# EFFECT OF CARBON-BASED NANOMATERIALS ON AEROBIC GRANULAR SLUDGE WASTEWATER TREATMENT PROCESS

PH.D. THESIS

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# 1. INTRODUCTION AND OBJECTIVES

Currently, carbon-based nanomaterials (CBNs), such as graphene (G), graphene oxide (GO), single-walled carbon nanotubes (SWCNT), and multi-walled carbon nanotubes (MWCNT), are being increasingly used in industry, due to their excellent electrochemical properties. According to some predictions, the global market for CBN production could reach approximately $1.3 billion by 2023, making their release into the environment inevitable. Due to their antimicrobial properties, they pose a risk to the process and efficiency of biological wastewater treatment. In a comprehensive study, researchers investigated the quantity of nanomaterials present in wastewater treatment plants. Their results indicated that the quantity of various nanomaterials in wastewater treatment plants can be as high as 150 mg/kg, making it essential to investigate their potential negative effects.

Previous studies that investigated the effect of CBNs on conventional activated sludge (CAS) wastewater treatment systems typically only conducted short-term (acute: 4-5 hours) experiments, with only a few examining the long-term (chronic: minimum 1 week) effects of nanomaterials. The experienced results are very different, during acute experiments, the nanomaterials did not cause a negative effect (if they did, only at extremely high concentrations, >100 mg/L), while the CBNs exerted a negative effect on the treatment efficiency of the bioreactors after a few days of exposure.

The aerobic granular sludge (AGS) process is a relatively new and promising technology in wastewater treatment, as the use of granular sludge has several advantages over traditional activated sludge due to the high extracellular polymeric substance (EPS) content and structure of the granules. Such advantages include resistance to certain toxic substances, excellent settling ability, rich microbial composition, as well as the removal of organic and inorganic matter simultaneously in space and time, and suitability for the treatment of industrial wastewater.

The chronic or long-term effects of CBNs on AGS have not yet been investigated. Two previous studies evaluated the effects of GO nanoparticle (NP) at a concentration of 60 mg/L, measuring only the system's nitrogen and phosphorus removal capacity for 4 hours. Thus, the chronic effects of G NP, GO NP, SWCNT, and MWCNT on AGS bioreactor performance, EPS quantity, and microbial community remain unknown.

Taking the above into consideration, the main aim of this dissertation was to investigate the chronic effects of G NP, GO NP, SWCNT, and MWCNT on the aerobic granular sludge biological wastewater treatment technology, by monitoring the removal efficiency of nutrients, the production of EPS, and changes occurring in the microbial community. The first goal of the research was to design and construct a bioreactor and produce granulated sludge, as well as to ensure the stable operation of the AGS bioreactor, which, according to the literature and our knowledge, has not yet been investigated with in Hungary. Afterwards, our aim was to investigate the effect of GO NPs at different concentrations and to assess the regenerative capacity of the AGS sequencing batch reactor (SBR). Finally, we examined and compared the chronic effects of G NP, GO NP, SWCNT, and MWCNT on the AGS operating in a sequencing batch mode

## 2. EXPERIMENTAL

We obtained the G NP (specific surface area of 300 m2/g) and SWCNT (≥90%) nanomaterials used in the experiments from Sigma-Aldrich Kft., while the GO NP and MWCNT nanomaterials were synthesized using modified Hummer's and catalytic chemical vapor deposition (CCVD) methods, applied at the Department of Applied and Environmental Chemistry at the University of Szeged. The nanomaterials were characterized using scanning electron microscopy (SEM), X-ray diffraction (XRD), and Raman spectroscopy.

The activated sludge (AS) was obtained from the Szeged Municipal Wastewater Treatment Plant, and the granular sludge was produced in a glass, sequenced batch reactor with a height-to-width ratio of 7 and a useful volume of 1.4 L. The bioreactor was operated with synthetic wastewater (SWW), with a hydraulic retention time (HRT) of 8 hours, divided into 4-hour cycles. Granule formation was achieved using a biomass washing method.

During the experiments, we measured the chemical oxygen demand (COD), ammonium-nitrogen ($NH_4$-N), nitrite-nitrogen ($NO_2$-N), nitrate-nitrogen ($NO_3$-N), and phosphorus ($PO_4$-P) concentrations of the effluent wastewater using HACH rapid tests and UV-VIS spectrophotometer. We determined the mixed liquor suspended solids (MLSS) and volatile suspended solids (MLVSS) content, and the sludge volume index (SVI) at 30 and 5 minutes ($SVI_{30}$ and $SVI_5$) using standard methods accepted in the literature. During the determination of EPS quantity in the granular sludge, EPS was extracted, and the amount of protein and polysaccharide (PN and PS) were measured using Anthrone and modified Lowry methods. The granular sludge was examined using scanning electron microscopy, where the sludge was stabilized, dehydrated, and then coated with gold before imaging.

In the first experiment, we operated 11 AGS SBRs (each with an initial biomass content of 6.2 g/L MLSS), and fed them with different concentrations of GO NP-containing synthetic wastewater (0, 5, 15, 25, 35, 45, 55, 65, 75, 85, and 95 mg/L GO NP) for 1 week (42 cycles each). At the end of every third cycle, we measured the nutrient concentration in the effluent, and at the end of the experiment, we examined the biomass and its EPS content, as well as the microbial composition changes. The potential changes in the microbial community were determined by denaturing gradient gel electrophoresis (DGGE) and 16S rRNA gene amplicon sequencing. To examine the relationship between environmental factors and the microbial community, redundancy analysis (RDA) was performed.

In the next experiment, we investigated the long-term effect of GO NP and the regenerative capacity of AGS SBR. The GO NP concentration in the fed SWW was continuously increased from 0 to 15, 35, 55, 75, 95, and 115 mg/L, with each experimental phase lasting 14 days. Finally, to test the regenerative capacity of the bioreactor, GO NP-free SWW was fed to the system for 14 days. The bioreactor was operated for a total of 112 days. We monitored continuously the water chemistry and sludge properties, as well as we determined the microbial activity of the AGS at the end of each phase, including specific oxygen uptake rate (SOUR), specific ammonia oxidation rate (SAOR), specific nitrite oxidation rate (SNOR), specific nitrite reduction rate (SNIRR), specific nitrate reduction rate (SNRR), specific phosphorus uptake rate (SPUR), and specific phosphorus release rate (SPRR).

Finally, we investigated the chronic effects of four types of carbon-based nanomaterials (CBN), namely G NP, GO NP, SWCNT, and MWCNT, on the AGS SBR. During the experiment, in addition to the control bioreactor (which was fed with nanomaterial-free synthetic wastewater), we fed other bioreactors with synthetic wastewater containing 1, 5, and 10 mg/L of nanomaterials for 10 days, examining the nutrient removal efficiency and sludge properties of the AGS SBRs. We determined the changes in the microbial community using next-generation sequencing (NGS), 16S ribosomal RNA metagenomic sequencing. Finally, we examined the alpha diversity of the microbial community and performed principal coordinate analysis (PCoA).

## 3. NOVEL SCIENTIFIC RESULTS

**T1. We have demonstrated that extracellular polymeric substances (mainly PN) play a key role in mitigating the toxic effects of GO NPs.**

1.1. We were the first to publish in Hungary about the formation of aerobic granules and the topic of granulated aerobic sludge wastewater treatment. In a bioreactor assembled with activated sludge from a wastewater treatment plant, granule formation took place during a 4-week operation period, when the granules were nearly spherical and their size varied between 0.5-10 mm. During this time, we observed several changes in the properties of the sludge. The MLSS increased from 2.3 to 6.2 g/L, while the $SVI_5$ decreased from 219 to 32 mL/g, resulting in the settling velocity increasing from 0.2 to 53 m/h. During granule formation, the amount of EPS in the sludge significantly increased from 2.25 to 5.95 mg/g MLVSS, which embedded various microorganisms as demonstrated by SEM images.

1.2. We have found that up to a concentration of 35 mg/L GO NP, AGS SBR can effectively remove $NH_4$–N and COD from wastewater. The amount of EPS increased compared to the initial control amount when we added 15, 25, and 35 mg/L of GO NP to the bioreactor. Therefore, the effective removal of $NH_4$–N and COD can be attributed to the increased EPS content of the granulated sludge. In contrast, GO NP had a negative effect on EPS secretion in larger amounts (55, 75 and 95 mg/L), which resulted in a decrease in the nutrient removal capacity of the bioreactors and the biomass quantity. We also observed that the change in EPS affected the efficiency of $NH_4$–N removal, as the decrease in PN content resulted in an increase in ammonia concentration in the treated wastewater.

1.3. We observed that the changes were closely related to the biomass quantity as well. The initial biomass quantity was around $6.2 \pm 0.1$ g/L MLSS in all cases, but after dosing with 15, 25, and 35 mg/L GO NP, the biomass increased significantly (7.9, 7.6, and 7.02 g/L MLSS). However, further increases in the GO concentration reduced the biomass quantity and settling velocity of the bioreactors, while the $SVI_5$ increased. These observations demonstrate that the AGS wastewater treatment process is capable of efficient nutrient removal up to a concentration of 35 mg/L of GO nanoparticles, but when GO NP is applied in larger quantities ($\geq$55 mg/L), it has a significant negative impact on the bioreactor operation (decreasing biomass and EPS content, resulting in decreasing nutrient removal).

1.4. We demonstrated that the GO nanoparticles used in the concentration applied by us did not affect the removal efficiency of $NO_2$-N and $NO_3$-N.

1.5. We demonstrated that increasing the concentration of GO nanoparticles resulted in a significant reduction in the microbial community composition, with the greatest change in microbial composition observed at a concentration of 95 mg/L GO NPs.

**T2. We confirmed that the AGS SBR has excellent regenerative properties, as the nutrient removal efficiency of the bioreactor significantly improved after 14 days when the synthetic wastewater did not contain GO nanoparticles.**

2.1. We have proven that the AGS SBR has excellent regenerative capacity. After discontinuing GO dosing and allowing 14 days to elapse with synthetic wastewater not containing nanoparticles, the effluent concentrations of COD and $NH_4$-N, as well as the SOUR and SAOR, were the same as the initial phase measurements.

2.2. We have shown that the effluent nitrite- and nitrate-nitrogen concentrations did not change even after dosing the SWW with 115 mg/L GO NPs, and the SNRR and SNIRR did not change significantly in the granular sludge. This phenomenon can be explained by the structure of the granules and the microenvironment within them, which results in the anaerobic microorganisms being present in the inner part of the granules, thereby protecting the denitrifying microorganisms within the inner part of the granules.

2.3. We have found that increasing the concentration of GO NPs caused a significant decrease in SOUR and SAOR. This explains the increased concentrations of COD and $NH_4$-N in the effluent, typically observed when the SWW contained more than 55 mg/L of GO NPs. The results are consistent with changes in EPS content, as the sludge showed a decreasing trend in EPS content after the addition of 55 mg/L of GO NPs.

2.4. We have shown that the total phosphorus removal efficiency and the SPUR decreased even at 15 mg/L GO NP concentration, indicating that grapheme-oxide primarily hindered phosphorus uptake under aerobic conditions. The GO NP also negatively affected anaerobic phosphorus release from the sludge, but only above 55 mg/L, which also contributed to the decrease in phosphorus removal efficiency.

2.5. In the last experimental phase, when we stopped the GO dosing, the amount of EPS increased. The accumulated GO in the sludge was not able to exert further negative effects, and long-term exposure to the nanoparticles did not cause permanent damage to the EPS producing microbial community.

**T3. We first compared the chronic effects of G NP, GO NP, SWCNT, and MWCNT at environmentally relevant concentrations in the case of AGS SBR, and based on their effects, we determined the toxicity order of these carbon-based nanomaterials.**

3.1. We have shown that the G NP, GO NP, SWCNT and MWCNT nanomaterials at concentrations of 1, 5, and 10 mg/L did not affect the nitrogen removal efficiency of the AGS SBRs. The abundance of *Comamonas*, *Dyadobacter*, *Lysobacter*, *Gemmobacter*, and *Pseudoxanthomonas* genera responsible for removing nitrogen compounds varied differently in response to CBN dosing. This observation explains the unchanged nitrification and denitrification processes, as different bacteria with similar functions respond differently to treatment (while the abundance of one genus decreased, that of another increased), thus averaging out the negative effect.

3.2. We found that AGS has good tolerance, as nano-particles at a concentration of 1 mg/L did not negatively affect the bioreactor operation. This observation suggests that AGS is more tolerant to CBN exposure than AS, since in the literature, researchers have observed a decrease in nutrient removal efficiency of CAS bioreactors even after a dose of 1 mg/L CBN.

3.3. We demonstrated that GO NP and SWCNT at a concentration of 5 mg/L slightly increased the concentration of COD and $PO_4^{3-}$ in the effluent, while we did not observe the same effect after dosing with G NP and MWCNT at the same concentration. This suggests that GO and SWCNT nanomaterials have a more toxic effect on the microbial community in the AGS reactor than G and MWCNT. We did not observe any changes in the removal of nitrite and nitrate.

3.4. We found that increasing the amount of G and MWCNT (1, 5, and then 10 mg/L) caused a continuous increase in EPS production in the AGS. In contrast, the addition of GO NP and SWCNT at a concentration of 10 mg/L resulted in lower EPS production compared to when their concentration in the SWW was 5 mg/L. GO NP and SWCNT at a concentration of 10 mg/L had a stronger negative impact on those microorganisms that could have produced the appropriate amount of EPS for protection, resulting in higher nutrient concentrations in the effluent than in the case of G NP and MWCNT.

3.5. After the addition of CBNs, the *Zoogloea* and *Flavobacterium* genera, which are known EPS producers, increased in abundance, making them the most important and tolerant microorganisms in the granular sludge. The abundance of these genera was higher in the presence of the nanoparticles compared to the initial control, which explains the higher EPS content in the granular sludge compared to the initial sludge.

3.6. Based on the water chemistry results, we determined that among the nanomaterials, GO was the most toxic, while G caused the mildest negative effects. According to our results, the toxicity of the CBNs was as follows: G<MWCNT<SWCNT<GO. We can attribute the differences in their effects to their structural and qualitative properties. Graphene-oxide exhibited higher toxicity than graphene, which can be attributed to G's hydrophobic properties, making it prone to aggregation, thereby significantly reducing its specific surface area. The greater toxicity of SWCNTs compared to MWCNTs is due to the "nano-darts" effect, which can cause more severe damage to cell membranes.

## 4. PUBLICATIONS RELATED TO THE PRESENT THESIS

Hungarian Scientific Bibliography (MTMT) identifier: 10056885

**1. Chronic responses of aerobic granules to the presence of graphene oxide in sequencing batch reactors.**

**Alfonz Kedves**, Levente Sánta, Margit Balázs, Péter Kesserű, István Kiss, Andrea Rónavári, Zoltán Kónya.

*Journal of Hazardous Materials*, Volume 389, 5 May 2020.

DOI: 10.1016/j.jhazmat.2019.121905

$IF_{2020}$ = 10.39 Independent citations: 24

**2. Long-term effect of graphene oxide on the aerobic granular sludge wastewater treatment process.**

**Alfonz Kedves**, Andrea Rónavári, Zoltán Kónya.

*Journal of Environmental Chemical Engineering*, Volume 9, Issue 1, February 2021.

DOI: 10.1016/j.jece.2020.104853

$IF_{2021}$ = 7.49 Independent citations: 12

## 5. CONFERENCE PRESENTATIONS RELATED TO THE PRESENT THESIS

**1. Effect of graphene-oxide on aerobic granular sludge wastewater treatment.**

**Alfonz Kedves**, Balázs Buchholcz, Orsolya Kedves, Levente Sánta, Andrea Rónavári, János Halász, Zoltán Kónya.

*XIVth Edition of the Conference on the Environmental Sciences in the Carpathian Basin*, Gödöllő, 2018 (poster presentation).

**2. Insight into the impact of graphene-oxide (GO) nanoparticles on aerobic granular sludge (AGS) under shock loading.**

**<u>Alfonz Kedves</u>**, Balázs Buchholcz, Tamás Varga, Andrea Rónavári, Zoltán Kónya.

*8th Szeged International Workshop on Advances in Nanoscience*, Szeged, 2018 (poster presentation).

**3. Impact of graphene-oxide nanoparticles on aerobic granular sludge under shock loading.**

**<u>Alfonz Kedves</u>**, Balázs Buchholcz, Tamás Varga, Andrea Rónavári, Zoltán Kónya.

*Annual Meeting of the Hungarian Society for Microbiology and XIII. Fermentation Colloquium*, Eger, 2018 (poster presentation).

**4. The effect of graphene oxide on the aerobic granular sludge bioreactor performance: organic matter-, nitrogen-, and phosphorus removal, EPS secretion, and microbial community shifts.**

**<u>Alfonz Kedves</u>**, Andrea Rónavári, Margit Balázs, Péter Kesserű, István Kiss, Zoltán Kónya.

*XXIII. Spring Wind Conference ai and the Future of Science*, Budapest (online conference), 2020 (oral presentation).

**5. Response of aerobic granular sludge to the presence of carbon-based nanomaterials in sequencing batch reactors: nutrient removal, extracellular polymeric substances, and microbial population.**

**<u>Alfonz Kedves</u>**, Andrea Rónavári, Zoltán Kónya.

*Annual Meeting of the Hungarian Society for Microbiology and XIV. Fermentation Colloquium*, Kecskemét, 2020 (oral presentation).

## 6. OTHER PUBLICATION

**1. Chaetomium and Chaetomium-like Species from European Indoor Environments Include Dichotomopilus finlandicus sp. nov.**

Kedves Orsolya, Kocsubé Sándor, Bata Teodóra, Andersson Maria A., Salo Johanna M., Mikkola Raimo, Salonen Heidi, Szűcs Attila, **Kedves Alfonz**, Kónya Zoltán, Vágvölgyi Csaba, Magyar Donát, Kredics László.

Phatogens, Volume 10, Issue 9, September 2021.

DOI: 10.3390/pathogens10091133

$IF_{2021}$ = 4.41 Independent citations: 7

## 7. OTHER CONFERENCE PRESENTATIONS

**1. Microbial biocontrol of *Armillaria* root rot.**

Orsolya Kedves, Chen Liqiong, Bettina Bóka, **Alfonz Kedves**, Zoltán Kónya, Csaba Vágvölgyi, György Sipos, László Kredics.

*XXIII. Spring Wind Conference ai and the Future of Science*, Budapest (online conference), 2020 (oral presentation).

**2. Studies on the diversity and physiology of *Chaetomium* species isolated from closed environments.**

Orsolya Kedves, Sándor Kocsubé, Donát Magyar, Maria A. Andersson, Johanna M. Salo, Raimo Mikkola, Heidi Salonen, **Alfonz Kedves**, Zoltán Kónya, Csaba Vágvölgyi, László Kredics.

*Annual Meeting of the Hungarian Society for Microbiology and XIV. Fermentation Colloquium*, Kecskemét, 2020 (poster presentation).

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