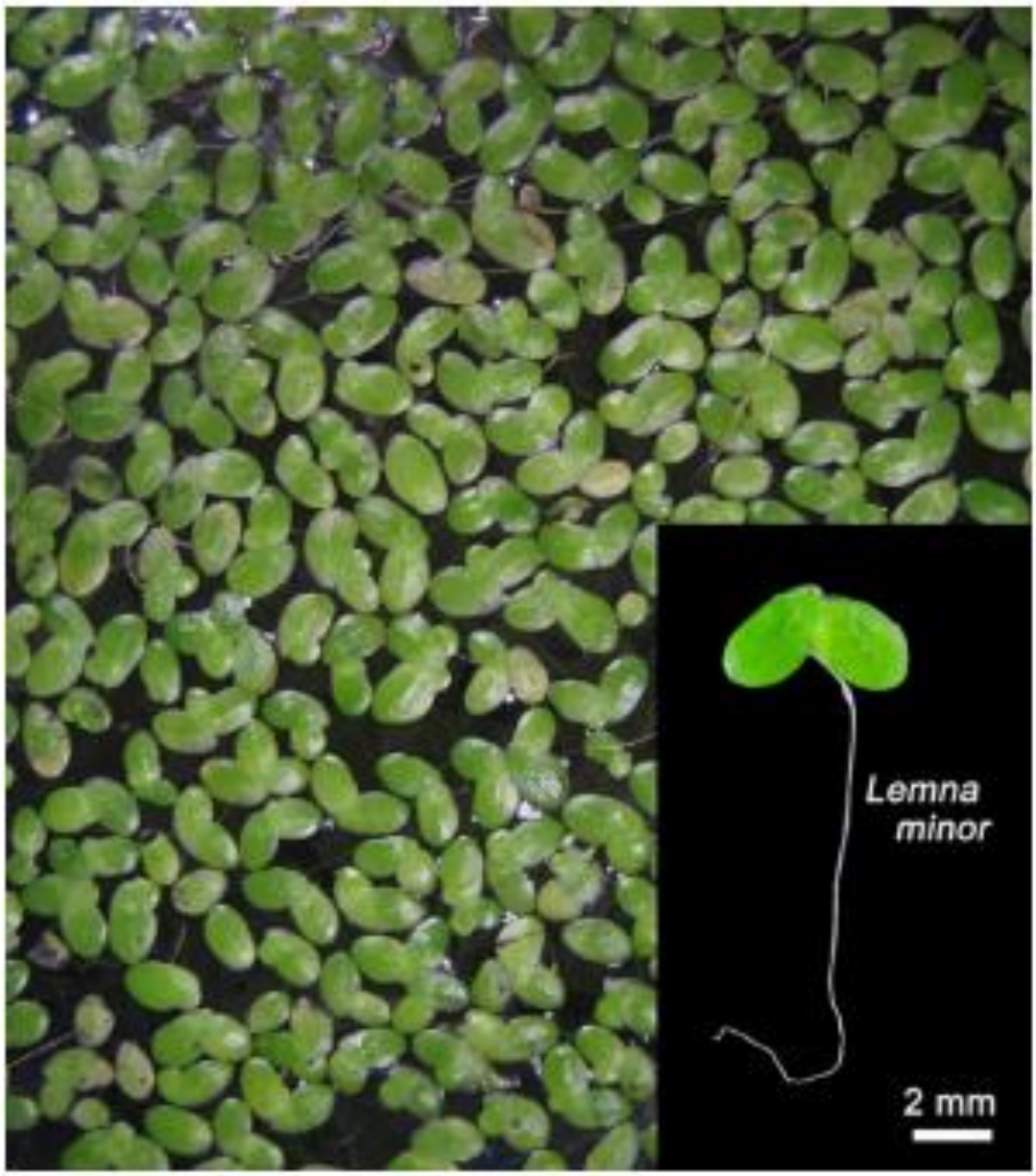


Figure 4.1: The common duckweed Lemna minor L., the type species of the genus. Numerous fronds are viewed from above, and one representative plant is shown in side view (inset). Figure taken from Kutschera & Niklas (2015).

Duckweed has been used for bioremediation of heavy metals (Verma & Suthar 2015, Alvarado *et al.* 2008), for wastewater treatment (Hasan *et al.* 2019, Sudiarto *et al.* 2019) and as a source of biofuel (Cheng *et al.* 2019, Ge *et al.* 2012). Duckweed has also been successfully farmed for use in aquaculture as fish feed, and for use as animal fodder (Goopy and Murray 2003, Huque *et al.* 1996, Beccera *et al.* 1995, Leng *et al.* 1995, Skillicorn *et al.* 1993, Culley *et al.* 1973). The numerous studies into duckweed species as animal fodder show its potential to be a sustainable, supplementary feed-crop rich in protein and amino acids. The protein and amino acid content in duckweed is comparable to soya meal, a well-known and commonly used protein supplement in animal feed. However, soya meal protein only comes from part of the plant, meaning that production of soya meal as a food supplement generates plant waste and ends up being expensive due to the need to extract the protein from the rest of the plant. In contrast, the entire duckweed plant can be used for the production of food supplements. This coupled with the high protein content makes duckweed an attractive alternative to soya that is cheaper to produce. Farmers in Ireland are currently dependent on imported animal feeds, generating an estimated cost of €1.6m annually (DAFM 2015, DAFM 2010). This highlights a particular need in Ireland right now for a cheap, locally produced animal feed supplement, a need which duckweed is ideally suited to fulfil as it is approved for use as a food supplement in the EU (EU Commission Novel Food Catalogue).

Cultivation of duckweed from wastewaters has not only been proven successful, it also contributes to sustainability by re-using nutrients and generating high nutritional value biomass in line with circular economy principals. Development of *Lemna minor* as a component of a waste management scheme will close the plant-nutrient cycle, thus turning waste into valuable food and protecting local surface-waters. It also has the added benefit of decreasing dependence on imported protein-rich feeds such as soy, thus saving the Irish economy millions each year. Another advantage of duckweed cultivation is that high crop yields can be generated on small plots of land, much less than would be needed for comparable amounts of soya or other feed crops. Use of dairy processing wastewater specifically is ideal for Ireland as dairy generates tonnes of wastewater each year and is a year-round process, providing a relatively unlimited, high-nutrient feedstock for *Lemna minor* production.

Lemna minor growth systems have been developed over the last decade, producing high yields, typically in the range of 10-40 tonnes DM/ha/year, with peaks close to 100 tonnes DM/ha/year in well managed systems (Huque *et al.* 1996, Goopy and Murray 2003). Growth systems need to be optimised for the physico-chemical characteristics of the waste

water stream, as well as local climate conditions. Climate is a particularly important consideration for outdoor systems. At the time of writing this MSc thesis, duckweed farming practices have not been undertaken in Ireland.

This chapter introduces the design and operation of a laboratory scale coupled system for growth of *Lemna minor* with pre-treated dairy processing wastewater, as well as the testing and experiment methods which were used in the research. Microbiological staining carried out on A/O and IASBR filaments during the research period are also covered in this chapter. All coupled system experiments were carried out in collaboration with Éamonn Walsh, a PhD candidate with the Newtrients project whose research is centred on the downstream cultivation process that will follow the initial wastewater treatment.

As stated in chapters 2 and 3, A/O and IASBR reactors were used to pre-treat the wastewater. It was determined over the course of these reactor operations and as a result of preliminary growth tests that an anoxic-only system was most suitable to carry forward for coupled system trials.

4.2 Materials and Methods

A laboratory scale *Lemna minor* cultivation system coupled to an anoxic wastewater treatment reactor, shown in Figure 4.2 below, was used to conduct the experiments outlined in this chapter. The cultivation system was designed to mimic part of an indoor industrial growth system at laboratory scale.

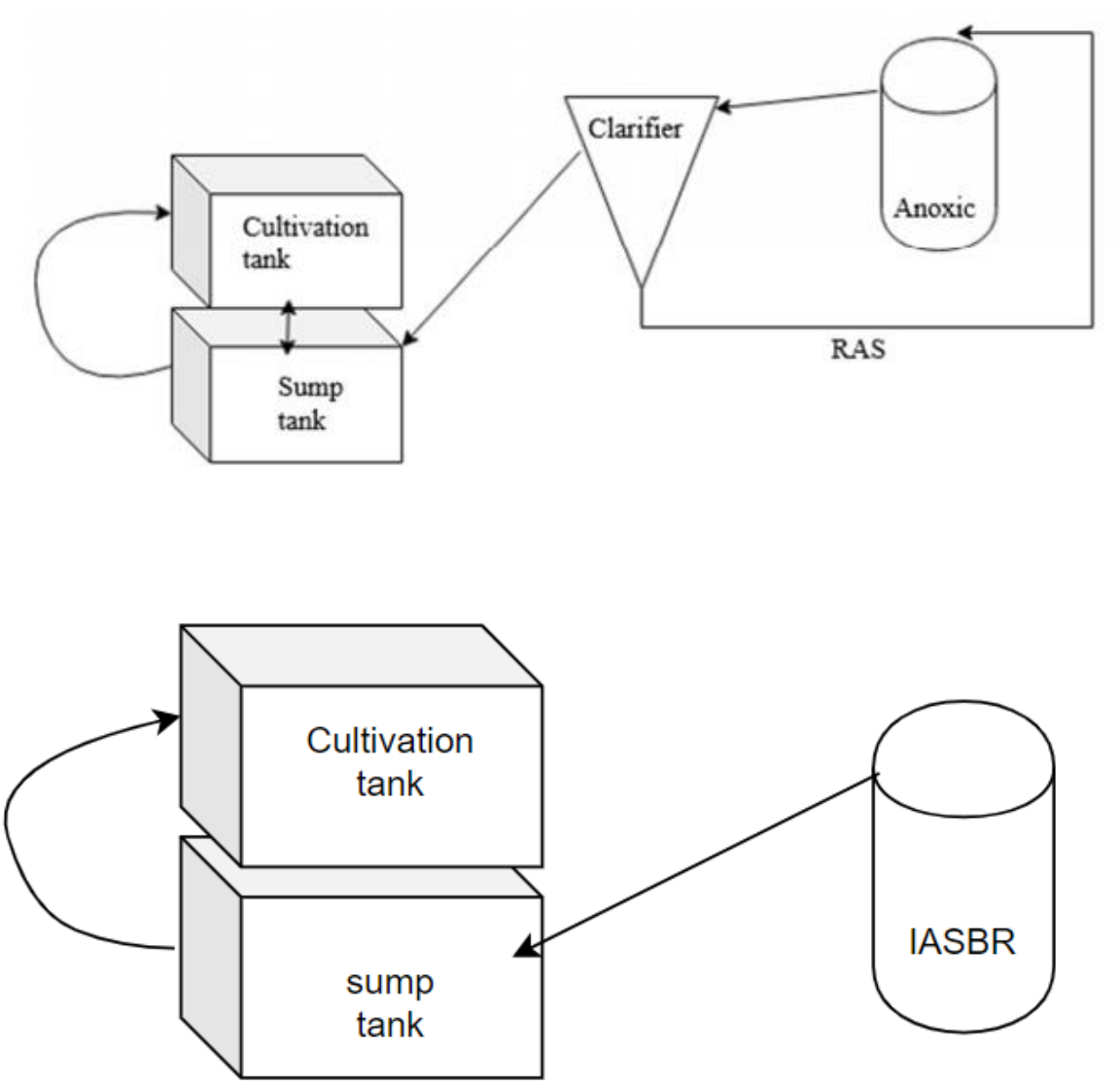


Figure 4.2: (a) Coupled system schematic with anoxic reactor. (b) Coupled system schematic with IASBR. Recycle of effluent between the cultivation and sump tanks was provided by a pump/drainage system inside the tanks.

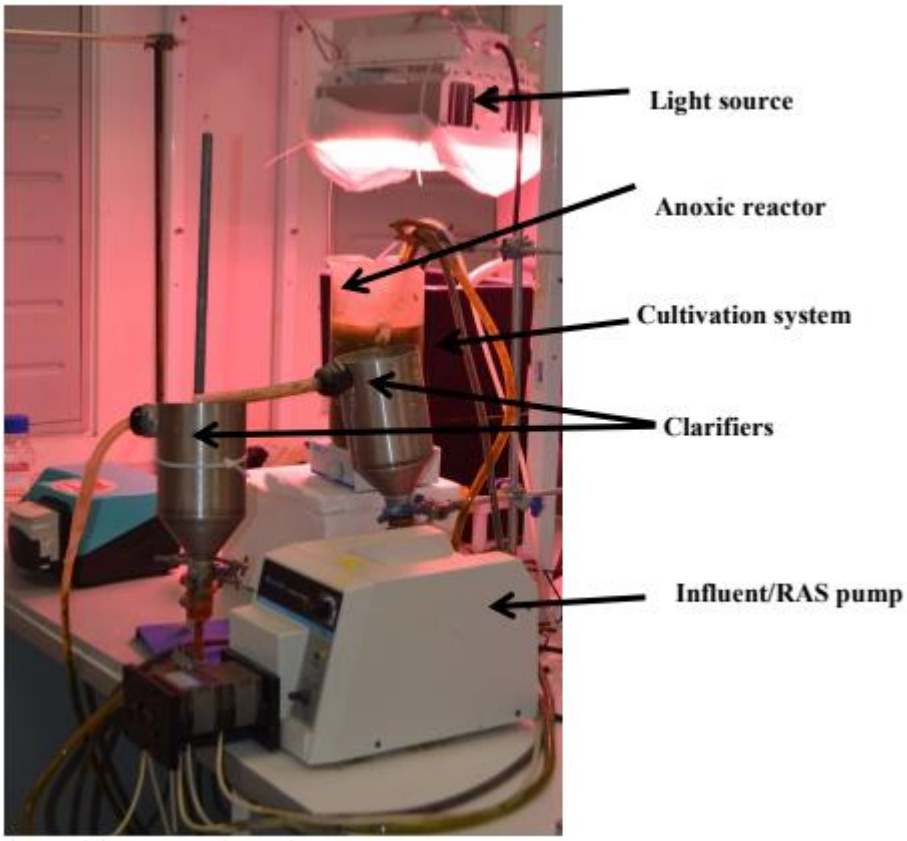


Figure 4.3: Image of anoxic coupled system.

The anoxic reactor was operated as per operational parameters outlined in chapter 2 for A/O operation. Effluent from the clarifier flowed into the sump tank at a flow rate (Q) of 200ml/h. The sump tank and cultivation tank were plastic aquarium tanks with the following dimensions; length: 30cm, width: 20cm and providing a surface area of 600cm^2 for plant coverage. Cycling of water between the sump tank and cultivation tank was created using a Boyu FP-150 submersible pump (Guangdong Boyu Group Co. Ltd, China). The cultivation system was placed under a Valoya R150 LED Grow Light AP673L (Valoya, Finland), providing light at an intensity of 300µmol/m^2/s. Tanks were wrapped in black electrical tape to prevent penetration of light from other sources and therefore algal growth. Samples of 50ml were taken 3 times a week from the anoxic reactor and from the sump tank. Samples were centrifuged before carrying out chemical analysis as per chapters 2 and 3.

A later trial growth system was also carried out using IASBR effluent. Two cultivation systems were established as part of the IASBR coupled system experiment which ran for 28 days. One contained effluent straight from the IASBR and the other contained autoclaved IASBR effluent. The purpose of this was to determine if nutrient removal was carried out by the plants or by microbial load from the reactor. This coupled system trial was

carried out during IASBR operation days 214 to 265. *Lemna* growth in both cultivation systems was recorded using Easy Leaf Area software (Easlon and Bloom 2014). pH in each cultivation system was maintained between 6 and 7 using 5M sulphuric acid. This was necessary to provide the ideal pH for *Lemna minor*. Samples of 50ml were taken twice a week for chemical analysis as per chapters 2 and 3. Gram stain, Neisser stain and DAPI staining were used to identify filamentous bacteria at certain stages of IASBR reactor operation. These stains are used to detect cell morphology and poly-P accumulation were carried out according to procedure in chapter 7 of Van Loosdrecht *et al*. (2016).

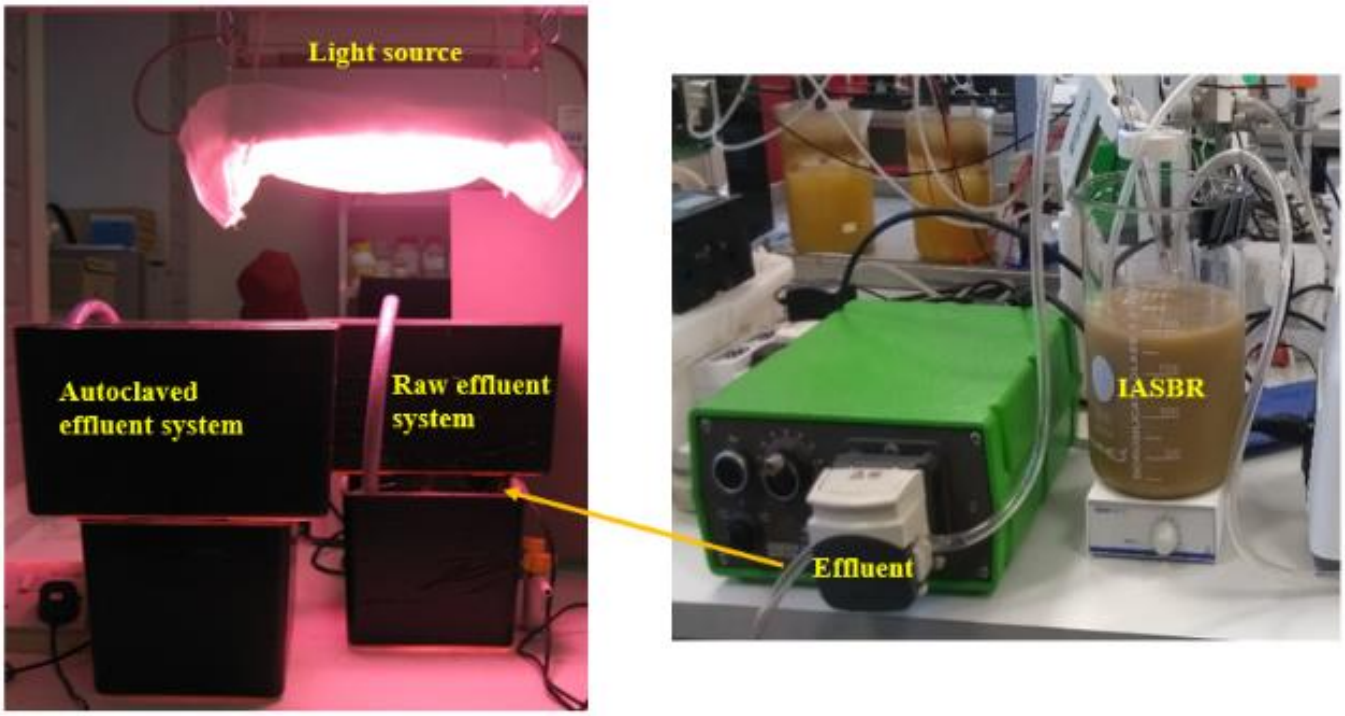


Figure 4.4: Image of IASBR/cultivation systems. Recycle of effluent between the cultivation and sump tanks was provided by a pump/drainage system inside the tanks. Cultivation systems were set up as outlined above for the anoxic coupled system.

4.3 Results and discussion

4.3.1 *Lemna* growth experiments.

Preliminary trials to assess effluent suitability for growth used effluent from each reactor that had been collected over time. The results of these initial growth experiments yielded little growth with IASBR effluent, and *Lemna* grown on A/O effluent showed symptoms of toxicity. The next growth experiments were carried out using effluent straight from each reactor. Samples were taken from both the Anoxic and Oxic zones of the A/O, and from the IASBR. This set of experiments yielded growth on all three samples, with Anoxic effluent showing the best results. These experiments were conducted after day 130 of IASBR activity, where MLSS levels were low and ammonia was being removed from the reactor rather than added. Growth experiments were carried out in triplicate and results from each experiment were averaged. The results of these preliminary trials (Figure 4.5), coupled with the N and P data from the anoxic zone of the A/O from day 160 -169, led to the decision to use an anoxic reactor for the coupled system trial.

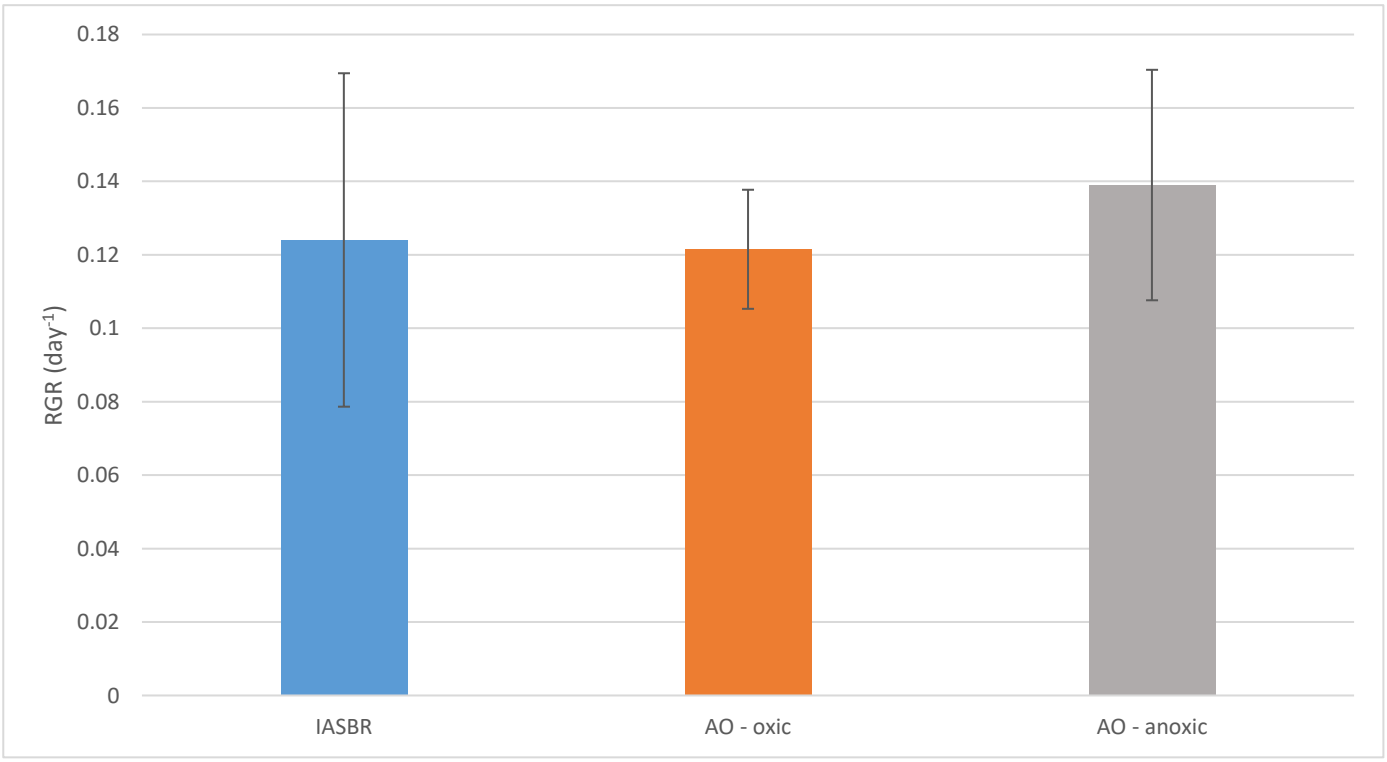


Figure 4.5: Average relative growth rate of *Lemna minor* on three different types of treated effluent.

The anoxic trial took place over 23 days, during which COD, NH_4^+, NO_3^-, NO_2^-, TN and PO_4^{3-} were measured to ascertain nutrient uptake by *Lemna minor*. Plant surface coverage was not measured during this trial. Figures 4.6 and 4.7 below show COD and PO_4^{3-} levels in the cultivation system. Day 1 includes measurements from the anoxic effluent and from the raw wastewater before treatment. These measurements are not shown for the remaining trial period as effluent was only added once at the beginning.

COD was removed to below 100mg/L over the course of the trial. This shows that COD removal is possible with a *Lemna* cultivation system. However, COD was still not removed to within EPA discharge limits. PO_4^{3-} removal fluctuated over the course of the trial, eventually being removed to below 50mg/L after day 10 until the end at day 23. However, PO_4^{3-} was not removed to within EPA discharge limits. It is unclear if COD and PO_4^{3-} were removed due to plant growth, microbial activity, or a combination of both.

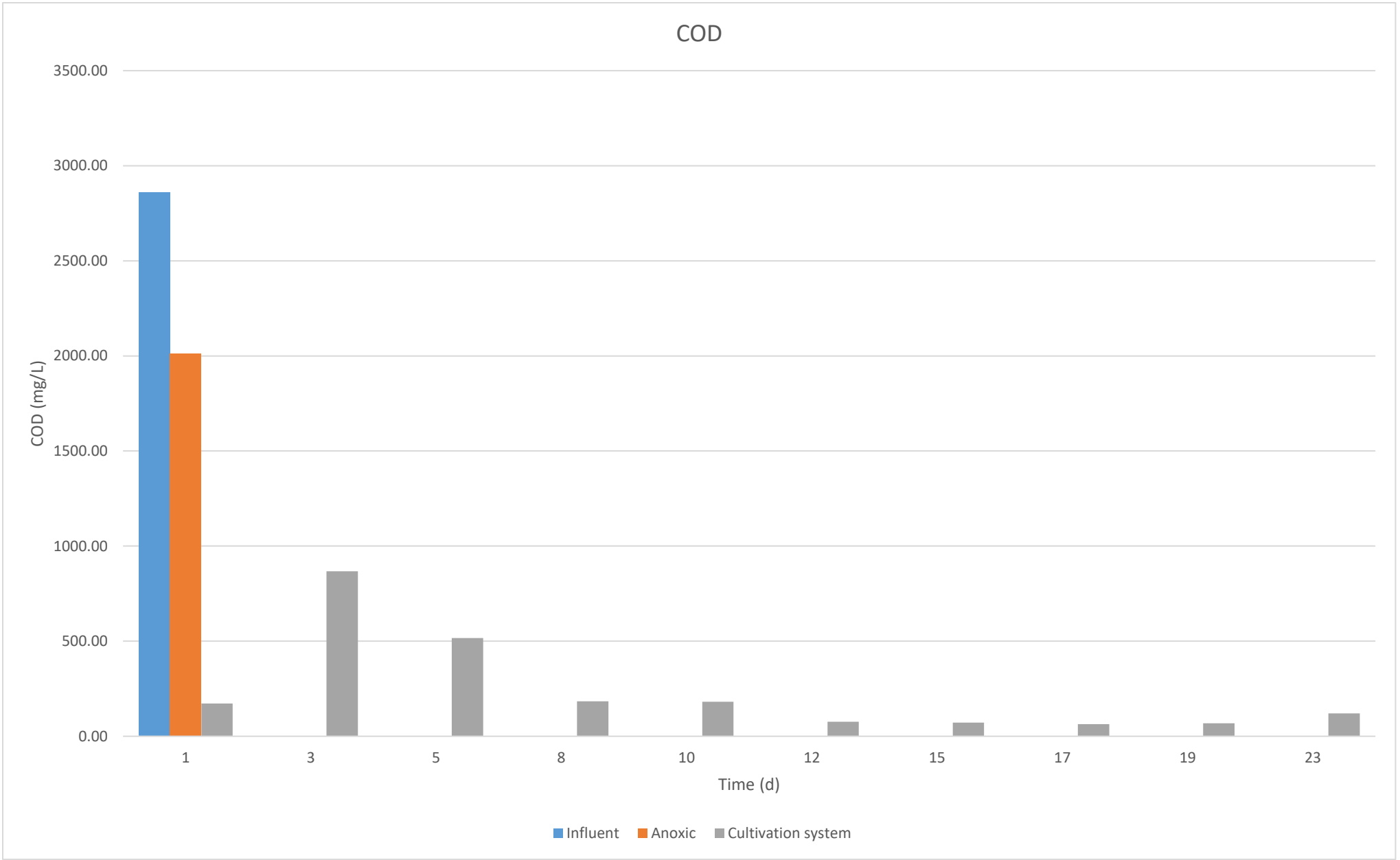


Figure 4.6: Anoxic/Cultivation system COD levels.

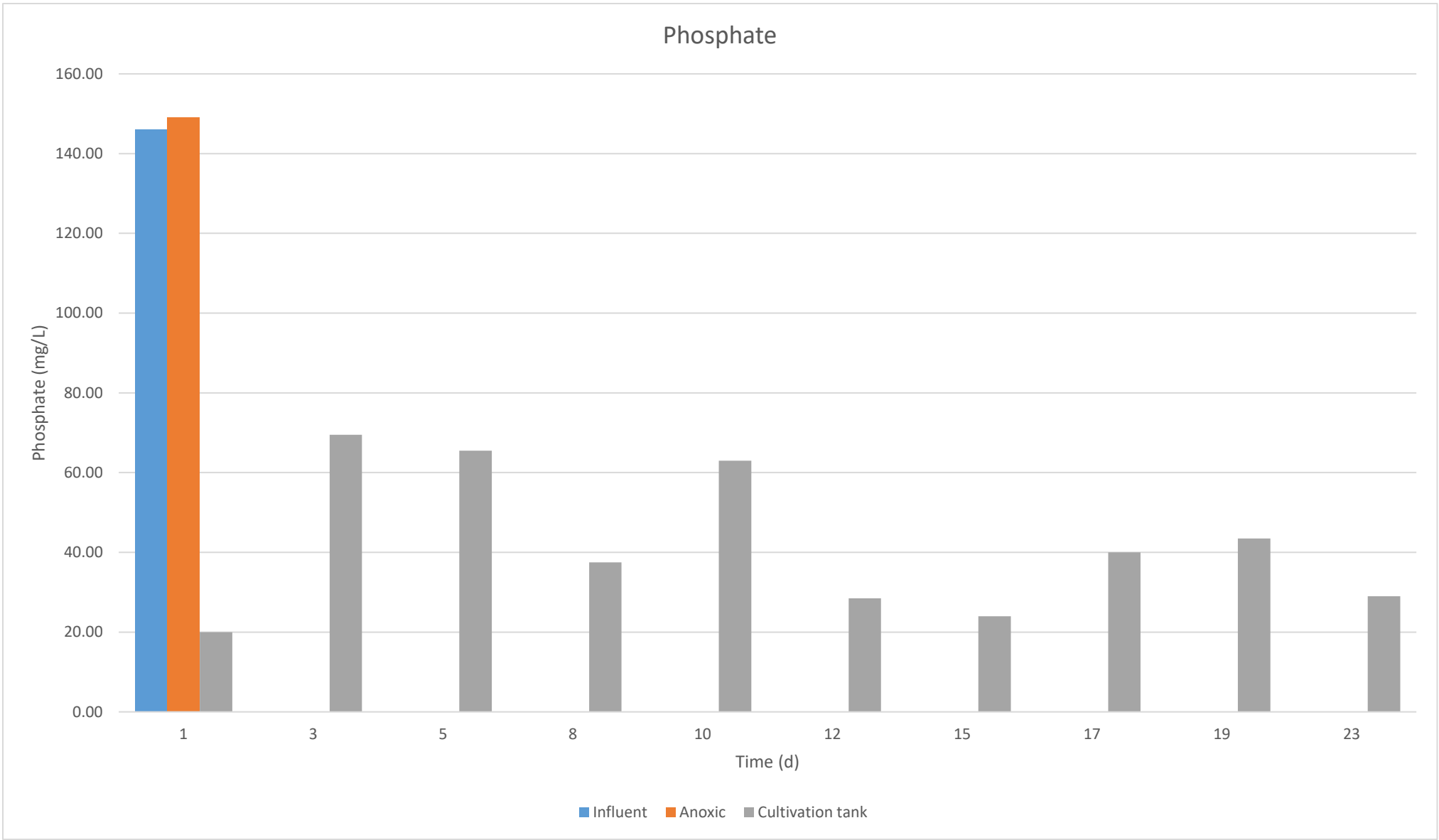


Figure 4.7: Anoxic/Cultivation system Phosphate levels.

Nitrogen removal was measured using NH_4^+, NO_3^-, NO_2^-, and TN. pH was taken into consideration for N removal as NH_4^+, the preferred form of N for *Lemna minor*, shifts to the unionised form NH_3 at pH 8 and above. It is important to closely monitor pH in duckweed cultivation systems, as free ammonia (FA) is toxic to duckweed, with plants unable to tolerate FA above 8mg/L (Körner *et al.* 2001). pH in the cultivation system remained between 8 and 9, which approaches the upper limit tolerated by duckweed (Körner *et al.* 2001, Skillicorn *et al.* 1993). pH was not adjusted during this trial. NH_4^+ levels generally decreased over time, with some increases at days 3 and 5 which may have been due to either ammonification, plant activity or a combination of both (Figure 4.9). Further experiments measuring plant growth and NH_4^+ uptake rates would need to be carried out to clarify this. NH_4^+ was removed to within EPA discharge limits during the trial, which shows promise for a successful coupled system once issues with COD and P removal are resolved.

NO_3^- and NO_2^- levels are shown in Figures 4.10 and 4.11. The y-axis for the graph in Figure 4.9 was adjusted, as raw influent nitrate levels were above 300mg/L on day 1, and cultivation system nitrate levels were below 60mg/L, making it difficult to see without changing the parameters of the y-axis. Low levels of NH_4^+ and NO_3^- combined with higher NO_2^- levels (3 – 60mg/L) indicate that partial nitrification occurred in the cultivation system. This partial nitrification was likely carried out by AOB, as some DO would have been provided by the recycling of water by the pump. Figure 4.12 shows NH_4^+, NO_3^-, NO_2^-, and Organic-N as a % of TN in order to show the change in nitrogen composition over time.

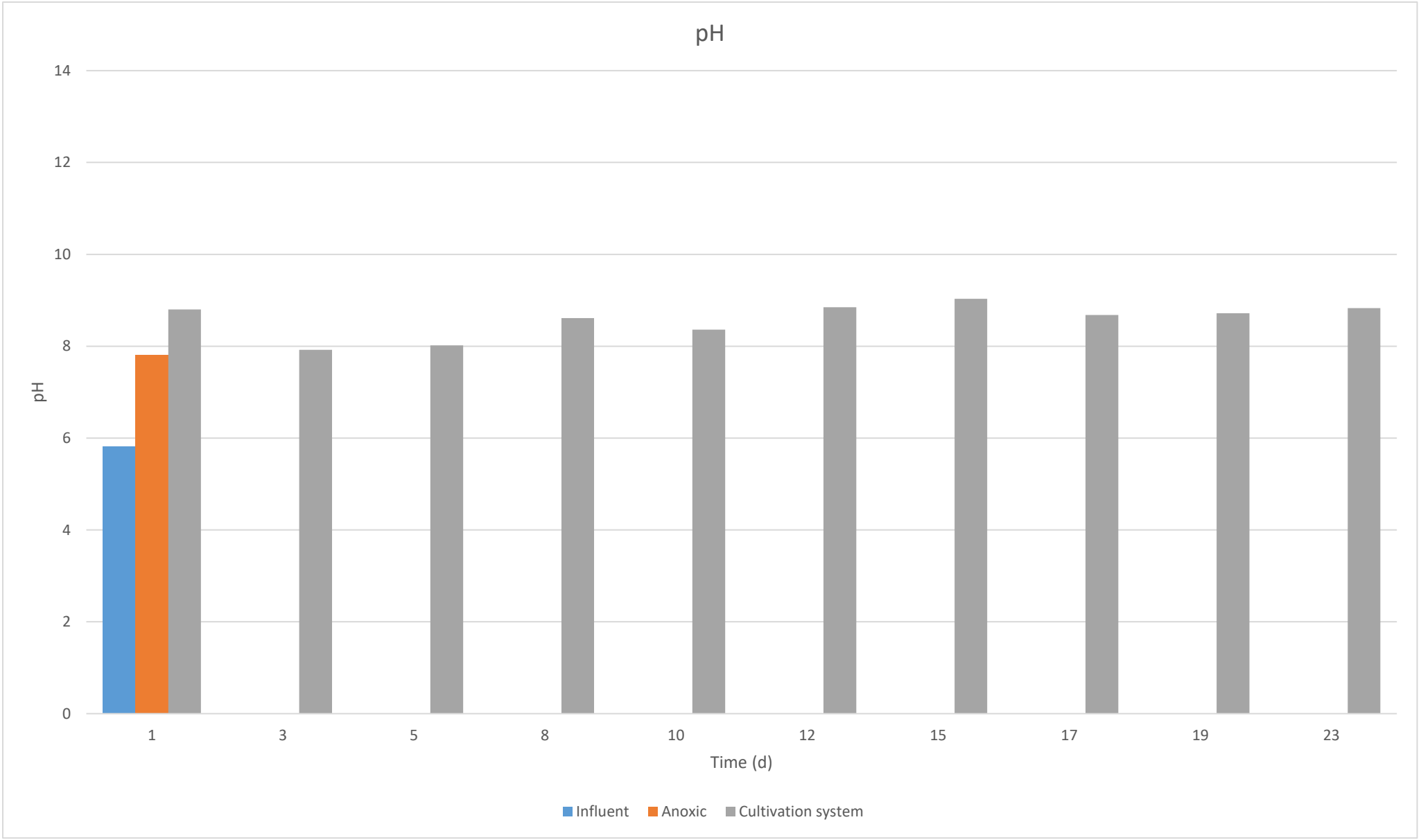


Figure 4.8: Anoxic/Cultivation system pH levels.

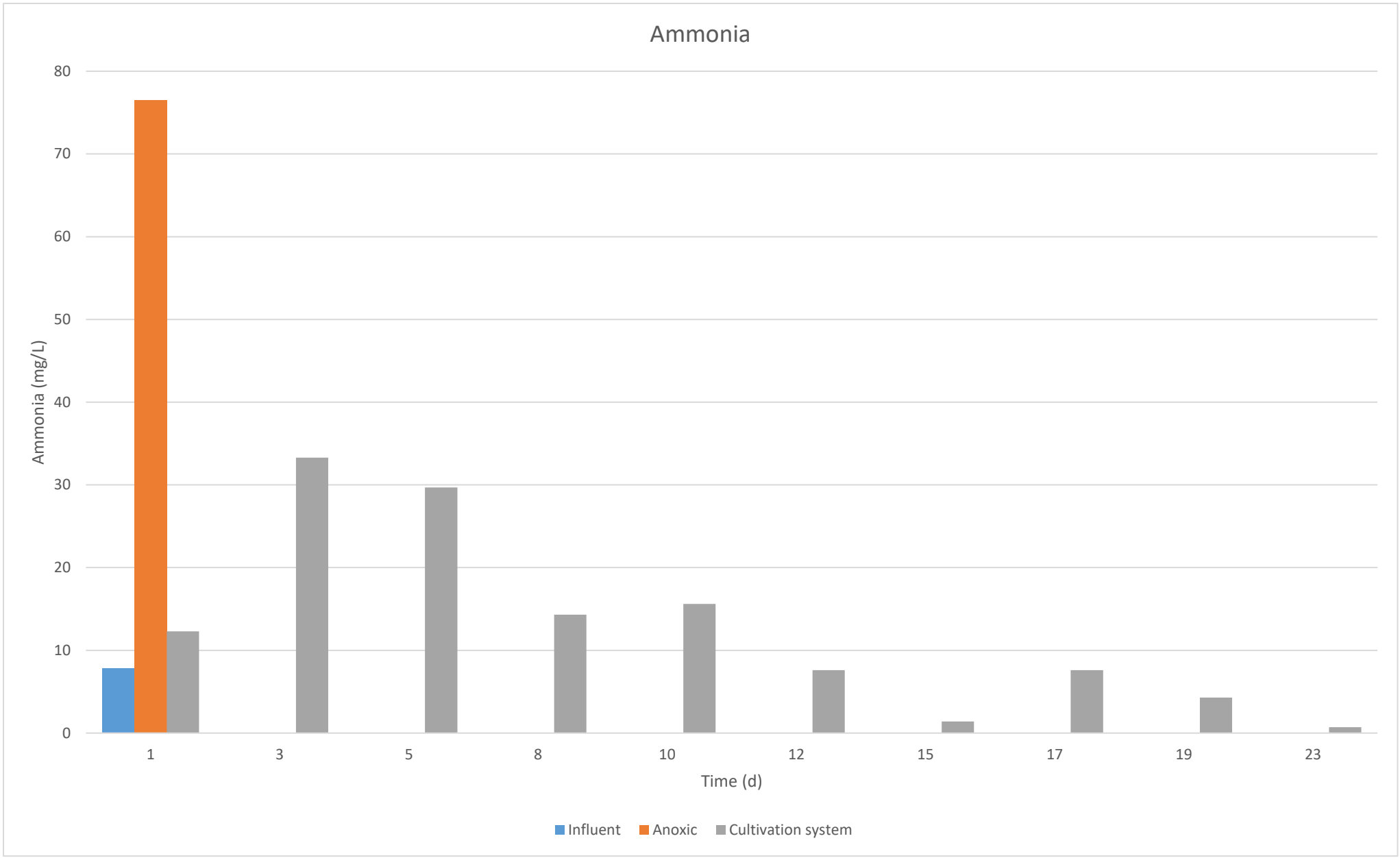


Figure 4.9: Anoxic/Cultivation system ammonia levels.

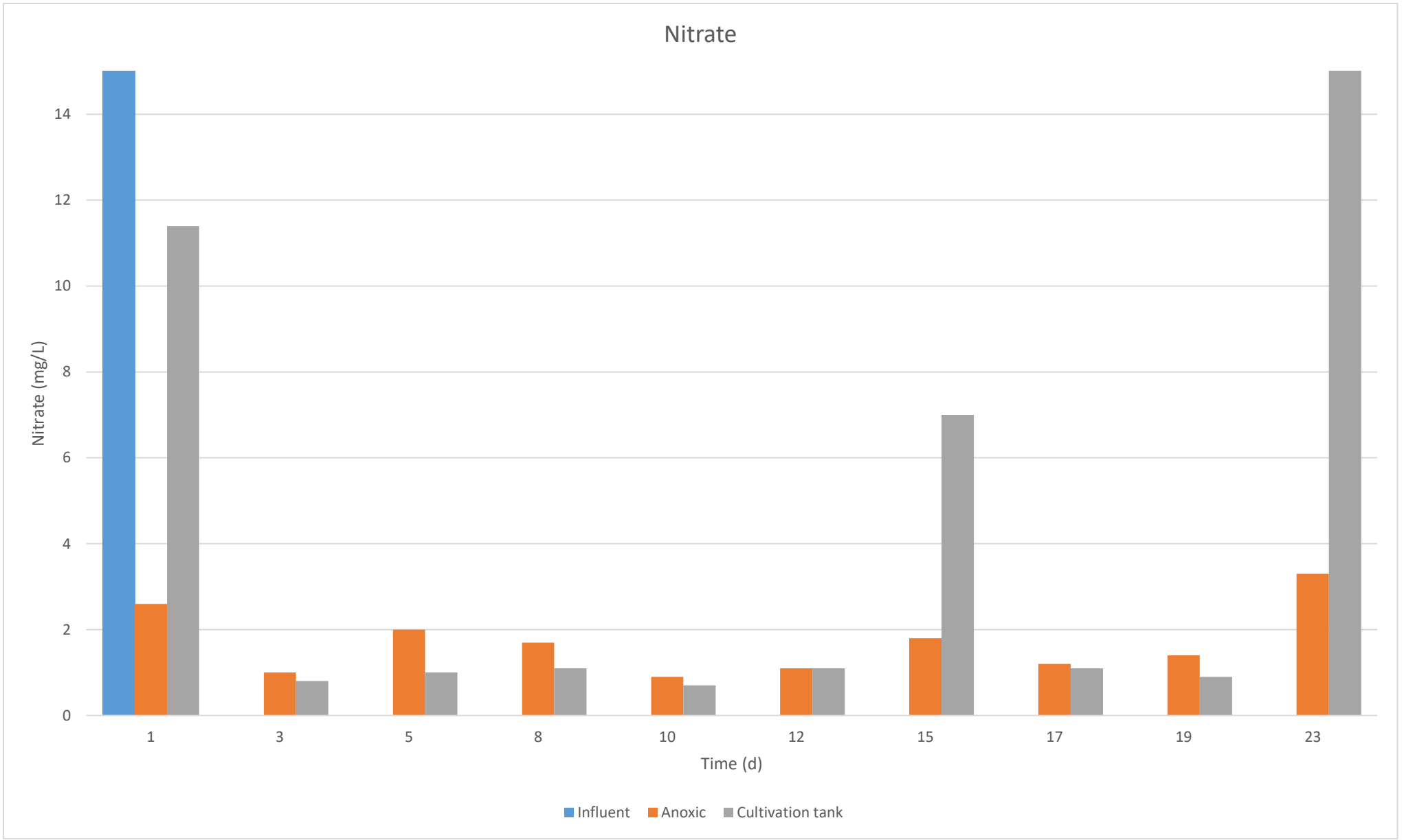


Figure 4.10: Anoxic/Cultivation system Nitrate levels.

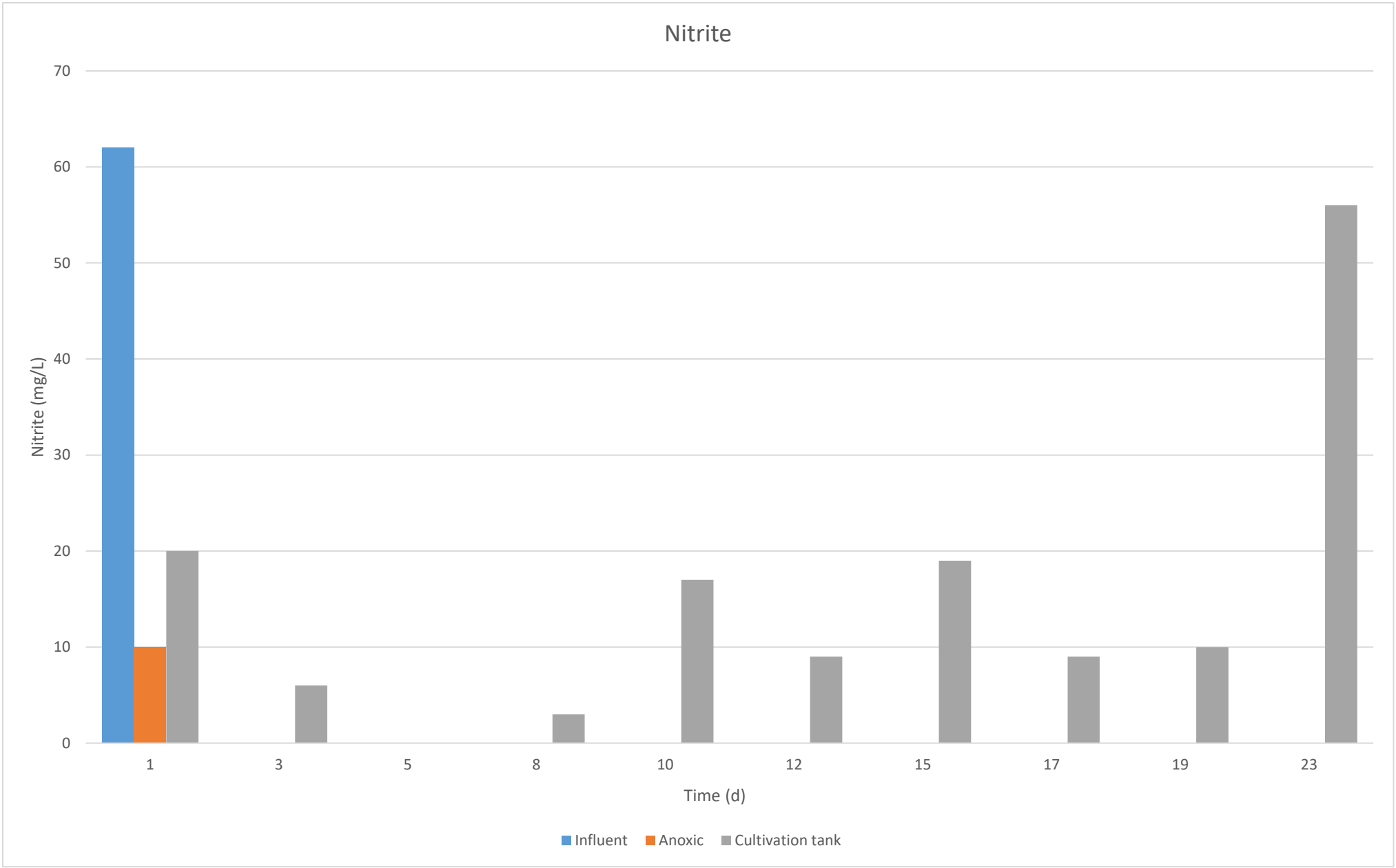


Figure 4.11: Anoxic/Cultivation system nitrite levels.

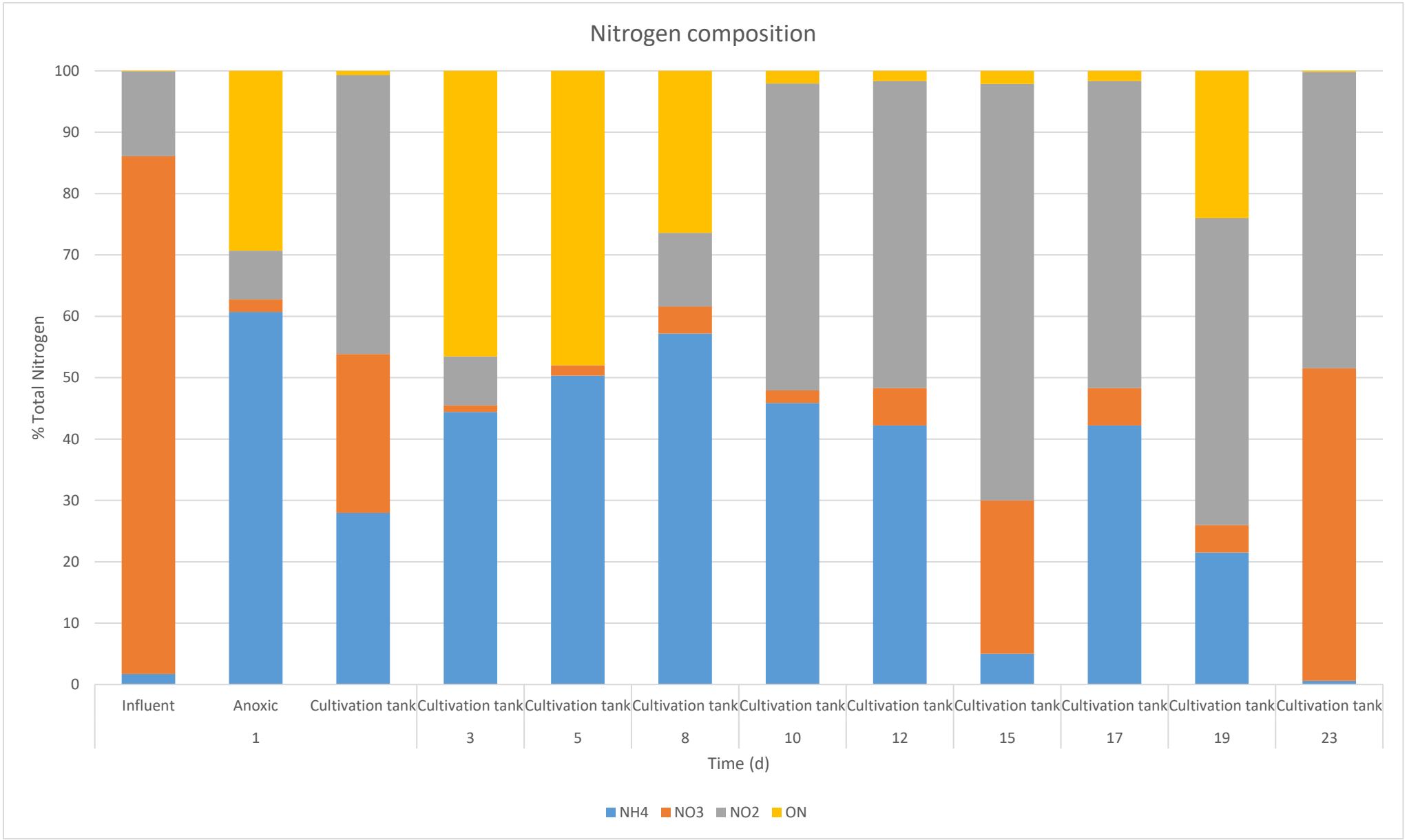


Figure 4.12: Anoxic/Cultivation system nitrogen levels as % total N.

Figures 4.13 and 4.14 below show COD and PO_4^{3-} levels during the IASBR coupled system trial. Day 1 was when effluent was added from the IASBR and from autoclaved effluent, therefore IASBR data was not included. Days 19 – 28 for autoclaved effluent represent a fresh batch of effluent, as plants in that system had died by day 14 and so a new batch of effluent and plants was required. Raw effluent was only added at day 1.

The y-axis on the graph for COD levels (Figure 4.13) was adjusted to maximum 600 mg/L to allow for all data to be visualised. As a result, COD for days 1 and 19 for autoclaved effluent surpass the bounds of the y-axis at 1620mg/L and 782mg/L respectively. COD removal does not appear to have been sustained in either cultivation system, although the raw effluent system did maintain COD below 300mg/L. The comparably high levels in the autoclaved effluent system were due to lysis of the microbial biomass that had been carried over from the IASBR.

PO_4^{3-} levels did not change significantly in either cultivation system after initial removal seen after day 1 on autoclaved effluent. Increases in PO_4^{3-} levels with autoclaved effluent seen at days 14, 26 and 28 are due to phosphate release caused by plant senescence and eventual death. The same can be said for days 26 and 28 on raw effluent. Overall P uptake by *Lemna* in both cultivation systems was poor due to lack of plant growth. This poor growth can be seen from surface area coverage data (Figure 4.15) and from Figures 4.16 to 4.26. The plants did not thrive in either cultivation system and even began to die after approximately 2 weeks on the autoclaved effluent.

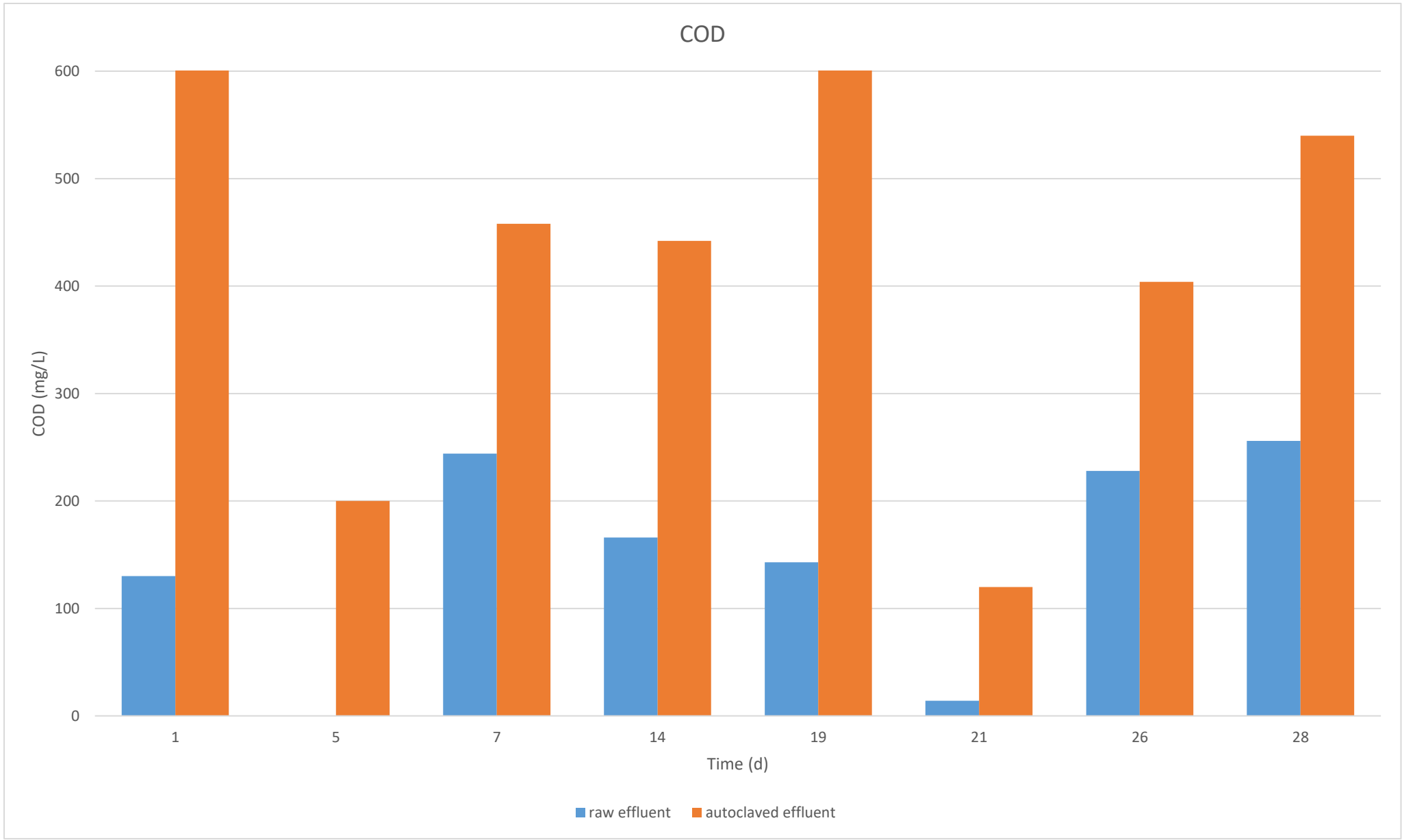


Figure 4.13: IASBR/Cultivation system COD levels.

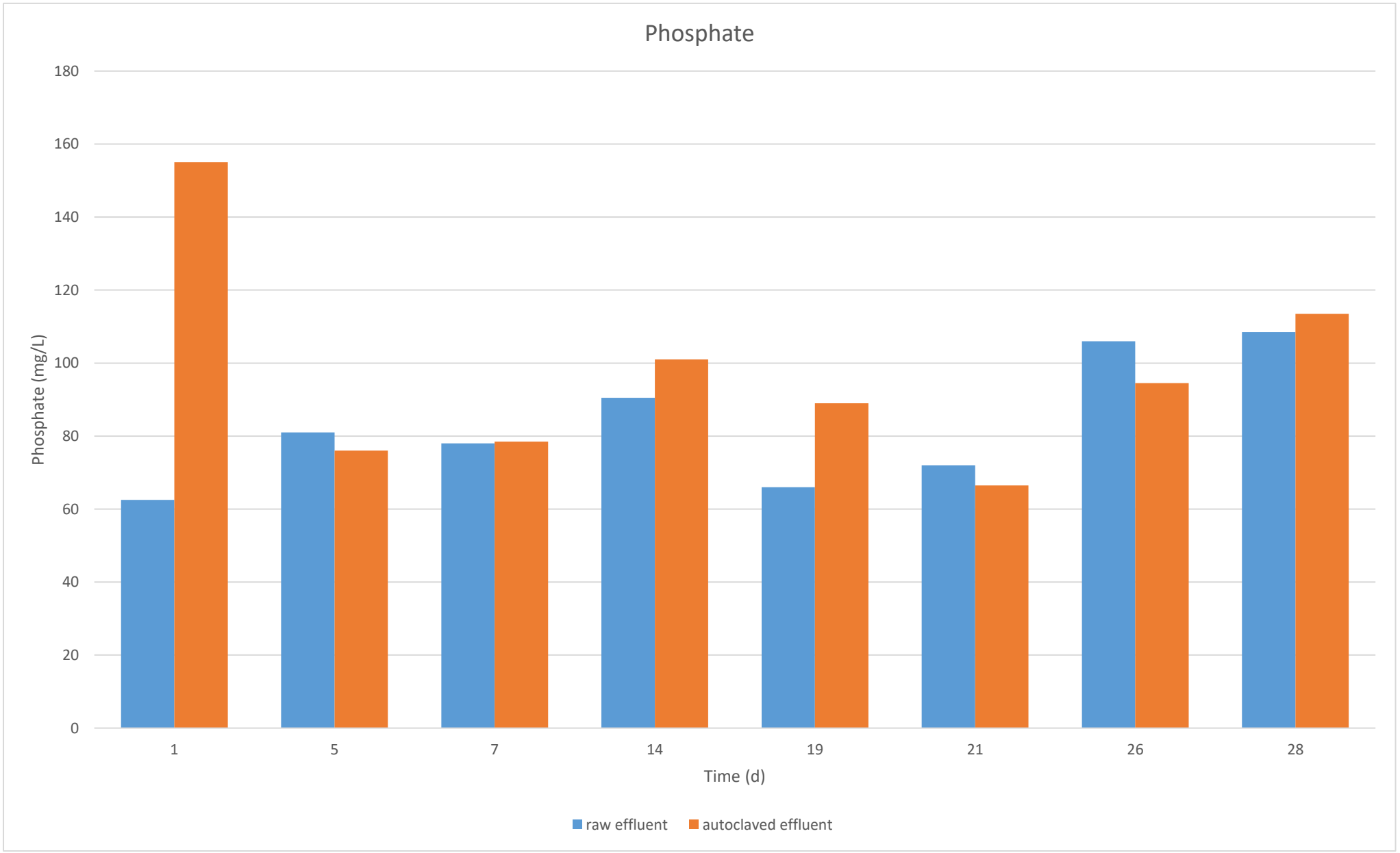


Figure 4.14: IASBR/Cultivation system Phosphate levels.

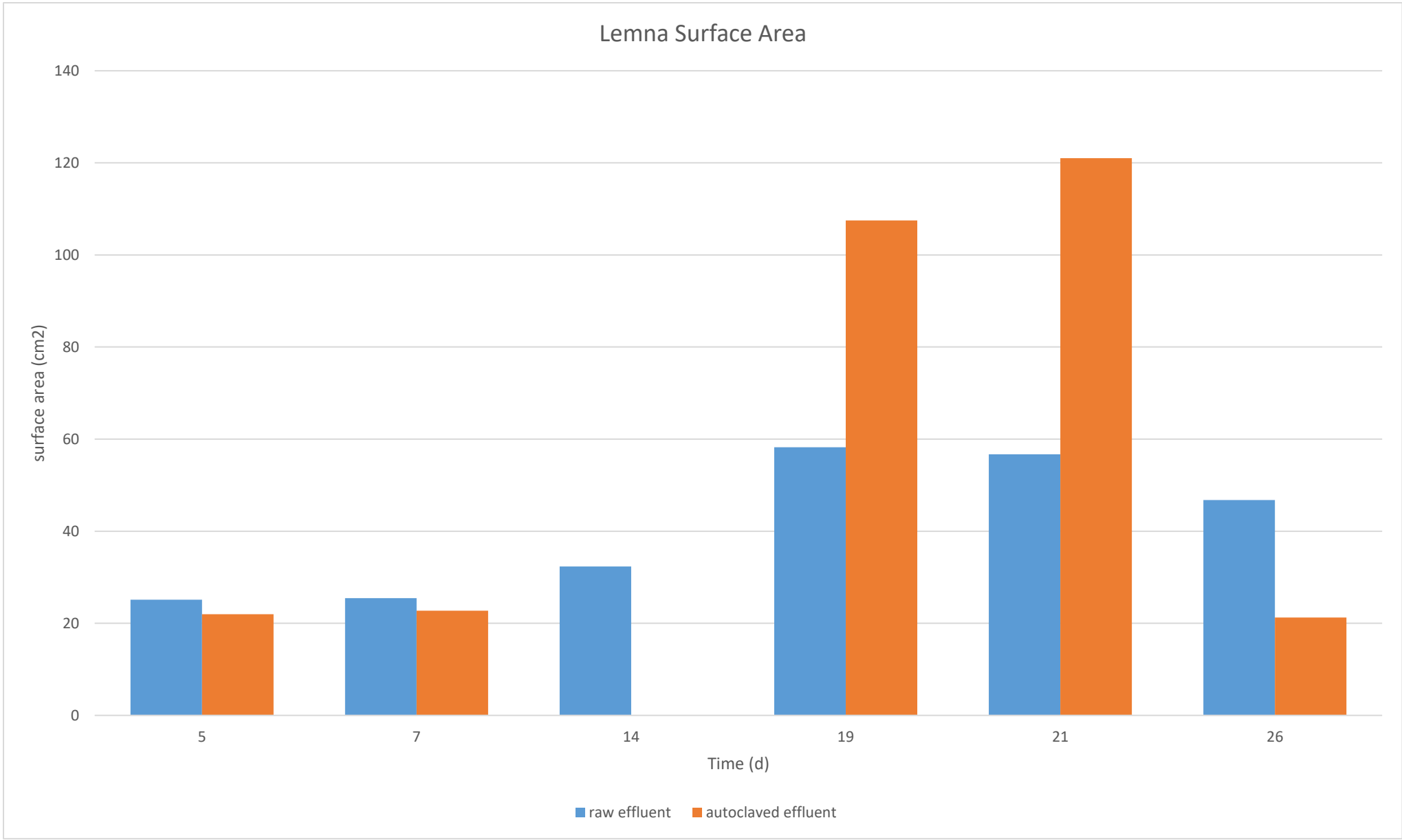


Figure 4.15: IASBR/Cultivation system *Lemna* surface area coverage. Cover ranged from 4 – 20% of total tank surface area (600cm^2).



Figure 4.16: Image of *Lemna minor* on autoclaved effluent day 1.

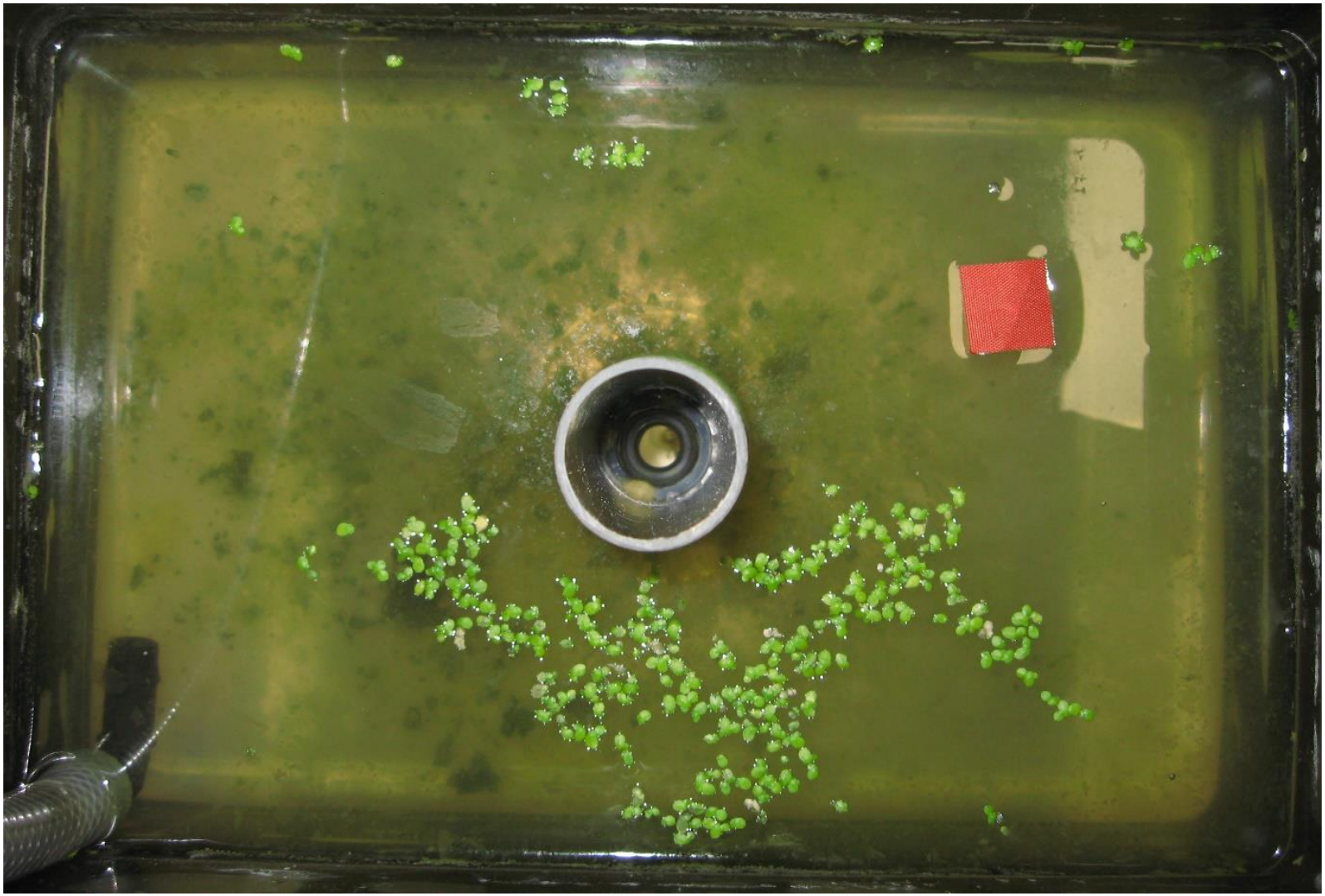


Figure 4.17: Image of *Lemna minor* on autoclaved effluent day 7. Red square is 1cm^2 for calculating surface area using EasyLeafArea software (Easlon & Bloom 2014).



Figure 4.18: Image of *Lemna minor* on autoclaved effluent day 14. Dead plants can be seen in right background and right foreground of the image. Green colour seen under the surface is algae.



Figure 4.19: Image of *Lemna minor* on autoclaved effluent day 19. A fresh batch of effluent and a larger plant inoculum was used. Coverage for this new batch was approximately 20% of tank surface area.



Figure 4.20: Image of *Lemna minor* on autoclaved effluent day 21.



Figure 4.21: Image of *Lemna minor* on autoclaved effluent day 26. Note the bleached leaves beginning to appear, indicative of senescence.



Figure 4.22: Image of *Lemna minor* on raw effluent day 1

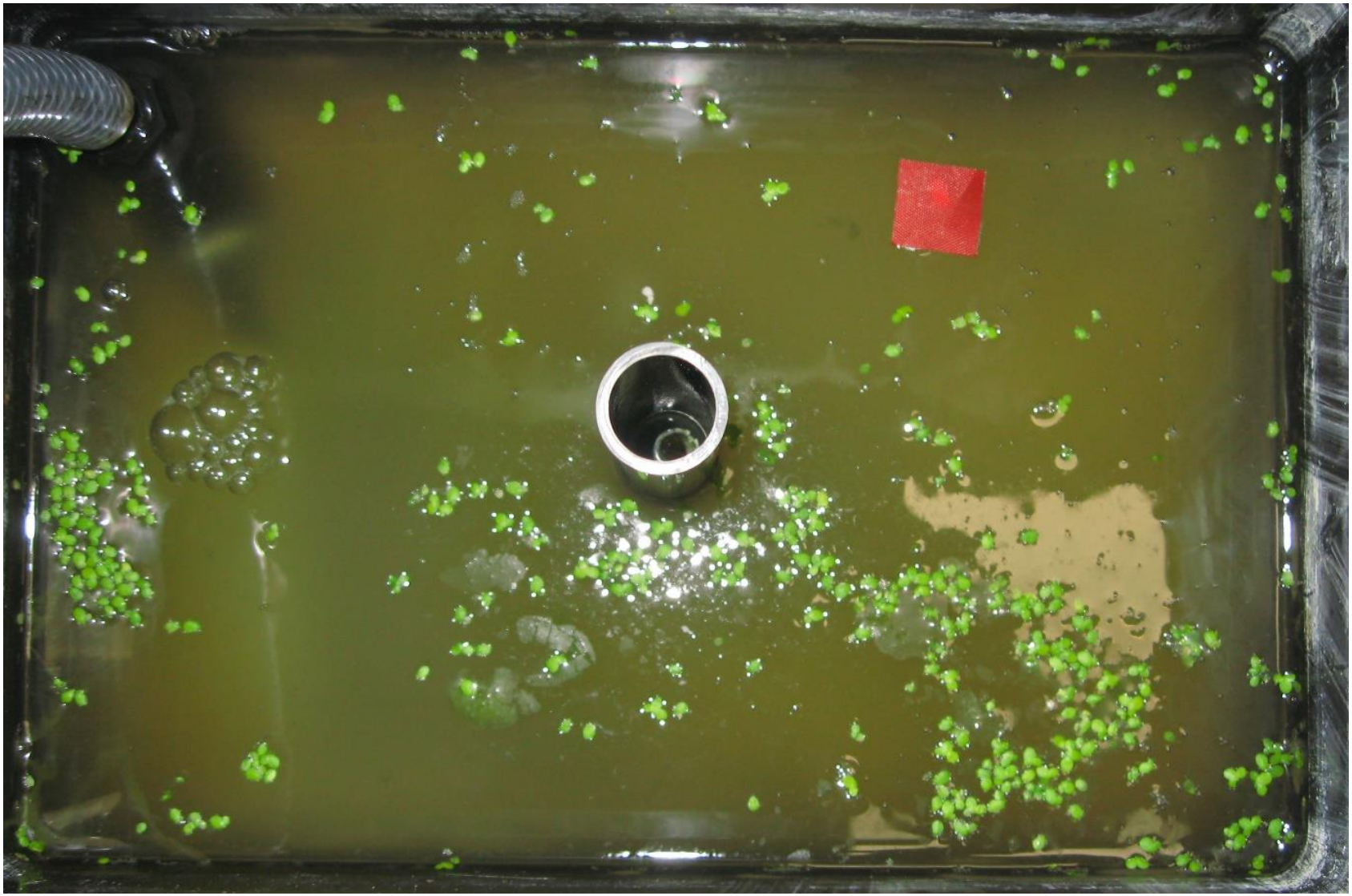


Figure 4.23: Image of *Lemna minor* on raw effluent day 7.

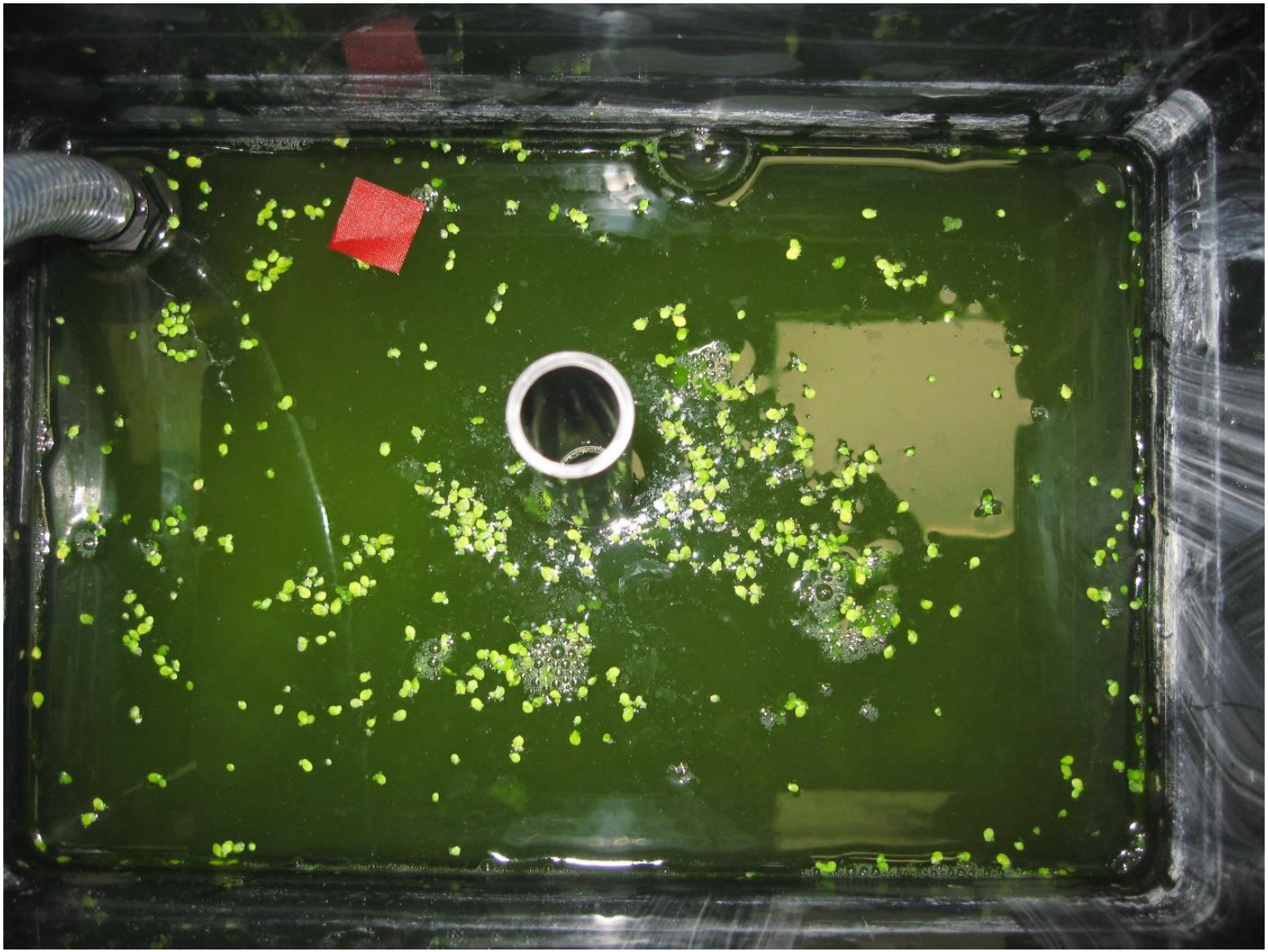


Figure 4.24: Image of *Lemna minor* on raw effluent day 14. Algae is now well established.



Figure 4.25: Image of *Lemna minor* on raw effluent day 19. Extra plants were added on this day in order to increase surface area coverage to 20%. Algal growth is clearly visible.

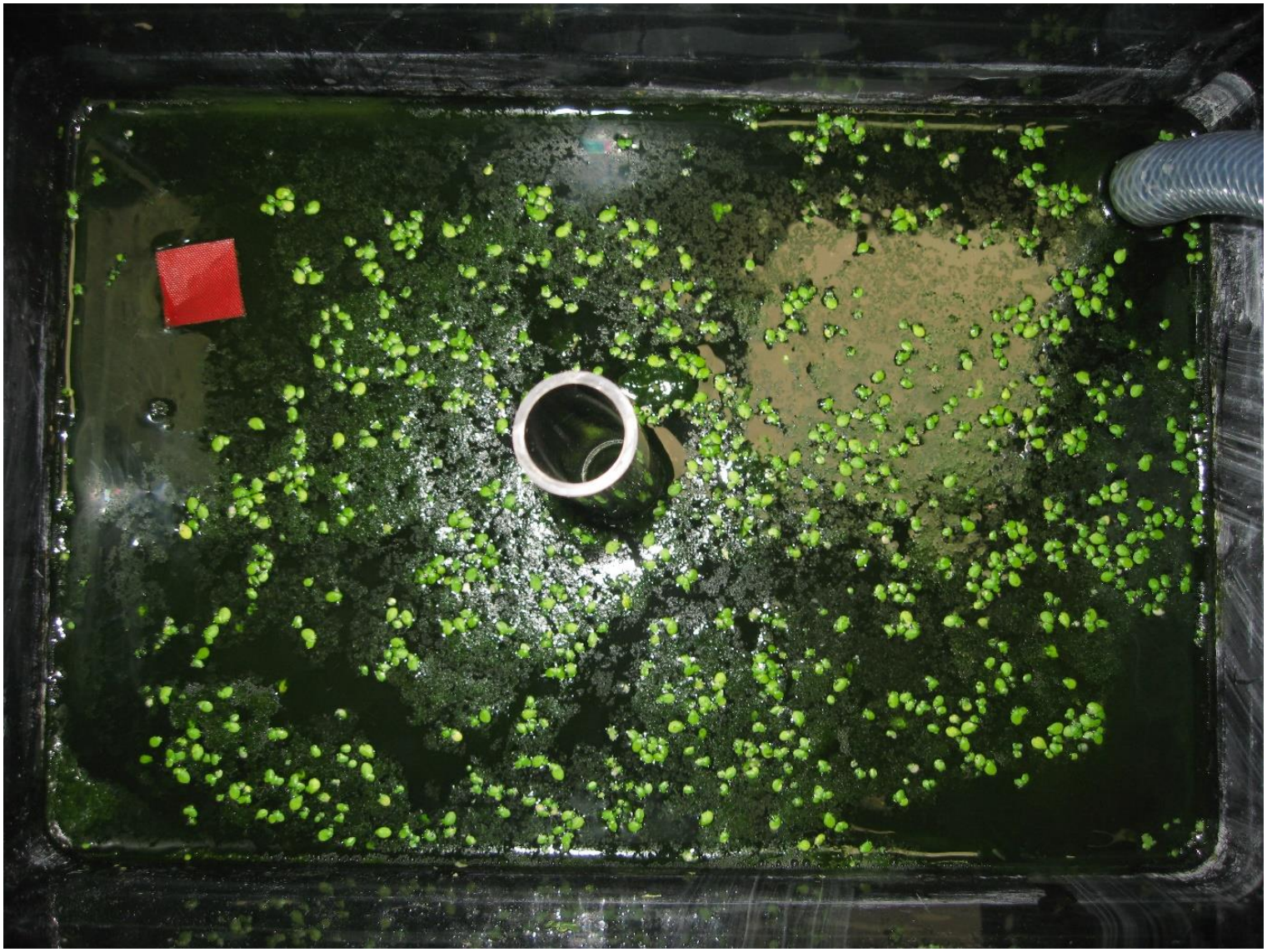


Figure 4.26: Image of *Lemna minor* on raw effluent day 26. No change in % coverage was observed at this point. Algae had completely taken over at this point, and plants had begun senescence by day 28 when the trial was ended.

The most likely explanation for the poor growth and rapid death on autoclaved effluent is high levels of free ammonia (NH_3), which is toxic to *Lemna minor* (Körner *et al.* 2001, Skillicorn *et al.* 1993). Figure 4.28 shows the relatively high ammonia level in the cultivation system with autoclaved effluent compared to the cultivation system with raw effluent. This can be attributed to ammonification of organic material, which was abundant after the effluent had been autoclaved. The pH in both cultivation systems remained relatively steady above pH 7 (Figure 4.27). This would have been sufficient to facilitate the equilibrium shift from ammonium (NH_4^+) ions to NH_3, therefore creating a supply of free ammonia.

As for the other cultivation system with raw effluent, the poor growth and eventual decline of the plants is most likely due to eutrophication. Unicellular algae are one of the few phototrophic eukaryotic organisms that can grow faster than *Lemna minor*. It also utilizes Nitrate more readily than *Lemna*, and there are some species of algae that can form a film around *Lemna* roots, preventing the plants from taking up nutrients and oxygen (Skillicorn *et al*. 1993). As can be seen from Figures 4.28, 4.29 and 4.30, the ammonia level in the cultivation system with raw effluent drops quite quickly and remains low over the course of the experiment, whereas the nitrate level in the same cultivation system is relatively high for the same period of time. This indicates that complete nitrification was occurring in the raw effluent system, reducing the ammonia available for *Lemna* and converting it to nitrate, which created a more ideal nitrogen source for algae. As a result, the algae outcompeted the *Lemna*, preventing its growth. The pH levels for most of the trial remained above 7, also as a result of algal growth.

In order to combat the above-mentioned issues, it would be necessary to vastly increase the surface area of the tank covered by *Lemna minor*. By increasing the plant coverage to about 90% of the tank surface area, it is possible to prevent competition from algae by blocking sub-surface exposure to light. More careful monitoring of pH would also be necessary to maintain pH between 6 and 7, which is ideal for *Lemna minor* and would prevent the equilibrium shift from NH_4^+ to NH_3, thus preventing toxicity. These changes would also improve COD removal and P uptake.

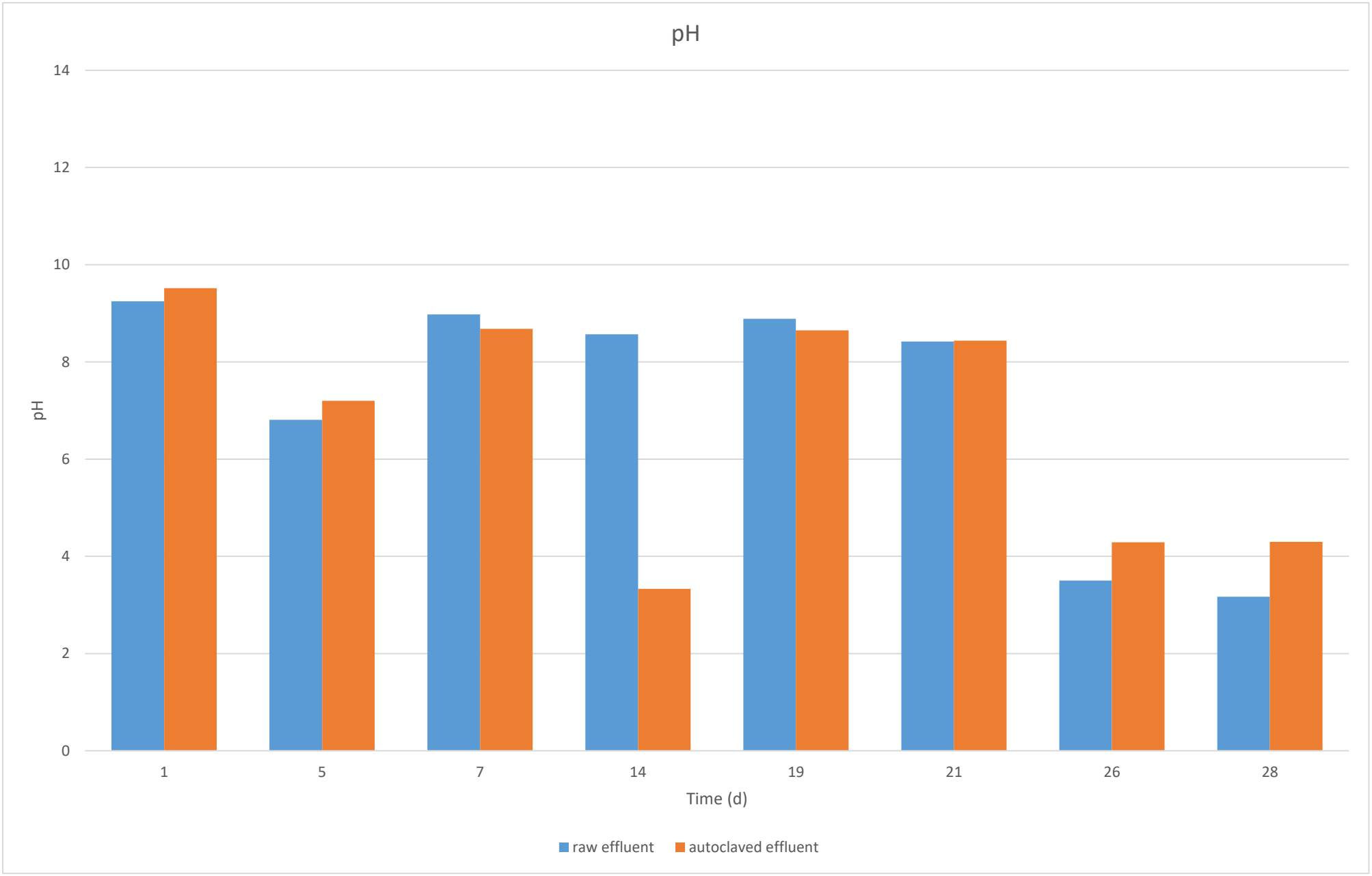


Figure 4.27: IASBR/Cultivation system pH levels.

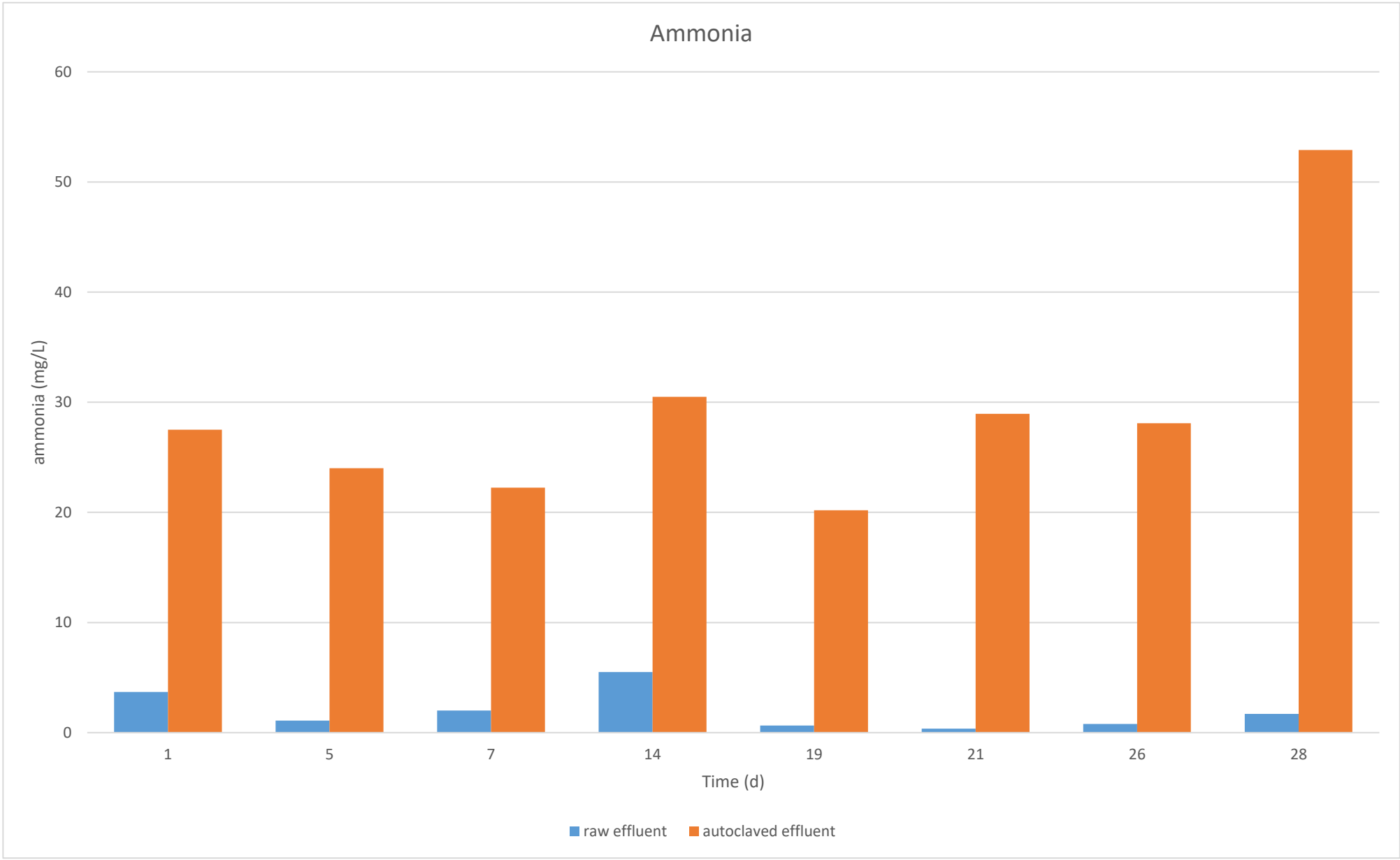


Figure 4.28: IASBR/Cultivation system ammonia levels.

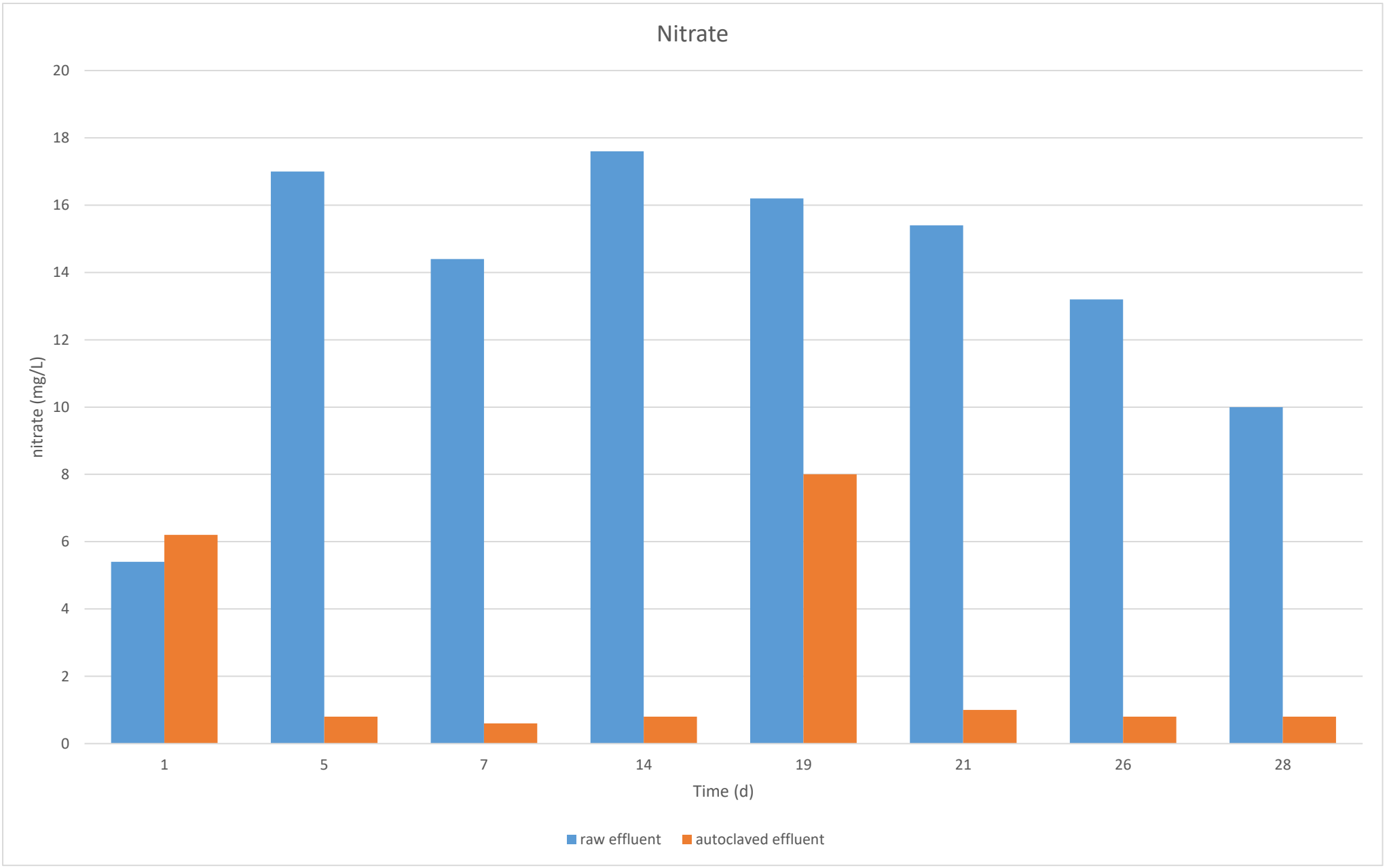


Figure 4.29: IASBR/Cultivation system nitrate levels.

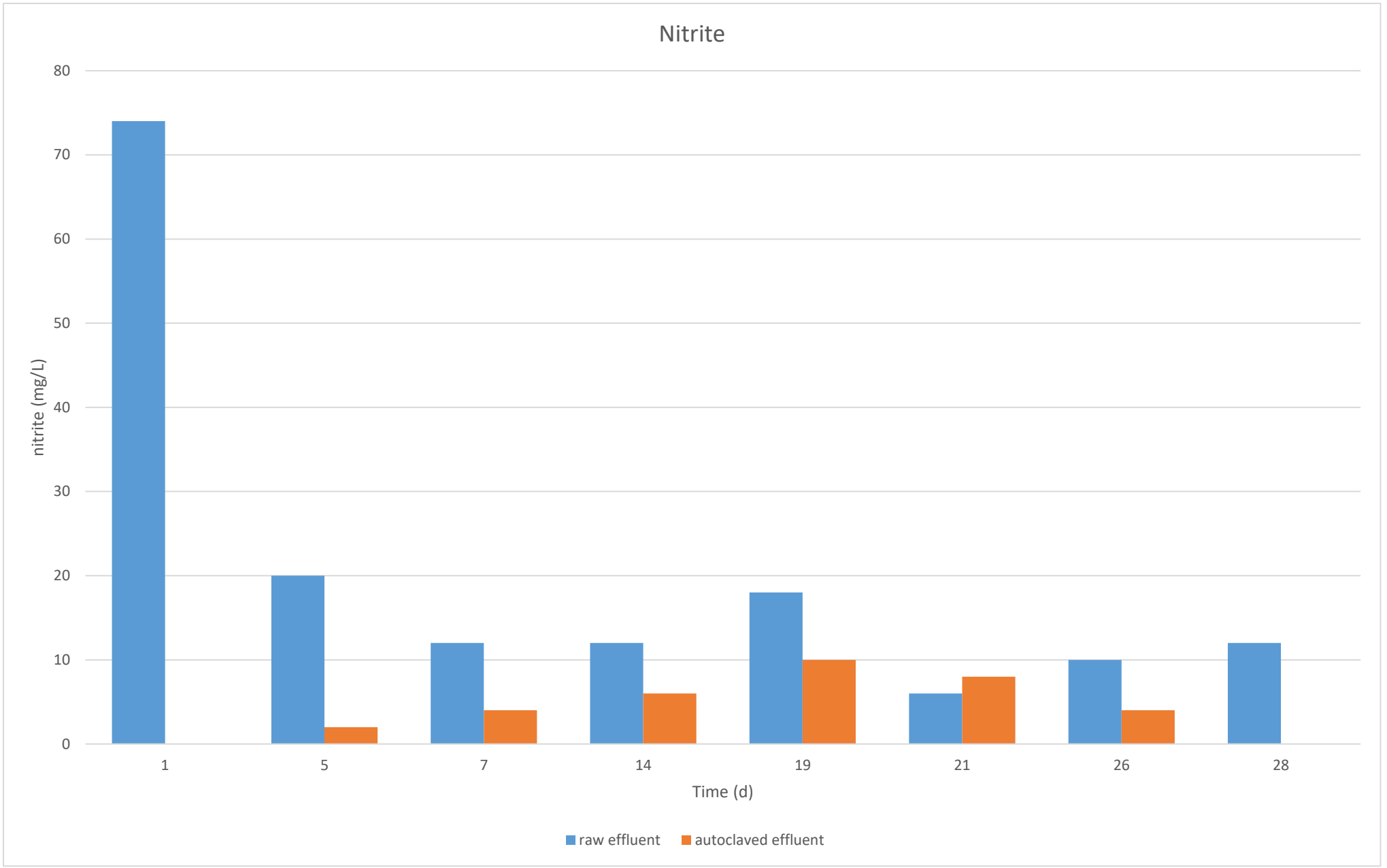


Figure 4.30: IASBR/Cultivation system nitrite levels.

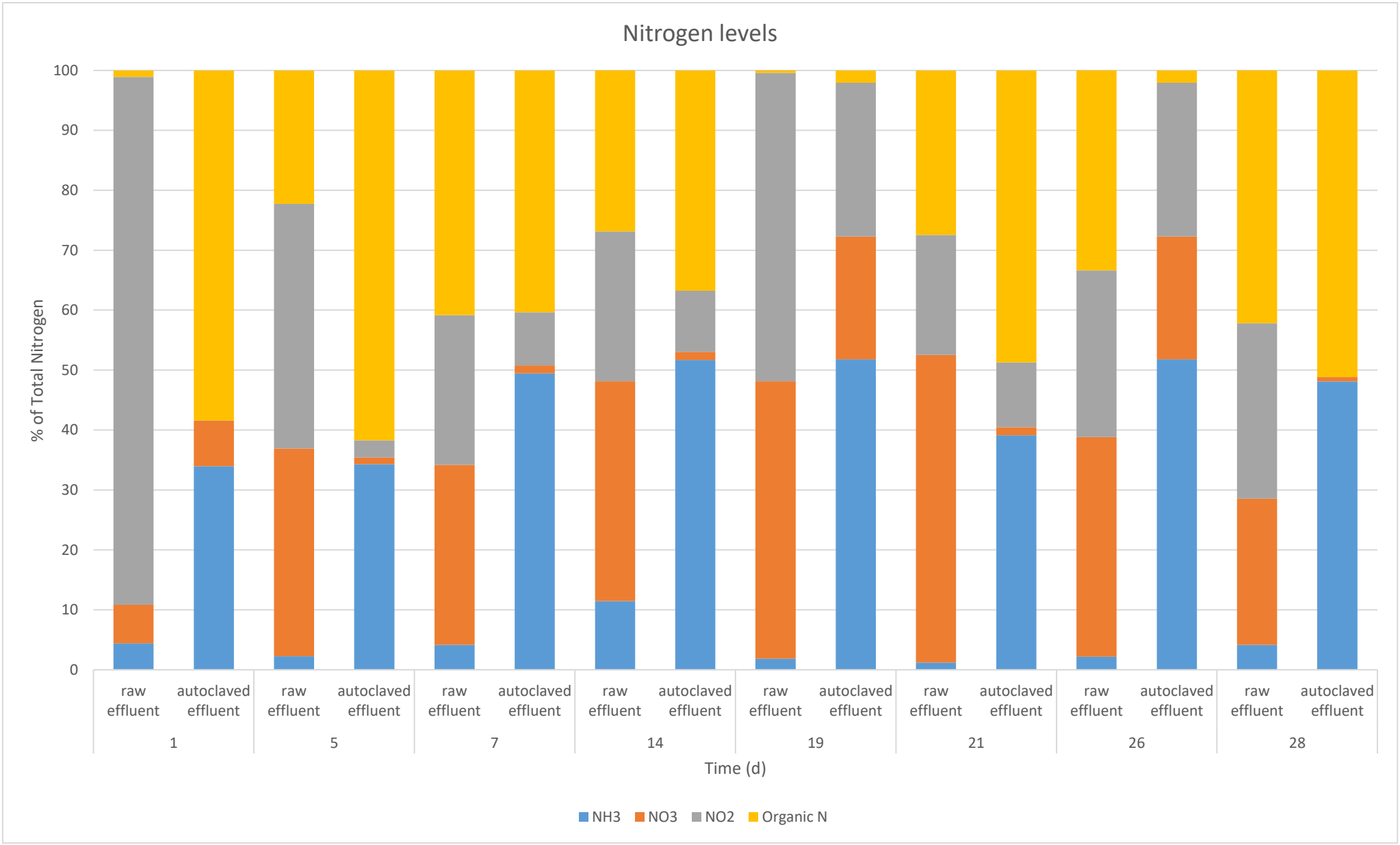


Figure 4.31: IASBR/Cultivation system nitrogen levels as % total N.

4.4 Conclusion.

The purpose of this chapter was to present and discuss research into the feasibility of using effluent from aerobic BNR systems to establish a coupled system for wastewater treatment and *Lemna minor* cultivation. The initial batch experiments with different effluents (Figure 4.5) showed a small increase in relative growth rates with A/O anoxic effluent. The anoxic reactor/cultivation system experiment was also more successful than the IASBR/cultivation system experiments in that no toxicity was observed with the A/O anoxic effluent. However, *Lemna* growth on anoxic effluent was not measured so it is difficult to compare the efficacy of the two systems in that respect. However, it can be concluded from the results of both coupled system experiments that there was insufficient plant biomass added at the beginning, and this allowed for eutrophication to occur and prevented successful plant growth.

As stated in chapters 3, the low F/M ratio observed during IASBR operation led to growth of filaments which caused issues with sludge bulking, which led to high levels of suspended solids in the effluent. This was particularly problematic during the coupled system trial, affecting COD, nutrient levels, and pH in the cultivation system with autoclaved effluent (Figures 4.27 – 4.31). As a result, FA built up to toxic levels and a biofilm formed on the tank surface, trapping plants, which prevented nutrient uptake and plant access to DO.

To rectify these issues in any future work a > 50% *Lemna* coverage must be established and maintained. It is also important to closely monitor pH and maintain it between 6 and 7 to keep NH_4^+ from shifting to NH_3, thus preventing toxicity. It is also important to ensure adequate COD removal and sludge settling in the pre-treatment system to prevent the formation of thick biofilms.

Chapter 5 – Overall Conclusions and Future Prospects.

The Dairy industry is one of the largest sources of income for the Irish economy, generating over €2bn per year between national sales and exports. This scale of production is set to continue increasing in line with government policies for national development such as Harvest 2020, FoodWise 2025 and Project Ireland 2040 (DAFM 2010, DAFM 2015, Government of Ireland 2018). These policies are committed to sustainable economic growth whilst also recognising industry responsibility to the environment by promoting EU circular economy principles. This poses a challenge to the dairy industry in particular, as continued growth will lead to generation of ever larger volumes of high-strength wastewater that must be treated to EPA mandated standards before release into water bodies. The increase in wastewater volume will assuredly increase the operational costs associated with treatment, particularly for aeration and P-removal. This is not sustainable.

Opportunities therefore exist to re-examine major agri-sector operators, such as dairy processing, to identify opportunities for waste capture in value-added products. This Thesis examines one such opportunity by refocusing biological treatment strategies to act as conditioners of dairy processing wastewater to generate feedstocks for production of *Lemna minor*, a species of duckweed native to Ireland. Duckweed is a high protein source that could potentially be returned to the dairy herds that produce the milk as a feed during the off-peak season (Leng *et al.* 1995). Such a system would allow for dairy processing companies to secure their milk supply, reduce their effluent output, reduce their water intake from the municipal supply, reduce operational costs, move away from chemical P removal and provide farmers with a competitive alternative to Soya protein. This type of process, by turning waste into a valuable product, would allow dairy industry to grow in a sustainable manner, while also lessening the environmental impact these industries currently have.

For this reason, Newtrients project work package 4 and therefore this MSc project was launched with the following objectives:

- Determination of optimal aerobic reactor parameters for conditioning of wastewater as *Lemna* feedstock.
- Development of coupled reactor system module for wastewater treatment and *Lemna* propagation.
- Characterization of coupled system resilience to nutrient challenges.

Chapters 2 and 3 of this thesis discuss the outcomes of using A/O and IASBR technology for conditioning of dairy processing wastewater from Dairygold, Mitchelstown, Co. Cork. Chapter 4 discusses the development of a coupled system with the above-mentioned technologies. The overall conclusion from this research is that aerobic treatment systems are

not suitable for conditioning dairy processing wastewater for *Lemna minor* cultivation. A/O systems have been used to treat high-strength industrial effluent with removal efficiencies of up to 99% for COD and 90% N removal (Carrera *et al.* 2004). IASBR systems have been utilized for treatment of dairy processing wastewater with removal efficiencies of >95% for COD, N and P (Tarpey 2016, Leonard *et al.* 2018(a), Leonard *et al.* 2018(b)). In this project the systems had to be operated inefficiently in order to retain the COD removal features of the above-mentioned systems, while diminishing the capacity for N and P removal – as organics can cause issues for duckweed growth. However, operational parameters imposed to achieve this caused destabilisation through filamentous growth.

It can also be concluded that *Lemna minor* cultivation on these effluents failed as a result of; the A/O and IASBR operational issues, insufficient seeding of cultivation tanks, and potential NH_3. Heavy metal toxicity may also have played a role in the failure of *Lemna minor* to grow on the effluent produced during the trials. It is known that *L. minor* species are sensitive to sodium chloride, with substantial inhibition of growth occurring at concentrations of 6 g/L (Keppler, 2009). *Lemna* sensitivity to cadmium (Cd), nickel (Ni), copper (Cu), zinc (Zn) and boron (B) has also been identified at or greater than; 0.64 mg/L, 0.5 mg/L, 0.2 mg/L, 5.64 mg/L and 22.4 mg/L respectively (Khellaf and Zerdaoui, 2009, Singh and Singh, 2006, Butterwick *et al.* 1989). It is also known that *L. minor* can tolerate Cu, Ni, Cd and Zn at concentrations of 0.4, 3.0, 0.4 and 15.0 mg/L respectively without showing any visible signs of toxicity (Khellaf and Zerdaoui, 2009). The concentrations of these metals in the effluent used for this study is unknown, as measurement of such was not within the remit of this project. However, given *Lemna* sensitivity to the metals listed above, future growth experiments should include assays for heavy metals.

Duckweed species, like all plants, have associated microbial communities which are beneficial to plant growth and survival. The failure of IASBR autoclaved effluent to support *Lemna minor* cultivation may also have been partly due to a lack of specific microbes that normally associate with the plant. As for the cultivation of *Lemna minor* on raw effluent, associated microbes may have been present, but perhaps not in sufficient numbers. However, this was not investigated during the project. For this reason, future work with coupled wastewater treatment/duckweed cultivation systems should involve investigation into plant-symbiont interactions, as well as profiling of wastewater microbial communities and their potential impacts on duckweed growth.

Most large-scale dairy processing plants employ BNR for tertiary treatment of wastewater (N and P removal) and use an anaerobic digestor for secondary treatment (COD

removal) and biogas production. These digestors achieve high rates of COD removal without affecting N and P levels the way BNR systems do. Effluent from these digestors also has a low pH (~4), which could potentially reduce the need to manually alter the pH of a downstream cultivation system. Optimal nutrient range for *Lemna minor* growth is 5-50 mg/L NH_4^+, 3-350 mg/L TN, 0.03 – 300 mg/L PO_4 and 0.01 – 100 mg/L TP (table 2.4). A study carried out by Caicedo *et al.* (2005) examined the effect of anaerobic digester effluent on the growth of duckweed in stabilization ponds. As part of the experiment, seven stabilization ponds were established in series, with the first pond receiving effluent from an Upstream Anaerobic Sludge Blanket reactor (UASB). The effluent contained 189 mg/L COD, 30.5 mg/L NH_4^+, and 6.8 mg/L TP. The COD level observed in the UASB effluent is lower than effluent COD levels observed for A/O (figure 2.17) but is comparable with COD levels obtained with IASBR (figure 3.9). However, much higher levels of NO_2^- and NO_3^- in the IASBR (figures 3.12 and 3.13) compared with the negligible amounts noted in the UASB (0.02 mg/L for both N species) along with the lower pH in AD effluent make it more suited to *Lemna* growth, as low levels of nitrate and nitrite will inhibit algae, while higher concentrations of NH_4^+ at a pH below 8 will suit *Lemna*.

This information, combined with the data from batch growth experiments outlined in chapter 4, suggests that anaerobic digestor effluent may be more suitable for *Lemna* cultivation. Having a cultivation system downstream of an anaerobic digestor would also reduce the need for retro-fitting the plant, therefore reducing start-up costs (Caicedo *et al.*, 2005). It is therefore recommended to focus future studies on the suitability of anaerobic treatment technologies for conditioning of dairy processing wastewater for *Lemna minor* cultivation.

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