https://doi.org/10.15255/KUI.2024.034
KUI-9/2025
Original scientific paper
Received August 2, 2024
Accepted September 14, 2024

# Denitrifying Dephosphatation *via* Nitrite under Anoxic Conditions

*A. Brozinčević*,[a] *D. Grgas*,[b*] *I. Landeka Jurčević*,[b] *M. Habuda-Stanić*,[c] *B. Zelić*,[d] and *T. Landeka Dragičević*[b]

[a] Plitvice Lakes National Park, Dr. Ivo Pevalek Scientific Research Centre, Josipa Jovića 19, 53 231 Plitvička Jezera, Croatia
[b] University of Zagreb, Faculty of Food Technology and Biotechnology, Pierottijeva 6, 10 000 Zagreb, Croatia
[c] J. J. Strossmayer University of Osijek, Faculty of Food Technology Osijek, F. Kuhača 18, 31 000 Osijek, Croatia
[d] University of Zagreb Faculty of Chemical Engineering and Technology, Trg Marka Marulića 19, 10 000 Zagreb, Croatia



**Abstract**

Denitrifying dephosphatation is a cost-effective method for simultaneously removing nitrogen and phosphorus due to its lower chemical oxygen demand (COD) requirement, reduced aeration, and decreased sludge production. In batch experiments, denitrifying dephosphatation of 20 ± 1 mg $PO_4$-P/l using nitrite was studied at N/P ratios of 2, 3, and 4. The effects of both limiting and non-limiting COD, provided by sodium acetate as a carbon source, on the activity of denitrifying phosphorus-accumulating organisms (DPAOs) were investigated. In all batch tests, at ratios N/P of 2, 3, 4, increasing the C/N ratio led to higher $NO_2$-N and P removal rates and efficiencies. In all batch tests, at ratios N/P of 2, 3, 4, the highest denitrification and anoxic phosphorus uptake rates were recorded under the following conditions: at C/N ratio of 4 and N/P ratio of 2, C/N ratio of 3 and N/P ratio of 3, and C/N ratio of 2 and N/P ratio of 4. In tests with limited carbon sources (N/P of 2 and C/N < 4, N/P of 3 and C/N < 3, and N/P of 4 and C/N < 2), both denitrification and P uptake rates and efficiencies increased with rising C/N ratios. However, the batch tests with COD overdose resulted in increased denitrification rate but deteriorated P removal rate and efficiency. In the tests with a C/N ratio of 7, the P removal efficiencies were 75 ± 3 %, 68 ± 2 %, and 62 ± 4 % at N/P ratios of 2, 3, and 4, respectively.

**Keywords**

*Denitrification, nitrite, phosphate uptake, anoxic conditions, denitrifying phosphorus-accumulating organisms*

## 1 Introduction

Denitrifying dephosphatation, an alternative to conventional nutrient removal, offers several advantages, such as approximately 50% lower sludge production, a 50% reduction in organic carbon requirements, and a 30 % decrease in oxygen demand.[1] Denitrifying phosphorus-accumulating organisms (DPAOs) in anaerobic-anoxic alternate environments have the ability for simultaneous denitrification and P uptake using nitrite ($NO_2$-N) and/or nitrate ($NO_3$-N) as terminal electron acceptors. Under anaerobic conditions, DPAOs absorb volatile fatty acids (VFAs) and store them as polyhydroxyalkanoate (PHA), which is accompanied by glycogen degradation and phosphate release. Under subsequent anoxic conditions, microbial growth occurs, where the DPAOs utilise nitrite or nitrate as electron acceptors to uptake phosphate, and restore the polyphosphate and glycogen pools with the energy provided from PHA oxidation.[1–6] Most DPAOs exhibit anaerobic-anoxic metabolism.[1,2,4–7] However, some DPAOs can perform phosphorus removal under purely anoxic conditions, without the need for an anaerobic phase, using $NO_3$-N as the electron acceptor.[8] Others function under anoxic-oxic conditios,[7,9] where the aerobic phase facilitates oxidation of residual chemical oxygen demand (COD) and P uptake. Various factors affecting DPAOs, such as anaerobic-aerobic or anaerobic-anoxic reaction times, carbon source, dissolved oxygen (DO), temperature, and pH, have been widely studied.[4,5,10–13] In denitrifying phosphorus removal, nitrite is known to act as an inhibitor of microbial metabolism.[14–16] High nitrite concentrations inhibit anoxic phosphate uptake.[5,17] Lower levels of nitrite (4–5 mg $NO_2$-N/l) have no significant effect on anoxic phosphorus uptake.[17] Nitrite concentrations up to 4 mg $NO_2$-N/l showed no effect on anoxic phosphate uptake, but levels as high as 12 mg $NO_2$-N/l reduced the anoxic phosphate uptake by 65 %.[3] For anoxic phosphate uptake, the nitrite threshold is around 2 mg $NO_2$-N/gVSS (volatile suspended solids), and it has been suggested that the somewhat higher tolerance of PAO's (phosphate-accumulating organisms) for nitrite is due to their ability to metabolise it anoxically, reducing its concentration near the cells.[3] The 20 mg of $NO_2$-N/l were completely used as electron acceptor in a denitrifying phosphate removal process for P uptake in an integrated fixed film activated sludge system in long-term operation, though nitrite was limiting the anoxic phosphorus uptake.[14] In an anaerobic-anoxic SBR with DPAOs acclimated to nitrate as an electron acceptor, the nitrite concentration up to 115 mg N/l showed no inhibition of anoxic P uptake.[18] In an anaerobic-anoxic-aerobic SBR (sequencing batch reactor), the DPAOs in anoxic conditions, used more than 20 mg $NO_2$-N/l as an electron acceptor.[19] Batch experiments with biomass from an anaerobic-aerobic SBR

* Corresponding author: Dijana Grgas, PhD
Email: dgrgas@pbf.hr

showed that PAOs could utilise nitrite as an electron acceptor, with the highest denitrification rate and highest anoxic phosphorus uptake rate observed at 20 mg $NO_2$-N/l.[20] However, experiments with biomass not acclimatised to nitrite showed that approximately 25 mg $NO_2$-N/l inhibited anoxic phosphorus uptake regardless of the concentration of the tested external carbon sources.[21] The batch experiments demonstrated that supplemental additions of nitrate (45–200 mg $NO_3$-N/l) mitigated nitrite toxicity (5–100 mg $NO_2$-N/l).[22] Another proposed strategy to overcome nitrite inhibition is step-feed approach. A concentration of 20 mg $NO_2$-N/l led to inhibition of anoxic phosphorus uptake by 64.85 %, and the inhibition of DPAOs denitrification by 61.25 %. The authors suggest a step-feed strategy using nitrite as an electron acceptor, while maintaining nitrite concentrations below 15 mg $NO_2$-N/l.[23] Free nitrous acid (FNA) is suggested as an inhibitor of phosphorus uptake, rather than nitrite.[16] For biomass not adapted to nitrite, concentrations exceeding 10 mg $NO_2$-N/l and 2.25 μg $HNO_2$-N/l were inhibitory to anoxic phosphorus uptake in denitrifying phosphorus removal processes.[24]

Since DPAOs activity is inhibited by nitrite, denitrifying dephosphatation is less stable compared to conventional biological P removal, posing challenges for its application in real systems.[25] Due to the conflicting conclusions about the effect of nitrite on denitrifying phosphorus removal, evaluating the efficiency and stability of this process with nitrite as an electron acceptor remains difficult. With these considerations in mind, the primary aim of this study was to investigate denitrifying dephosphatation *via* nitrite under anoxic conditions, with acetate and nitrite simultaneously present as electron donor and acceptor, respectively. The study also sought to determine the effect of limiting and non-limiting carbon source concentrations on DPAO activity, and consequently, on the N and P removal rates and efficiencies.

## 2 Experimental

### 2.1 Batch tests of DPAOs activity for denitrification and P uptake under anoxic conditions

Batch tests were conducted under anoxic conditions in laboratory beakers with a working volume of 600 ml, at 20–25 °C, using sludge enriched with DPAOs. The pH was not controlled. The sludge enriched with DPAOs was obtained from a sequencing batch (SBR) parent reactor operated in an 8-hour cycle with a hydraulic retention time (HRT) of 12 h and a sludge retention time (SRT) of 20 days. Dissolved oxygen (DO), pH, and temperature were monitored using an oxygen and pH probe (WTW Multi 3420 SET KS1, Germany). At the start of each anoxic batch test, carbon sources to provide COD, as well as nitrite and phosphate solutions were added simultaneously. The mixed liquor was stirred at 150 rpm (Thermo Scientific Super-nuova, SP88857195, USA). All experiments were conducted with 2.1 ± 0.1 g MLSS/l (mixed liquor suspended solids), in triplicate. The results are expressed as the mean value ± standard deviation.

The batch tests (Table 1) were conducted to investigate:

The effects of limited and non-limited COD values, as well as COD overdose from sodium acetate as an electron donor (with C/N ratios ranging from 0.5 to 7), and the effect of nitrite concentrations (with N/P ratios from 2 to 4) as an electron acceptor on DPAO activity, with an initial concentration of 20 ± 1 mg $PO_4$-P/l.

The efficiency of DPAOs in the simultaneous processes of denitrification and P uptake under anoxic conditions, by determining the efficiency and rate of denitrification and P uptake.

*Table 1* – Experimental design
*Tablica 1* – Dizajn eksperimenata

| $NO_2$-N / mg l$^{-1}$ | $PO_4$-P / mg l$^{-1}$ | N/P | C/N ratio range<br>Raspon omjera C/N |
|---|---|---|---|
| 40 ± 2 | 20 ± 1 | 2 | 0.5 – 7 |
| 60 ± 3 | 20 ± 1 | 3 | 0.5 – 7 |
| 80 ± 3 | 20 ± 1 | 4 | 0.5 – 7 |

### 2.2 Electron donors and acceptors

Sodium acetate ($CH_3COONa$) was used as the carbon source (electron donor), while $NaNO_2$ served as the electron acceptor, and $K_2HPO_4$ was used as the phosphate source. All chemicals were supplied by Merck, Germany.

### 2.3 Analytical methods

COD, $PO_4$-P, $NO_2$-N, and MLSS were analysed according to standard methods.[26] Dissolved oxygen, pH, and temperature were determined using an oxygen probe (WTW Multi 3420 SET KS1, FDO 925, Germany), and a pH probe (WTW Multi 3420 SET KS1, SenTix940-3, Germany). The fluorescence *in situ* hybridisation (FISH) was performed following the method of *R. I. Amann*[27] for the characterisation of DPAOs in sludge samples (Table 2), using Cy-5-labelled EUBMIX probes for most bacteria, and Cy-3-labelled PAOMIX probes. FISH preparations were visualised using a Leica confocal laser scanning microscope (CLSM). Polyphosphate (PolyP) was determined by staining the activated sludge samples according to *Neisser*[28] and visualized with CLSM.

COD, N, and P removal rates were calculated by subtracting the final value from the initial value and dividing by process duration. The results are expressed in mg/lh. Free nitrous acid (FNA) was calculated according to *Anthonisen et al*.[31]

*Table 2* – Probes used for FISH
*Tablica 2* – Probe primijenjene za FISH

| Probe name<br>Naziv probe | | Target group<br>Ciljana skupina | Sequence<br>Sekvenca (5′–3′) | Formamide<br>Formamid / % | Ref.<br>Lit. |
|---|---|---|---|---|---|
| Acc-I-444 | DPAOmix | DPAOs | CCCAAGCAATTTCTTCCCC | 35 | 2 |
| Acc-II-444 | | | CCCGTGCAATTTCTTCCCC | 35 | |
| EUB338 | EUBmix | All bacteria<br>Sve bakterije | GCTGCCTCCCGTAGGAGT | 0–50 | 29 |
| EUB338-II | | | GCAGCCACCCGTAGGTGT | 0–50 | 30 |
| EUB338-III | | | GCTGCCACCCGTAGGTGT | 0–50 | |

## 3 Results and discussion

### 3.1 DPAO activity with $NO_2$-N as an electron acceptor under anoxic conditions

To gain a deeper understanding of metabolic behaviour of denitrifying phosphorus-removal organisms, batch tests were conducted to investigate DPAO activity for simultaneous N and P removal using $NO_2$-N as an electron acceptor under anoxic conditions. The objective was to determine the minimum C/N ratio required for selected N/P ratios of 2, 3, and 4, with C/N ratios as limiting and non-limiting carbon sources for a concentration of 20 ± 1 mg $PO_4$-P/l.

The batch tests were conducted under anoxic conditions with electron acceptors and donors present simultaneously, omitting the anaerobic phase of the process, as previous anaerobic-anoxic regime experiments showed no acetate uptake into the cells or P release during the anaerobic phase. *Jena et al.*[9] demonstrated in anoxic-aerobic SBR experiments that simultaneous nitrate, P, and COD removal is achievable under anoxic conditions without the need for an anaerobic phase. Their research demonstrated that DPAOs enrichment over ordinary heterotrophic organisms (OHOs) under anoxic conditions benefited from an excess of electron donors and acceptors. They suggested that the presence of an electron acceptor in both the anoxic and aerobic phases eliminates the need for an anaerobic system.[9]

The batch tests measuring DPAO activity in anoxic conditions for simultaneous denitrification and P uptake at minimal required C/N ratio are shown in Table 3. Detailed variations of COD, $NO_2$-N, $PO_4$-P, FNA, temperature, and pH during the batch test with 80 mg $NO_2$-N/l at an N/P ratio of 4 and a C/N ratio of 2 are shown in Fig. 1, while data for the C/N ratio of 7 are provided in Table 4. The denitrification rate (rN) is expressed as mg N/lh, and the phosphate uptake rate (rP) is expressed as mg P/lh.

*Table 3* – COD, N, and P removal efficiency, and N and P removal rates under anoxic conditions for an initial concentration of 20 ± 1 mg $PO_4$-P/l at the minimum required C/N ratio
*Tablica 3* – Učinkovitost uklanjanja KPK, N i P, i brzine uklanjanja N i P pri anoksičnim uvjetima pri početnih 20 ± 1 mg $PO_4$-P/l, pri minimalno potrebnom omjeru C/N

| $PO_4$-P / mg $l^{-1}$ | $NO_2$-N / mg $l^{-1}$ | N/P | C/N | rN / mgN/lh | rP / mgP/lh | N removal<br>N uklonjeno / % | P removal<br>P uklonjeno / % | COD decrease<br>Smanjenje KPK / % |
|---|---|---|---|---|---|---|---|---|
| 20 ± 1 | 40 ± 2 | 2 | 4 | 20.0 ± 1.3 | 8.3 ± 0.5 | 100 | 84.0 ± 1.2 | > 97 |
| 20 ± 1 | 60 ± 3 | 3 | 3 | 23.7 ± 2.0 | 6.7 ± 0.7 | 100 | 83 ± 1 | > 97 |
| 20 ± 1 | 80 ± 3 | 4 | 2 | 25.9 ± 1.7 | 4.8 ± 0.7 | 100 | 74 ± 2 | > 97 |

*Table 4* – COD, N, and P removal efficiency, and N and P removal rates under anoxic conditions for an initial concentration of 20 ± 1 mg $PO_4$-P/l at a C/N ratio of 7
*Tablica 4* – Učinkovitost uklanjanja KPK, N i P, i brzine uklanjanja N i P pri anoksičnim uvjetima pri početnih 20 ± 1 mg $PO_4$-P/l, pri C/N 7

| $PO_4$-P / mg $l^{-1}$ | $NO_2$-N / mg $l^{-1}$ | N/P | C/N | rN / mgN/lh | rP / mgP/lh | N removal<br>N uklonjeno / % | P removal<br>P uklonjeno / % | COD decrease<br>Smanjenje KPK / % |
|---|---|---|---|---|---|---|---|---|
| 20 ± 1 | 40 ± 2 | 2 | 7 | 26 ± 2 | 7 ± 1 | 100 | 75 ± 3 | 45 ± 8 |
| 20 ± 1 | 60 ± 3 | 3 | 7 | 27 ± 3 | 4.9 ± 0.9 | 100 | 68 ± 2 | 49 ± 10 |
| 20 ± 1 | 80 ± 3 | 4 | 7 | 29 ± 2 | 3.5 ± 0.4 | 100 | 62 ± 4 | 55 ± 11 |

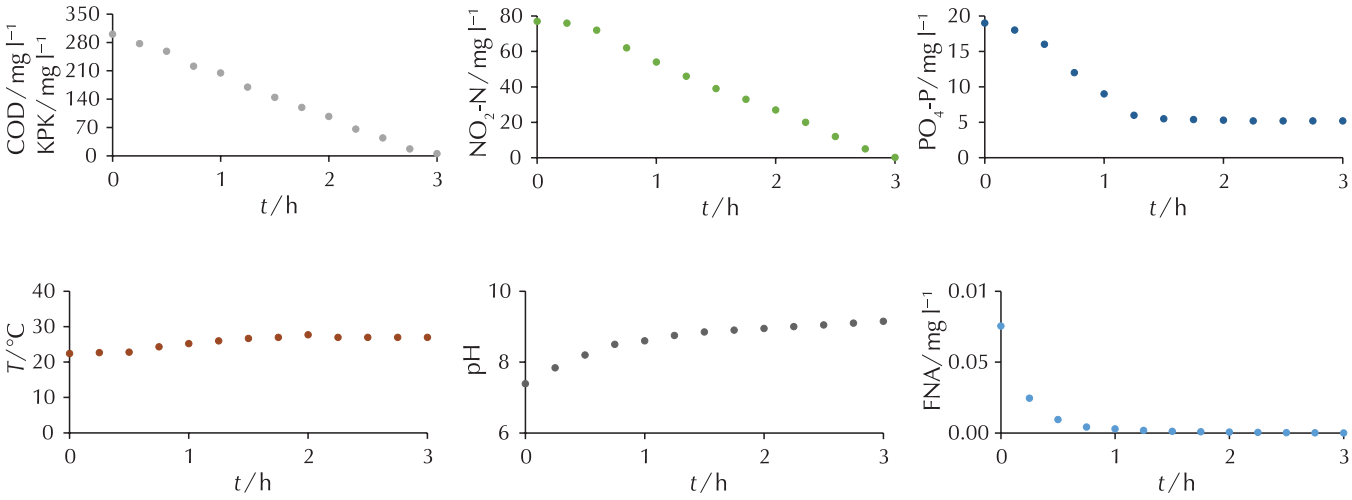


*Fig. 1* – Variations of COD, $NO_2$-N, $PO_4$-P, FNA, temperature, and pH in batch test with 80 mg $NO_2$-N/l at N/P ratio of 4 and C/N ratio of 2

*Slika 1* – Varijacije KPK, $NO_2$-N, $PO_4$-P, FNA, temperature i pH u šaržnim ispitivanjima od 80 mg $NO_2$-N/l pri N/P 4 i C/N 2

In all the batch tests, at N/P ratios of 2, 3, 4, an increase in the C/N ratio resulted in higher $NO_2$-N and P removal rates and efficiencies. The highest denitrification and anoxic phosphorus uptake rates were recorded at 40 ± 2 mg $NO_2$-N/l with a C/N of 4 (N/P of 2), at 60 ± 3 mg/l $NO_2$-N with a C/N of 3 (N/P of 3), and at 80 ± 3 mg $NO_2$-N/l with a C/N of 2 (N/P of 4) (Table 3).

The literature points out the contradictory effects of $NO_2$-N as an inhibitor of anoxic P uptake. It has been reported that concentrations greater than 10 mg $NO_2$-N/l inhibited anoxic phosphorus uptake in biomass not adapted to nitrite.[24] At 12 mg $NO_2$-N/l the anoxic phosphate uptake was reduced by 65 %,[3] and at 20 mg $NO_2$-N/l anoxic phosphorus uptake was inhibited by 64.85 % with a 61.25 % inhibition of DPAO denitrification.[23] Additionally, 25 mg $NO_2$-N/l inhibited anoxic phosphorus uptake, regardless of the concentration of external carbon sources, in non-acclimated process biomass.[21] The nitrite threshold for anoxic phosphate uptake is around 2 mg $NO_2$-N/gVSS.[17] Conversely, concentrations of 4–5 mg $NO_2$-N/l,[3,17] 20 mg $NO_2$-N/l,[14] and even up to 115 mg $NO_2$-N/l[18] were not detrimental to anoxic phosphorus uptake.

In the batch tests (Table 3), with 40 ± 2 mg $NO_2$-N/l (N/P of 2), denitrification efficiency reached 100 % at a C/N of 4, with P removal efficiency of 84.0 ± 1.2 %. For 60 ± 3 mg $NO_2$-N/l (N/P of 3), denitrification efficiency was also 100 % at a C/N of 3, with P removal efficiency of 83 ± 1 %. For 80 ± 3 mg $NO_2$-N/l (N/P of 4), denitrification efficiency remained 100 % at a C/N of 2, with P removal efficiency of 74 ± 2 %. Denitrification dephosphatation for 40 ± 2 mg $NO_2$-N/l (N/P of 2) at a C/N of 4 resulted in a denitrification rate of 20.0 ± 1.3 mg $NO_2$-N/lh, and a P uptake rate of 8.3 ± 0.5 mg $PO_4$-P/lh. For 60 ± 3 mg $NO_2$-N/l (N/P of 3) at a C/N of 3, the denitrification rate was 23.7 ± 2.0 mg $NO_2$-N/lh and the P uptake rate was 6.7 ± 0.7 mg $PO_4$-P/lh. For 80 ± 3 mg $NO_2$-N/l (N/P of 4) at a C/N of 2, the denitrification rate was 25.9 ± 1.7 mg $NO_2$-N/lh, with a P uptake rate of 4.8 ± 0.7 mg $PO_4$-P/lh (Table 3). Available COD for denitrification and simultaneous P uptake in these experiments was not a limiting factor for either process, nor was it added in excess. However, increasing $NO_2$-N concentrations from 40–80 mg $NO_2$-N/l (N/P 2-4), resulted in a decrease in both the P uptake rate and the P removal efficiency. By increasing the $NO_2$-N concentration from 40 ± 2 mg $NO_2$-N/l to 80 ± 3 mg $NO_2$-N/l (N/P 2–4), the effect of P removal decreased from 84.0 ± 1.2 % to 74 ± 2 %, as did the P uptake rate from 8.3 ± 0.5 mg $PO_4$-P/lh to 4.8 ± 0.7 mg $PO_4$-P/lh (Table 3). Since both denitrifiers and DPAOs require organic carbon for denitrification and P release, it is important to ensure a sufficient amount of organics for an effective process.[32] *Zhang et al.*[32] conducted batch experiments under anaerobic conditions with the simultaneous presence of acetate (360 mg COD/l), phosphate (9.7 mg $PO_4$-P/l), and nitrite (0–10 mg $NO_2$-N/l), and observed reduced phosphate release as the initial nitrite concentrations increased. In our experiments, where acetate, nitrite, and phosphate were present simultaneously, no phosphate release occurred; however, COD consumption, nitrite reduction, and phosphate uptake were recorded from the start of the experiment. *Zhang et al.*[32] suggested that DPAOs coexist with ordinary heterotrophic denitrifiers, competing for organic carbon during the anaerobic phase in the presence of nitrite, which led to reduced organic availability for DPAOs, and less effective phosphate release, thus lowering subsequent anoxic P uptake. They noted that nitrite inhibited anaerobic P release and that nitrite concentrations in the anaerobic phase should be kept below 2 mg N/l to ensure efficient P release and subsequent P uptake. *Jena et al.*[9] suggest that some of the acetate was used for intracellular PHB (polyhydroxybutyrate) storage, while some COD was used for $NO_3$-N reduction, in addition to biomass maintenance and growth in denitrifying dephosphatation with $NO_3$-N under an anoxic-aerobic regime. They showed that anoxic P uptake was not dependent on PHB metabolism. *Hu et al.*[33] reported that, in the presence of excess nitrite in anoxic conditions, anoxic P uptake by DPAOs becomes feasible, surpassing the denitrifying potential of other heterotrophic organisms.

In all experiments with both limiting and non-limiting COD, with an increase in C/N ratio, the P uptake rate exhibited two phases: an initial phase with a higher rate where around 70% of $NO_2$-N and $PO_4$-P were removed, followed by a second phase with a significantly lower P uptake rate, although $NO_2$-N reduction continued at the same denitrification rate (Fig. 1). *Zhang et al.*[32] observed a similar trend, with rapid phosphate uptake and nitrite reduction rate in the first 30 minutes of the anoxic phase, followed by a slowdown.

FNA variations showed a consistent trend in all experiments – FNA was the highest at the start of the experiment with the initial $NO_2$-N concentration, and decreased as $NO_2$-N concentrations dropped and pH increased (Fig. 1). The highest FNA concentration, 0.008 mg/l, was recorded in the experiment with 20 ± 1 mg $PO_4$-P/l at an N/P ratio of 4 and C/N ratio of 2. The absence of inhibitory effects from such high FNA concentrations on denitrifying dephosphatation may be due to the long-term exposure of the sludge to nitrite as an electron acceptor. *Zhou et al.*[16] reported that 0.02 mg $HNO_2$-N/l completely inhibited anoxic P uptake, while for biomass not adapted to nitrite, 2.25 μg $HNO_2$-N/l was enough to inhibit anoxic phosphorus uptake.[24]

In our experiments, the pH profile (Fig. 1) was characteristic of the denitrifying dephosphatation process: from the start of the process, as available COD was consumed, the pH value increased. In all experiments, initial pH was 7.2 ± 0.2. Once total COD was consumed, the pH either remained constant or slightly decreased until the end of the experiment, indicating that pH could serve as an indirect indicator of the process. The reason for the two distinct rates of anoxic P uptake could be related to pH value. The critical pH value for anoxic P uptake was 8.75 ± 0.05. Significant P removal occurred when the pH was at or below 8.75 ± 0.05, while additional P uptake was observed at higher pH levels (Fig. 1). At the end of all batch tests, $PO_4$-P concentrations ≥ 3 mg $PO_4$-P/l were recorded, suggesting that P removal can be entirely attributed to DPAO activity, rather than P precipitation.

The enhanced biological phosphorus removal (EBPR) process is characterised by a decrease in pH during the anaerobic phase and an increase in pH during the aerobic/anoxic phase. The pH value influences microbial growth and reproduction, affecting the microbial cell membrane's surface charge and permeability.[34–36] *Guo et al.*[37] suggested that lower phosphorus release at higher pH levels could be due to precipitation of phosphate adhered to the surface of zooglea, which blocked carbon adsorption and PAOs P release. *Peng*[38] pointed out that higher pH might reduce the proton motive force, causing PAOs to hydrolise more polyP, thus higher pH leads to higher P discharge. *Li et al.*[39] demonstrated that under anaerobic conditions, phosphorus release increased as the pH value rose from 6.5–8, but decreased when pH reached 8.5. They proposed that acetic acid actively diffuses into cell membranes and is converted to PHB. Since acetic acid degradation requires energy, this energy is replenished through the disintegration of intercellular polyP, which is subsequently released from the cell. In their experiments, under subsequent anoxic conditions, P uptake increased with the increase in pH from 6.5 to 8.0, but declined when the pH was elevated to 8.5. They emphasised that the rate of P release was superior to the rate of P uptake in response to the change in pH value, and maintaining pH within the range of 6.5–8.0 supports stable operation, promoting anaerobic P release and anoxic P uptake.[39]

In batch tests with COD overdose (Table 4), DPAO activity showed a slight increase in the $NO_2$-N denitrification rate, while P uptake rate and efficiency decreased. In these batch tests, the residual COD remained in the treated water.

*Jena et al.*[8] reported that a high COD/N ratio could cause the dominance of heterotrophs over DPAOs. *Zhang et al.*[32] reported that both very high and very low initial COD levels can negatively affect denitrifying dephosphatation. They explained that higher initial COD promotes ordinary heterotrophic denitrifiers over DPAOs, resulting in complete nitrite reduction but low P uptake. Conversely, low initial COD led to most of the COD being consumed by DPAOs, with insufficient PHA stored for anoxic P uptake, while ordinary denitrifiers did not use the COD. They concluded that for effective denitrifying dephosphatation, the initial COD should be high enough to ensure adequate PHA formation.[32]

### 3.2 Microbial analysis of the DPAOs

FISH images of the activated sludge enriched with DPAOs are shown in Fig. 2, while polyP, identified by Neisser staining, is shown in Fig. 3.

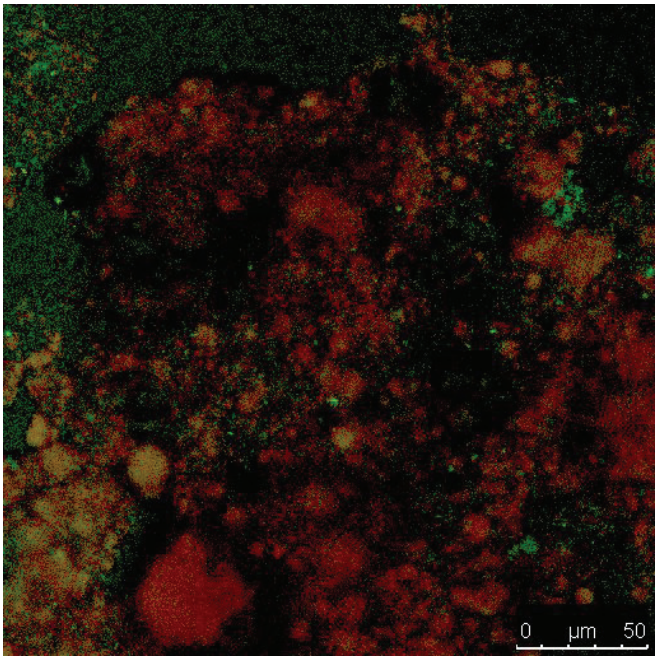


*Fig. 2* – CLSM of activated sludge enriched with DPAOs. All bacteria are shown in green and DPAOs in red.
*Slika 2* – CLSM aktivnog mulja obogaćenog s DPAOs. Sve bakterije su prikazane zeleno, a DPAOs crveno.

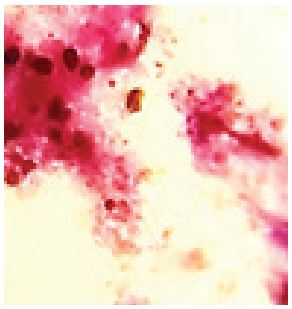

*Fig. 3* – PolyP stained by Neisser, 400× magnification
*Slika 3* – PoliP obojen po Neisseru, povećanje 400×

The FISH method[27] was used to visualise and confirm the presence of microbial clusters specifically stained with probes that target organisms involved in anoxic phosphorus removal using $NO_2$-N as an electron acceptor (DPAOmix, Fig. 2). Through CLSM analysis, the presence of microbial clusters responsible for N and P removal processes was documented. The presence of PolyP, stained by the Neisser method,[28] was confirmed in the denitrifying dephosphatation process, visible as dark purple granules (Fig. 3).

## 4 Conclusion

Denitrifying dephosphatation of 20 ± 1 mg $PO_4$-P/l under anoxic conditions resulted in simultaneous $NO_2$-N removal, with 70 ± 2 % $NO_2$-N denitrification achieved regardless of the initial $NO_2$-N concentration (40 ± 2, 60 ± 3, and 80 ± 3 mg $NO_2$-N/l). At the same time, the P removal efficiency of 81 ± 2 %, 79 ± 3 %, and 70 ± 4 % were achieved at N/P of 2 and C/N of 4, N/P of 3 and C/N of 3, and N/P of 4 and C/N of 2. The remaining $NO_2$-N was fully reduced with the residual COD, accompanied by a slight increase in P uptake, resulting in total P removal efficiency of 84.0 ± 1.2 % for N/P of 2 and C/N of 4, 83 ± 1 % for N/P of 3 and C/N of 3, and 74 ± 2 % for N/P of 4 and C/N of 2. In these batch tests, COD removal exceeded 97 %. In the tests with limited carbon source (N/P of 2 and C/N < 4, N/P of 3 and C/N < 3, and N/P of 4 and C/N < 2), both denitrification and P uptake efficiencies and rates increased with increasing C/N ratio. Batch tests with COD overdose resulted in higher denitrification rates but reduced P removal efficiencies and rates. In batch tests with C/N ratio of 7, the P removal efficiency were 75 ± 3 %, 68 ± 2 %, and 62 ± 4 % at N/P ratios of 2, 3, and 4, respectively. For future research, it is recommended to investigate the effects of alternative carbon sources on the efficiency of the denitrifying dephosphatation process, as well as their influence on the functionality and structure of the microbial community.

ACKNOWLEDGEMENTS

This research was funded by the University of Zagreb through the Scientific and Artistic Research Support grant, funding No. 2440.

### List of abbreviations
### Popis kratica

CLSM – confocal laser scanning microscope
COD – chemical oxygen demand
DO – dissolved oxygen
DPAOs – denitrifying phosphate accumulating organisms
DPAOs – nitrate denitrifying phosphorus-accumulating organisms over nitrate
DPAOs – nitrite denitrifying phosphorus-accumulating organisms over nitrite
EBPR – enhanced biological phosphorus removal
FISH – fluorescence *in situ* hybridisation
FNA – free nitrous acid
HRT – hydraulic retention time
KPK – kemijska potrošnja kisika
$NO_3$-N – nitrate
$NO_2$-N – nitrite
OHO – ordinary heterotrophic organisms
PAOs – phosphate accumulating organisms
PHAs – polyhydroxyalkanoates
PHB – polyhydroxybutyrate
SBR – sequencing batch reactor
SRT – sludge retention time
VFAs – volatile fatty acids
VSS – volatile suspended solids

### References
### Literatura

1. *T. Kuba, M. C. M. van Loosdrecht, J. J. Heijnen*, Phosphorus and nitrogen removal with minimal COD requirement by integration of denitrifying dephosphatation and nitrification in a two-sludge system, Water Res. **30** (1996) 1702–1710, doi: https://doi.org/10.1016/0043-1354(96)00050-4.
2. *J. J. Flowers, S. He, S. Yilmaz, D. R. Noguera, K. D. McMahon*, Denitrification capabilities of two biological phosphorus removal sludges dominated by different '*Candidatus accumulibacter*' clades, Environ. Microbiol. Rep. **1** (2009) 583–588, doi: https://doi.org/10.1111/j.1758-2229.2009.00090.x.
3. *T. Saito, D. Brdjanović, M. C. M. van Loosdrecht*, Effect of nitrite on phosphate uptake by phosphate accumulating organisms, Water Res. **38** (2004) 3760–3768, doi: https://doi.org/10.1016/j.watres.2004.05.023 .
4. *T. Kuba, M. C. M. van Loosdrecht, J. J. Heijnen*, Biological dephosphatation by activated sludge under denitrifying conditions: pH influence and occurrence of denitrifying dephosphatation in a full-scale waste water treatment plant, Water Sci. Technol. **36** (1997) 75–82, doi: https://doi.org/10.1016/S0273-1223-(97)00713-0.
5. *T. Kuba, M. C. M. van Loosdrecht, J. J. Heijnen*, Effect of cyclic oxygen exposure on the activity of denitrifying phosphorus removal bacteria, Water Sci. Technol. **34** (1996) 33–40, doi: https://doi.org/10.1016/0273-1223(96)00492-1.
6. *T. Kuba, E. Murnleitner, M. C. M. van Loosdrecht, J. J. Heijnen*, A metabolic model for biological phosphorus removal by denitrifying organisms, Biotechnol. Bioeng. **52**

(1996) 685–695, doi: https://doi.org/10.1002/(SICI)1097-0290(19961220)52:6<685::AID-BIT6>3.0.CO;2-K.

7. *J. Ahn, T. Daidou, S. Tsuneda, A. Hirata*, Transformation of Phosphorus and Relevant Intracellular Compounds by a Phosphorus-Accumulating Enrichment Culture in the Presence of Both the Electron Acceptor and Electron Donor, Biotechnol. Bioeng. **79** (2002) 83–93, doi: https://doi.org/10.1002/bit.10292.
8. *J. Jena, S. Ray, S. Pandey, T. Das*, Effect of COD/N ratio on simultaneous removal of nutrients and COD from synthetic high strength waste water under anoxic conditions, J. Sci. Ind. Res. **72** (2013) 127–131.
9. *J. Jena, R. Kumar, M. Saifuddin, A. Dixit, T. Das*, Anoxic-aerobic SBR system for nitrate, phosphate and COD removal from high-strength wastewater and diversity study of microbial communities, Biochem. Eng. J. **105** (2016) 80–89, doi: https://doi.org/10.1016/j.bej.2015.09.007.
10. *Y. Wang, Y. Peng, T. Stephenson*, Effect of influent ratios and hydraulic retention time (HRT) on simultaneous phosphorus and nitrogen removal in a two-sludge sequencing batch reactor process, Bioresour. Technol. **100** (2009) 3506–3512, doi: https://doi.org/10..1016/j.biortech.2009.02.026.
11. *Y. Y. Wang, Y. Z. Peng, C. Y. Peng, S. Y. Wang, W. Zeng*, Influence of ORP variation, carbon source and nitrate concentration on denitrifying phosphorus removal by DPB sludge from dephanox process, Water Sci. Technol. **50** (2004) 153–161, doi: https://doi.org/10.2166/wst.2004.0632.
12. *K. Soejima, S. Matsumoto, S. Ohgushi, K. Naraki, A. Terada, S. Tsuneda, A. Hirata*, Modeling and experimental study on the anaerobic/aerobic/anoxic process for simultaneous nitrogen and phosphorus removal: the effect of acetate addition, Process Biochem. **43** (2008) 605–614, doi: https://doi.org/10.1016/j.procbio.2008.01.022.
13. *M. Merzouki, N. Bernet, J. P. Delgenes, R. Moletta, M. Benlemlih*, Effect of operating parameters on anoxic biological phosphorus removal in anaerobic-anoxic sequencing batch reactor, Environ. Technol. **22** (2001) 397–408, doi: https://doi.org/10.1080/09593332208618269.
14. *P. Jabari, G. Munz, J. A. Oleszkiewicz*, Selection of denitrifying phosphorous accumulating organisms in IFAS systems: comparison of nitrite with nitrate as an electron acceptor, Chemosphere **109** (2014) 20–27, doi: https://doi.org/10.1016/j.chemosphere.2014.03.002.
15. *Y. Zhou, L. Ganda, M. Lim, Z. Yuan, W. J. Kjelleberg*, Free nitrous acid (FNA) inhibition on denitrifying poly-phosphate accumulating organisms (DPAOs), Appl. Microbiol. Biotechnol. **88** (2010) 359–369, doi: https://doi.org/10.1007/s00253-010-2780-3.
16. *Y. Zhou, M. Pijuan, Z. Yuan*, Free nitrous acid inhibition on anoxic phosphorus uptake and denitrification by polyphosphate accumulating organisms, Biotechnol. Bioeng. **98** (2007) 903–912, doi: https://doi.org/10.1002/bit.21458.
17. *J. Meinhold, E. Arnold, S. Isaacs*, Effect of nitrite on anoxic phosphate uptake in biological phosphorus removal activated sludge, Water Res. **33** (1999) 1871–1883, doi: https://doi.org/10.1016/S0043-1354(98)00411-4
18. *J. Y. Hu, S. L. Ong, W. J. Ng, F. Lu, X. J.* Fan, A new method for characterizing denitrifying phosphorus removal bacteria by using three different types of electron acceptors, Water Res. **37** (2003) 3463–3471, doi: https://doi.org/10.1016/S0043-1354(03)00205-7.
19. *M. Vargas, A. Guisasola, A. Artigues, C. Casas, J.A. Baeza*, Comparison of a nitrite-based anaerobic-anoxic EBPR system with propionate or acetate as electron donors, Process Biochem. **46** (2011) 714–720, doi: https://doi.org/10.1016/j.procbio.2010.11.018
20. *Y. Zhou, L. Ganda, M. Lim, Z. Yuan, W. J. Ng*, Response of poly-phosphate accumulating organisms to free nitrous acid inhibition under anoxic and aerobic conditions, Bioresour. Technol. **116** (2012) 340–347, doi: https://doi.org/10.1016/j.biortech.2012.03.111
21. *X. Hu, D. Sobotka, K. Czerwionka, Q. Zhou, L. Xie, J. Makinia*, Effects of different external carbon sources and electron acceptors on interactions between denitrification and phosphorus removal in biological nutrient removal processes, J. Zhejiang Univ. Sci. B **19** (2018) 305–316, doi: https://doi.org/10.1631/jzus.B1700064
22. *I. Zekker, A. Mandel, E. Rikmann, M. Jaagura, S. Salmar, M. M. Ghangrekar, T. Tenno*, Ameliorating effect of nitrate on nitrite inhibition for denitrifying P-accumulating organisms, Sci. Total Environ. **797** (2021) 149133, doi: https://doi.org/10.1016/j.scitotenv.2021.149133.
23. *B. Ma, S. Wang, G. Zhu, S. Ge*, Denitrification and phosphorus uptake by DPAOs using nitrite as an electron acceptor by step-feed strategies, Front. Env. Sci. Eng. **7** (2013) 267–272, doi: https://doi.org/10.1007/s11783-012-0439-2.
24. *W. Zeng, B. Li, Y. Yang, X. Wang, L. Li, Y. Peng*, Impact of nitrite on aerobic phosphorus uptake by poly-phosphate accumulating organisms in enhanced biological phosphorus removal sludges, Bioprocess Biosyst. Eng. **37** (2014) 277–287, doi: https://doi.org/10.1007/s00449-013-0993-4.
25. *Y. Y. Wang, G. Gang, H. Wang, T. Stephenson, J. H. Guo, L. Ye*, Long-term impact of anaerobic reaction time on the performance and granular characteristics of granular denitrifying biological phosphorus removal systems, Water Res. **47** (2013) 5326–5337, doi: https://doi.org/10.1016/j.watres.2013.06.013
26. *APHA, AWWA, WPCF,* Standard methods for examination of water and wastewater, American Public Health Association, Washington, DC, 1998.
27. *R. I. Amann*, Fluorescently labelled, rRNA-targeted oligonucleotide probes in the study of microbial ecology, Molec. Ecol. **4** (1995) 543–554, doi: https://doi.org/10.1111/j.1365-294X.1995.tb00255.x.
28. *D. H. Eikelboom, H. J. J. van Buijsen*, Microscopic Sludge Investigation Manual, 2nd ed. Delft: TNO Research Institute for Environmental Hygiene, 1983.
29. *R. I. Amann, B. J. Binder, R. J. Olson, S. W. Chisholm, R. Devereux, D. A. Stahl*, Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations, Appl. Environ. Microbiol. **56** (1990) 1919–1925, doi: https://doi.org/10.1128/aem.56.6.1919-1925.1990.
30. *H. Daims, A. Bruhl, R. Amann, K. H. Schleifer, M. Wagner*, The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: Development and evaluation of a more comprehensive probe set, Syst. Appl. Microbiol. **22** (1999) 434–444, doi: https://doi.org/10.1016/S0723-2020(99)80053-8.
31. *A. C. Anthonisen, R. C. Loehr, T. B. Prakasam, E. G. Srinath*, Inhibition of nitrification by ammonia and nitrous acid, J. Water Pollut. Control Fed. **48** (1976) 835–852.
32. *S.-H. Zhang, Y. Huang, Y.-M. Hua*, Denitrifying dephosphatation over nitrite: Effects of nitrite concentration, organic carbon, and pH, Bioresour. Technol. **101** (2010) 3870–3875, doi: https://doi.org/10.1016/j.biortech.2009.12.134.
33. *Z. R. Hu, M. C. Wentzel, G. A. Ekama*, Anoxic growth of phosphate-accumulating organisms (PAOs) in biological nutrient removal activated sludge systems, Water Res. **36** (2002) 4927–4937, doi: https://doi.org/10.1016/s0043-1354(02)00186-0.

34. *I. Zafiriadis, S. Ntougias, C. Nikolaidis, A. G. Kapagiannidis, A. Aivasidis*, Denitrifying polyphosphate accumulating organisms population and nitrite reductase gene diversity shift in a DEPHANOX-type activated sludge system fed with municipal wastewater, J. Biosci. Bioeng. **111** (2011) 185–192, doi: https://doi.org/10.1016/j.biosc.2010.09.016.
35. *W. C. Ding, Q. Li, F. Y. Ji, X. M. Tian, D. Wu, Y. Du*, Short-cut nitrification-denitrification and denitrifying phosphorus removal in two sludge SBR system, China Water Wastewater **26** (2010) 11–14.
36. *Y. Y. Yang, W. Zeng, J. R. Liu, L. Li, X. D. Wang*, Effect of nitrite on biological phosphorus removal in wastewater, Microbiol. China **37** (2010) 586–593, doi: https://doi.org/10.1016/j.biortech.2011.03.091
37. *X. Guo, Z. H. Meng, J. H*. Dong, Study on pH value effect on removal of biological phosphorus in anaerobic pool, J. Harbin Univ. Commerce (Natur. Sci. Ed.) **21** (2005) 292–293.
38. *Y. Z. Peng*, SBR Method for Sewage Biological Phosphorus Removal and Process Control. Science Press, 2011, pp. 188-190.
39. *W. Li, H. Zhang, H. Sun, F. Zeng, Y.-N. Gao, L. Zhu*, Influence of pH on short-cut denitrifying phosphorus removal, Water Sci. Eng. **11** (2018) 17–22, doi: https://doi.org/10.1016/J.WSE.2018.03.006.

## SAŽETAK

### Denitrificirajuća defosfatacija preko nitrita pri anoksičnim uvjetima

*Andrijana Brozinčević*,[a] *Dijana Grgas*,[b*] *Irena Landeka Jurčević*,[b] *Mirna Habuda-Stanić*,[c] *Bruno Zelić* [d] i *Tibela Landeka Dragičević* [b]

Denitrificirajuća defosfatacija je ekonomski povoljniji izbor za istodobno uklanjanje dušika i fosfora, zbog manjeg zahtjeva za kemijsku potrošnju kisika (KPK), manje potrebe za aeracijom i manje proizvodnje mulja. U šaržnim eksperimentima denitrificirajuće defosfatacije 20 ± 1 mg $PO_4$-P/l preko nitrita pri omjerima N/P 2, 3 i 4, istražen je učinak ograničavajućeg i neograničavajućeg KPK-a iz natrijeva acetata kao izvora ugljika na aktivnost denitrificirajućih fosfor akumulirajućih organizama (DPAOs). U svim šaržnim eksperimentima, pri omjerima N/P 2, 3 i 4, povećanje omjera C/N rezultiralo je povećanjem učinkovitosti i brzine uklanjanja $NO_2$-N i P, a najveća brzina denitrifikacije te najveći anoksični unos P zabilježen je pri C/N 4 i N/P 2, C/N 3 i N/P 3 te pri C/N 2 i N/P 4. U šaržnim eksperimentima s ograničenim izvorom ugljika (N/P 2 i C/N < 4, N/P 3 i C/N < 3, N/P 4 i C/N < 2) učinkovitost i brzina denitrifikacije povećavaju se s povećanjem omjera C/N, dok eksperimenti s prekomjernim KPK-om rezultiraju povećanjem brzine denitrifikacije, ali je brzina i učinkovitost uklanjanja P narušena. U šaržnim eksperimentima pri C/N 7 učinkovitost uklanjanja P iznosi 75 ± 3 % pri omjeru N/P 2, 68 ± 2 % pri omjeru N/P 3 i 62 ± 4 % pri omjeru N/P 4.

**Ključne riječi**
*Denitrifikacija, nitrit, uklanjanje fosfora, anoksični uvjet, denitrificirajući fosfor akumulirajući organizmi*

[a] *Nacionalni park Plitvička jezera, Znanstveno-stručni centar "Dr. Ivo Pevalek", Josipa Jovića 19, 53 231 Plitvička Jezera*
[b] *Sveučilište u Zagrebu Prehrambeno-biotehnološki fakultet, Pierottijeva 6, 10 000 Zagreb*
[c] *Sveučilište J. J. Strossmayera, Prehrambeno-tehnološki fakultet Osijek, Zavod za kemiju, F. Kuhača 18, 31 000 Osijek*
[d] *Sveučilište u Zagrebu Fakultet kemijskog inženjerstva i tehnologije, Trg Marka Marulića 19, 10 000 Zagreb*

Izvorni znanstveni rad
*Prispjelo 2. kolovoza 2024.*
*Prihvaćeno 14. rujna 2024.*