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Dynamics of occurrence and metabolic activity of microorganisms involved in the removal of nutrients in urban sequential sedimentation-biofiltration systems

Dynamika występowania oraz aktywność metaboliczna mikroorganizmów zaangażowanych w usuwanie biogenów w miejskich sekwencyjnych systemach sedymentacyjno-biofiltracyjnych

PhD Thesis

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I. Introduction

1. Sequential Sedimentation Biofiltration-Systems (SSBSs) as nature-based solutions for the treatment of water

The expansion of urban settlements represents a constant threat for the contamination of water resources, such as lakes, rivers, and reservoirs (Nurk et al., 2005; Adyel et al., 2016). Point sources, such as effluents from wastewater treatment plants (WWTPs), municipal, and industrial discharges, are the most common. However, diffuse, non-point sources are of increasing concern in cities due to the more difficult control for pollutant load. Storm water runoff and snowmelts are the most important non-point sources that transport pollutants to water bodies, e.g.: plastics, fertilizers, faecal matter, pharmacy residues, heavy metals, etc., being also important sources for nutrient discharge (e.g.: NH_4^+, NO_2^-, NO_3^-, and PO_4^{3-}) (**Fig 1.a,b**). The above mentioned nitrogen and phosphorus compounds are toxic when they are accumulated in higher concentrations in freshwater bodies, and furthermore, they are crucial nutrients for the proliferation of **cyanobacterial harmful algal blooms (CyanoHABs; Fig 1.c,d)** (Zanchett and Oliveira-Filho 2013; Paerl *et al*., 2018; Gilbert *et al*., 2020). Cyanobacteria are associated to the production of several types of toxins that target the liver (hepatotoxins, such as microcystin, cylindrospermopsin, and nodularin), the nervous system (neurotoxins, such as anatoxin and saxitoxin), the skin (dermatotoxins), and other cell-tissues (cytotoxins and endotoxins) (Rzymski and Poniedziałek 2012; Paerl et al., 2018). Moreover, strong blooms significantly reduce the light entrance in the vertical profile of water, and promote anaerobic environments in pelagic and benthic zones (Gilbert et al., 2020). Therefore, CyanoHABs could not only affect drastically the aquatic ecosystems and human health, but also impact the economic sector with the inability to use water resources for energy production, water supplies, recreation, and transportation, among others (Wang et al., 2017; Codd et al., 2017; Paerl et al., 2018; Gilbert et al., 2020).

Due the above described problem, the **Sequential Sedimentation Biofiltration-Systems (SSBSs)** have been implemented in urban rivers that are located upstream water reservoirs, to aid in water treatment for the removal of pollutants, including nutrients (**Fig 1.e**) (Zalewski 2012). The idea of these systems was designed according to the **principles in Ecohydrology**, as biotechnological solutions that aim to increase the resilience of aquatic environments in urban areas (Zalewski 2012; Zalewski 2014). SSBSs are described as **multi-zone biofilters**, since their horizonal profile is composed of different sequential compartments that perform

independent functions for the treatment of water: (i) the *sedimentation zone* (SEDz) for the accumulation of large particles; (ii) the *geochemical zone* (GEOz) - constructed with a rock barrier containing dolomite or limestone - dedicated to the adsorption of phosphorus; (iii) the *biofiltration zone* (BIOz) containing a constructed wetland with different macrophyte species (e.g.: *Glycera maxima*, *Acorus calamus*, *Typha latifolia*, or *Phragmites australis*) devoted to the absorption of nutrients and heavy metals (Zalewski et al., 2012; Negussie et al., 2012; Szklarek et al., 2018; Jurczak et al., 2019); and (iv) the *denitrification zone* (DENz) with the addition of brown coal as a carbon substrate to enhance the microbial metabolic activity for the removal of nitrogen compounds (**Fig 1.f,g**) (Serwecinska et al., 2017). SSBSs are included to **nature-based solutions (NBS)**, since they use ecological processes that naturally occur in the environment to improve the nutrient transformations (Zalewski 2018). Furthermore, NBS are strongly recommended as cost-effective and environmental-friendly solutions to treat contaminated water by non-point sources in urban settlements, especially under unpredictable climate change scenarios where restoration of ecosystem services are needed (Krauze and Wagner et al., 2018).

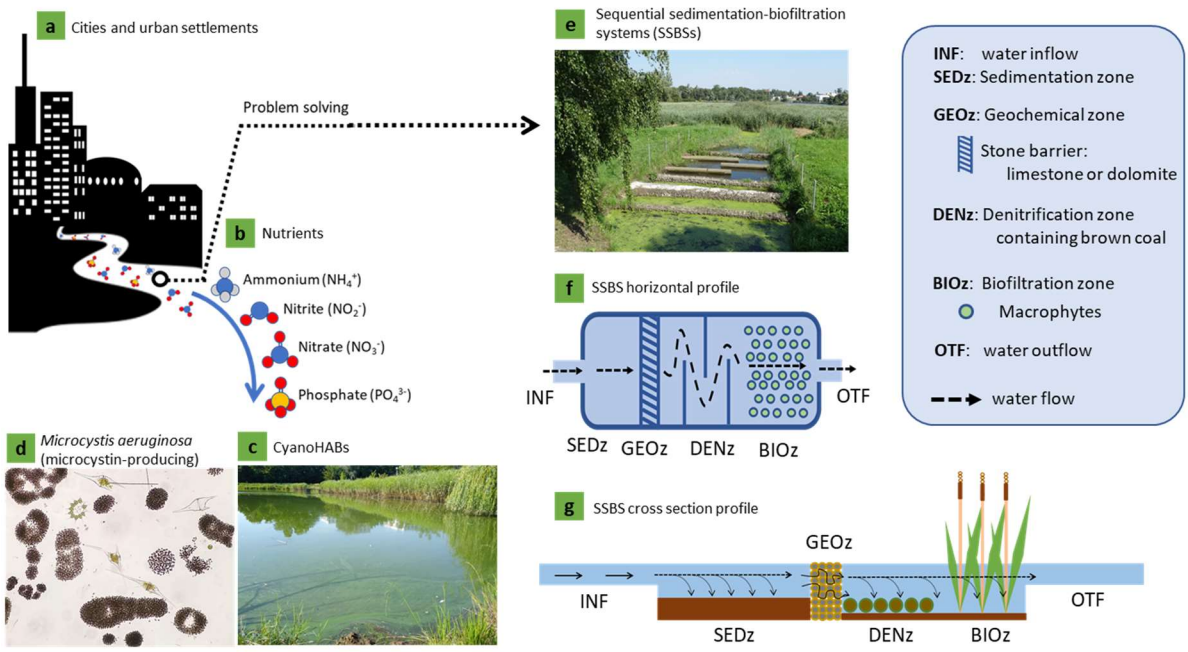

Fig 1. The threat from an urban river and applied nature-based solutions - Sequential Sedimentation-Biofiltration Systems (SSBSs).

Operation of SSBSs has been monitored in different systems constructed in urban rivers for the past decade. Their average nutrient removal efficiency has been observed up to 59.8% of NH_4^+ (Jurczak et al., 2019), 55% of NO_2^- (Zalewski et al., 2012), 91.3% of NO_3^-, 56.9% of total nitrogen (TN; Szklarek et al., 2018), 49% of PO_4^{3-}(Jurczak et al., 2019), and 93.0% of total phosphorus (TP; Szklarek et al., 2018). However, **there is no information on the quality, quantity, and activity of microbial communities involved in the transformations and (or) accumulation of the above mentioned nutrients for SSBSs.**

2. The role of microbial communities in the cycling of nutrients

Microbial communities, with special consideration on bacteria, are well known to have important ecological roles in the cycling of nutrients (nitrogen and phosphorus). Nitrogen transformations have been attributed to dynamics between nitrification-denitrification biochemical pathways, assimilation, under special conditions by anaerobic oxidation of NH_4^+ (ANAMMOX) (Faulwetter et al., 2009), and recently discovered by complete aerobic oxidation of NH_4^+ (COMAMMOX; Holger et al., 2015; Hu et al., 2017) (**Fig 2a**). The **nitrification process** usually involves the transformation of NH_4^+ to hydroxylamine (NH_2OH), which is accomplished by the enzyme ammonia monooxygenase (AMO) coded by the gene *amo*A (Rotthauwe et al., 1997), with subsequent oxidations to NO_2^- and NO_3^- (**Fig 2a**). The first nitrifying reaction is performed by ammonia oxidizing bacteria (AOB), with representatives from the genera *Nitrosomonas* spp., *Nitrosospira* spp., and *Nitrosococcus* (Kowalchuk et al., 2001; Guo et al., 2013), and the second reaction by nitrite oxidizing bacteria (NOB), with members of the genera *Nitrobacter* spp., *Nitrococcus* spp., and *Nitrospina* spp (Prosser 2005). All above strains are known to be aerobic autotrophic bacteria (Lukumbuzya et al., 2020), which are difficult to isolate, and therefore, have been studied using culture-independent methods (Rotthauwe et al,. 1997; Ibekwe et al., 2003; Dong and Reddy 2010). In contrast, the **denitrification process** involves at least four sequential transformations, where NO_3^- is usually reduced to the di-nitrogen gas (N_2) with the formation of different intermediates [NO_2^-, nitric oxide (NO), and nitrous oxide (N_2O) in order of reduction]. Many genes have been identified to code for the synthesis of the enzymes involved in the denitrifying process: periplastic and respiratory nitrate reductases (*nap*A and *nar*G), nitrite reductases (*nir*S and *nir*K), nitric oxide reductases (*cnor*B and *qnor*B), and nitrous oxide reductase (*nos*Z) in order of reduction (**Fig 2a**) (Faulwetter et al., 2009; Levy-Booth et al., 2014; Yang et al., 2020). Denitrifying bacterial communities are more diverse than nitrifying communities, with several

representatives of heterotrophic bacteria within the genera *Hyphomicrobium*, *Comamonas*, *Flavobacterium* (Repert et al., 2014; Palacin-Lizarbe et al., 2019; Ji et al., 2019), *Pseudomonas*, *Micrococcus*, *Bacillus*, *Aeromonas*, *Vibrio* (Heylen et al., 2006; Huang et al., 2011; Jones et al,. 2013), among others, and autotrophic bacteria such as *Thiobacillus* and *Thiomicrospira* (Chung et al., 2014). However, culture dependent-methods have limited the study from both of the above-mentioned bacterial communities, since most environmental bacteria do not grow in laboratory controlled experiments, and therefore may account for considerable underestimation from real community abundances (Torsvik and Ovreas 2002). Nowadays, it is preferable to study these bacterial communities using molecular markers (genes) that are associated to any of the particular nutrient transformations (Truu et al., 2009; Correa-Galeote et al., 2013; Banach-Wiśniewska et al., 2021).

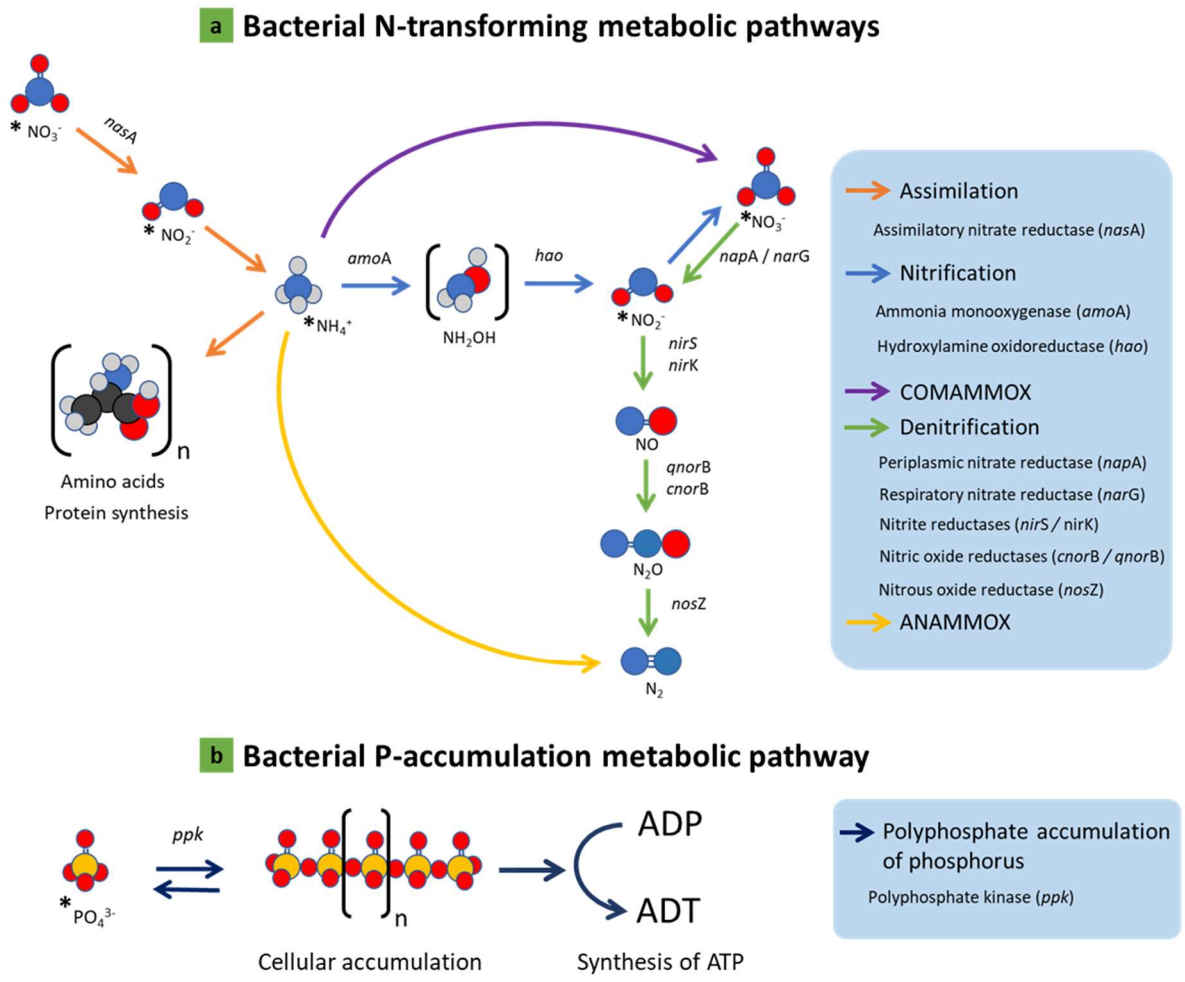

Fig 2. Bacterial nutrient transforming biochemical pathways. Genes involved in the coding of enzymes were written above / next to the arrow representing the specific biochemical reaction.

Although the biochemical processes for nitrifying/denitrifying pathways are well described, the dynamics between bacterial communities involved in these functions are still not well understood in natural and constructed wetlands (CWs), and in the case of multi-zone biofilters, these microbial communities have not been described in previous research. Seasonal studies have confirmed that warm periods present the highest rates of nutrient removal in CWs, which is largely associated to higher bacterial biomass. However, studies with functional gene abundances (*amo*A, *nar*G, *nir*S, and *nos*Z) have generally presented no significant correlation with seasonal variability, and in some cases genes were more abundant during cold periods (Chon et al., 2009; Chon and Cho et al., 2015; Huang et al., 2018). In a contrasting study from Sims et al., (2012) the gene *amo*A – representing **nitrifying bacterial communities** - was found with higher abundance during summer periods, positively confirming correlation with seasonal variability. Despite the above description, the gene *amo*A is a popular marker used to describe AOB nitrifying communities (family *Nitrosomonadaceae*), since they are known to perform an important role in nitrogen transformation for natural environments, and are usually dominant in freshwater sediments (Lukumbuzya et al., 2020). In studies concerning **denitrifying bacterial communities**, Huang et al., (2011) determined that gene *nos*Z abundance was attributed to higher total nitrogen concentrations in water and sediments from rivers, while Laverman et al., (2010) concluded the opposite. The individual abundance of genes in many different natural and CWs has also been described, generally resulting in higher gene copies for *nar*G and *nir*S from the denitrifying communities (Chon et al., 2009; Huang et al., 2011). New studies have re-designed molecular markers for the gene *nos*Z detection indicating that previous research may have sub-estimated the real abundance of these denitrifying communities. Nowadays, the gene *nos*Z is one of the most used molecular markers to study denitrifying communities, mainly because they are associated with the transformation of nitrous gas (a strong greenhouse gas) and completes the cycle of nitrogen back to the atmosphere as the non-harmful di-nitrogen gas (Ma et al., 2008; Jones et al., 2013).

Probably the great habitat diversity found in natural freshwater ecosystems, different designs in biofilters or CWs, and the variability in physico-chemical parameters pose a great challenge to study these communities. Furthermore, the previous literature contradicting many observations, regarding bacterial community descriptions using key functional genes, increases complicity in the elucidation of some conclusion; however some generalities can be observed. Natural freshwater ecosystems have shown similar dynamics for nitrifying and denitrifying

bacterial communities; however, though gene abundance might be higher in CWs, microbial diversity is generally higher in more natural environments (Bothe et al., 2000; Sims et al., 2012; Chon et al., 2009). In contrast, the greater gene abundances have been observed in natural areas that have been transformed into arable land (Jones et al., 2013) or paddy soils (Bannert et al, 2011) for agricultural purpose. Lower diversity in CWs has been partly attributed to the higher dominance and stability of AOB during operational times (Truu et al., 2009). Nitrifying communities have been largely dependent on ammonium load, which is largely obtained from urban wastewater in CWs (Paredes et al., 2007). On the contrary, denitrifying communities have been largely dependent on nitrate input and water residence time (Sirivedhin and Gray 2006; Truu et al., 2009).

In the specific case for bacteria involved in **phosphorus removal**, the research is focused in polyphosphate accumulating organisms (PAOs). These bacteria accumulate phosphorus from the environment as an inorganic polyphosphate chain (biological polymer containing many phosphorus residues) that eventually can be used to produce energy in the form of ATP (**Fig 2b**). The accumulation of polyphosphate in the bacterial cell is facilitated when environmental conditions change gradually between anaerobic and aerobic conditions. Many different bacteria have been described as PAOs, including *Escherichia coli*, *Pseudomonas, Neisseria, Acinetobacter* and Candidatus *Accumulibacter phosphatidis* (Kornberg et al., 1999; McMahon et al., 2002). Due to their capacity in phosphorus removal, the research is focused principally in WWTPs through the process known as enhanced biological phosphoral removal (EBPR) (Oehmen et al., 2007; Mielczarek et al., 2013). The polyphosphate kinase gene (*ppk*), responsible for the synthesis of polyphosphate from ADP, has been used as a molecular marker to study their dynamics (Maqbool *et al*., 2007; McMahon *et al.,* 2007; He et al., 2007; Zhang *et al*., 2016). However, all markers up to date have principally targeted bacterial communities from Candidatus *A. phophatidis*, which is the most dominant PAO in WWTPs. The *ppk* gene is known to be highly polymorphic, making it difficult to be used as marker to target universal communities of bacteria (Mehlig et al., 2009). In contrast to N-cycling bacterial communities, PAOs have been scarcely addressed in the research for freshwater ecosystems and biofilters (Alas et al., 2003; Edwards et al., 2006; Huang et al., 2011). Although CWs and SSBSs have also been designed to reduce phosphorus concentrations in water, this has been largely attributed to abiotic factors such as precipitation, absorption by sediments, molecular ion binding to calcium in limestone rocks and plant nutrient uptake, rather than bacterial absorption (Faulwetter et al., 2009; Jia et al., 2014).

3. Biotechnological potential of bacteria in the removal of nitrogen in contaminated water

Culturable bacteria with the ability to transform nitrogen compounds have been also principally isolated and characterized from waste water, sludge, and biofilms from bioreactors in WWTPs or scale experiments installed in laboratories. Most of the research is focused on the isolation of aerobic nitrifying-denitrifying bacteria, with several strains belonging to the genera: *Bacillus* (Rout et al., 2017), *Alcaligenes* (Wen et al., 2011), *Enterobacter* (Padhi et al., 2017), *Acinetobacter* (Zhao et al., 2010), *Klebsiella* (Pal et al., 2015), *Janthinobacterium* (Chen et al., 2019), and probably the most important genus is represented by *Pseudomonas* (Li et al., 2015; Sun et al., 2017; Zhang et al., 2019). Laboratory controlled experiments, with selective media (nitrifying, denitrifying, and simultaneous nitrifying-denitrifying media) have been particularly important to describe the kinetics on their dynamics for the transformation of nitrogen compounds (NH_4^+, NO_2^-, and NO_3^- among others). The above experiments have allowed to corroborate and study the biochemical pathways of N-cycling processes. Moreover, bacterial strains with the highest rates of nitrogen transformation have been selected as candidates with potential applications to increase the efficiency in nutrient removal in contaminated water. Several strains have been already proposed in the literature with high transformation rates for $N\text{-}NH_4$ and $N\text{-}NO_3$, e.g.: *Klebsiella pneumoniae* CF-S9 (3.3 and 2.6 mg L^{-1} h^{-1}, respectively; Pahdi et al., 2013), *Pseudomonas tolaasii* Y-11 (2.13 and 0.52 mg L^{-1} h^{-1}, respectively; He et al., 2016), *Bacillus cereus* GS-5 (2.69 and 2.94 mg L^{-1} h^{-1}, respectively; Rout et al., 2017), and *Janthinobacterium svalbardensis* F19 (0.62 and 0.52 mg L^{-1} h^{-1}, respectively; Chen et al., 2019).

4. Importance of the present study

Although the current state of knowledge in nutrient transformations by bacterial communities has grown in the last decade, it is still limited to complete our understanding in the elucidation of their dynamics. At the same time, the great majority of research has been focused in CWs that provide tertiary treatment to effluents in WWTP. Therefore, research should also be focused on biofilters directly constructed in natural freshwater ecosystems (such as rivers or stream courses) for the direct treatment of polluted urban rivers. **Thus, the present study contributed in our understanding of cause-effect relationships between the bacterial communities and specific parameters involving seasonal variation, SSBSs design, physic-**

chemical parameters, and nutrient composition in three biofilters constructed along the natural course of three rivers contaminated with urban wastewaters. This was the first time that microbial communities were characterized in SSBSs. The results allowed to identify particular conditions within the horizontal structure of SSBSs that are important to enhance the abundance of such communities. Moreover, isolated bacterial strains were identified to perform an important role in nitrogen transformation processes, and therefore they could be proposed as candidates with potential biotechnological application to future NBS. These results allowed to suggest better planning on the construction of SSBSs in order to increase their overall efficiency in nutrient removal.

II. Hypotheses

The present work was divided in two main chapters. The **first chapter** is dedicated to describe the dynamics of microbial communities in SSBSs that are involved in nutrient transformation pathways, and their sensitivity to environmental parameters marked by the periodical change in season and the design of the systems. The **second chapter** is dedicated to the isolation of bacterial strains that are involved in nitrogen transforming processes, their kinetic activity in N-transformation and genetic characterization. Therefore, the following hypothesis were defined:

1. *Dynamics of microbial metabolic activity and the abundance of nutrient-transforming bacteria in SSBSs are subject to change according to specific environmental conditions observed through the seasons and the different designs in the SSBSs multi-complex structure.*
2. *Culturable heterotrophic bacteria isolated from SSBSs have biotechnological potential to improve the efficiency in nutrient removal.*

III. Objectives

The previous hypotheses were investigated with the definition of the following objectives:

1. Monitor the environmental conditions, nutrient concentrations, and removal efficiency in the horizontal profile of three SSBSs.
2. Describe the heterotrophic microbial metabolic activity with the use of community level physiological profile (CLPP) approach.
3. Analyse the quantity of key functional genes involved in bacterial processes of nitrogen transformation (nitrification and denitrification).
4. Elucidate bacterial communities and estimate their relative abundance in biofilm formations with the use of high-throughput sequencing of the gene 16S rRNA.
5. Evaluate the relationship between the abundance of key functional genes and the relative abundance of bacterial communities with the different environmental conditions in the horizontal structure of SSBSs, and the change in the season.
6. Isolate potential N-transforming bacteria from SSBSs using selective media.
7. Characterize the genetic and kinetic activity of N-transforming pathways for selected bacteria.

IV. Publications

Publications constituting the doctoral dissertation

In accordance with the Law on Higher Education and Science of 20 July 2018 Article 187(3) this doctoral dissertation consists of a set of published and related scientific articles:

Article 1: Font Nájera A., Serwecinska L., Szklarek S., Mankiewicz-Boczek J. 2020. *Characterization and comparison of microbial communities in sequential sedimentation-biofiltration systems for removal of nutrients in urban rivers*. Ecological Engineering, 149, 105796, doi.org/10.1016/j.ecoleng.2020.105796 (**Appendix 1**).

Impact Factor: 3.512

5-Year Impact Factor: 3.844

List of journals of the MNiSW: 100 points

Article 2: Font Nájera A., Serwecinska L., Szklarek S., Mankiewicz-Boczek J. 2021a. *Seasonal and spatial changes of N-transforming microbial communities in sequential sedimentation-biofiltration systems - Influence of system design and environmental conditions.* International Biodeterioration & Biodegradation, 159, 105203, doi.org/10.1016/j.ibiod.2021.105203 (**Appendix 2**).

Impact Factor: 4.074

5-Year Impact Factor: 4.046

List of journals of the MNiSW: 140 points

Article 3: Font Nájera A., Serwecinska L., Mankiewicz-Boczek J. 2021b. *Culturable nitrogen-transforming bacteria from sequential sedimentation biofiltration systems and their potential for nutrient removal in urban polluted rivers.* Scientific Reports, 11, 7448, doi.org/10.1038/s41598-021-86212-3 (**Appendix 3**).

Impact Factor: 3.998

5-Year Impact Factor: 4.576

List of journals of the MNiSW: 140 points

Supplementary material 1: *Characterization and comparison of microbial communities in sequential sedimentation-biofiltration systems for removal of nutrients in urban rivers.* doi.org/10.1016/j.ecoleng.2020.105796, (**Appendix 4**).

Supplementary material 2: *Seasonal and spatial changes of N-transforming microbial communities in sequential sedimentation-biofiltration systems - Influence of system design and environmental conditions.* doi.org/10.1016/j.ibiod.2021.105203, (**Appendix 5**).

Supplementary material 3: *Culturable nitrogen-transforming bacteria from sequential sedimentation biofiltration systems and their potential for nutrient removal in urban polluted rivers.* doi.org/10.1038/s41598-021-86212-3, (**Appendix 6**).

V. Materials and methods

1. Study site

The present study was performed in three Sequential Sedimentation-Biofiltration Systems (SSBSs) established for the treatment of urban storm water (**Fig 3**):

a. Sokołówka SSBS (Sok-SSBS): located along the course of Sokołówka River at the park Adam Mickiewicz in the city of Lodz. The SSBS has a 65x16 m dimension, comprising a total area of 0.00104 km^2. It principally treats storm water originating from a 5.72 Km^2 draining catchment located above the biofilter, which is comprised of high-density residential areas with impermeable soil. It was constructed in 2011 as part of the SWITCH project (6 FP EU, GOCE 018530) in close cooperation with the Municipality of Lodz. The biofilter is part of a greater number of reservoirs that were constructed to treat the storm water, in order to provide better water quality for more attractive recreational zones. This SSBS contains the SEDz, GEOz – containing limestone as rock barrier - and BIOz (**Fig 3a**) (see chapter 2.1 in **Appendix 1:** Font Nájera et al., 2020; and **Appendix 2:** Font Nájera et al., 2021a).

b. Bzura SSBS (Bzura SSBS): is the upper reservoir within the course of the river Bzura at the park Arturówek in the city of Lodz. The SSBS has maximal capacity of 10 000 m^3 of water in an area of 1.08 ha. It is also part of a series of reservoirs constructed to treat storm water originating from an upper catchment comprised of an urban area. It was constructed in 2013 as part of the ER-REK project for the revitalization of recreational areas in the same park (Szulc et al., 2015). This SSBS contains the SEDz, GEOz and BIOz, which operate very similar to the Sokołówka SSBS; including four principal modifications: (a) the reservoir was constructed in the original course of the river, (b) the GEOz rock barrier is constructed with dolomite, (c) the BIOz is subdivided in a wetland containing floating algae followed by macrophytes, (d) the SSBS dimensions are larger than the other two systems (**Fig 3b**) (**Appendix 2:** Font Nájera et al., 2021a; see chapter 2.1).

c. Struga Gnieźnieńska SSBS (Str-SSBS): is located within the course of the river Struga Gnieźnieńska in the city of Gniezno. The SSBS was constructed during the year 2016. It is also part from a sequence of reservoirs constructed to control CyanoHABs in subsequent reservoirs used for urban recreational areas (Lake Jelonek). This SSBS contains the SEDz, GEOz - containing limestone as rock barrier -, DENz – containing brown coal -, and BIOz, which operate very similar to the Sokołówka BSS (Fig. 1), with the exception from the

DENz. The Str-SSBS was also constructed in the original course of the river (**Fig 3c**) (see chapter 2.1 in **Appendix 1:** Font Nájera et al., 2020; and **Appendix 2:** Font Nájera et al., 2021a).

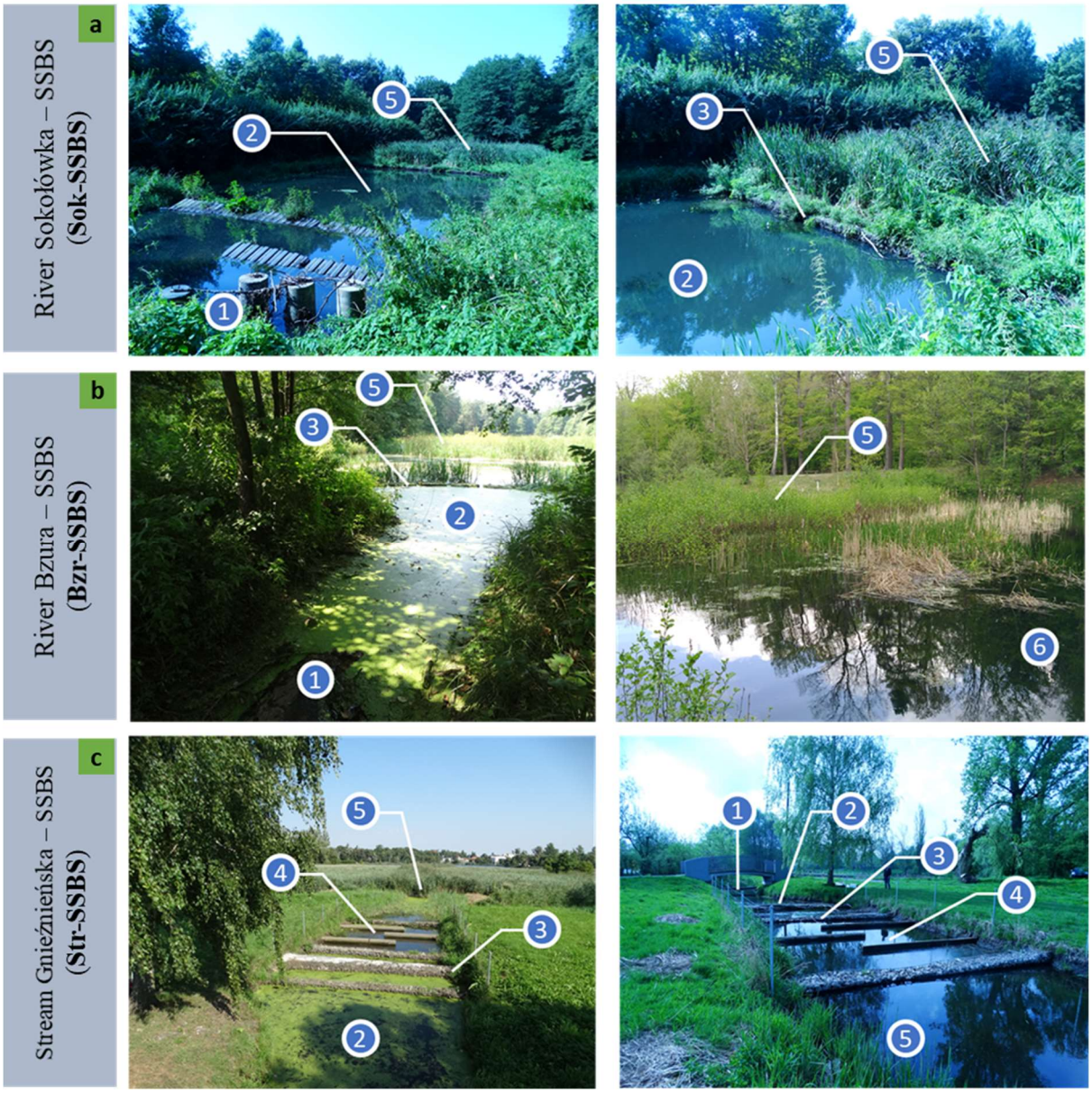

Fig 3. Description of horizontal profiles of SSBSs. (1) INF - inflow, (2) SEDz,- sedimentation zone (3) GEOz,- geochemical zone, (4) DENz – denitrification zone, (only constructed in Str-SSBSs), (5) BIOz – biofiltration zone, and (6) OTF - outflow. Pictures were taken by Arnoldo Font Nájera.

2. Sample collection

Sediment and surface water samples were aseptically collected along the horizontal profile of the three SSBSs, starting from the OFT, and continuing upstream in the BIOz, SEDz, and the INF. In the case of GEOz and DENz (constructed only for Str-SSBS), biofilm from limestone, dolomite, or brown coal were collected. Five different periods were collected for each SSBS, with each period representing a different season during the years 2017 and 2018:

a. Summer 2017: the 2nd of August for Sok-SSBS and Bzr-SSBS, and the 3rd for Str-SSBS (**Appendix 1:** Font Nájera et al., 2020; see chapter 2.2),
b. Autumn 2017: the 19th of October for Sok-SSBS and Bzr-SSBS, and the 24th for Str-SSBS (**Appendix 1:** Font Nájera et al., 2020; see chapter 2.2),
c. Spring 2018: the 25th of April for Str-SSBS, and the 26th for Sok-SSBS and Bzr-SSBS (**Appendix 2:** Font Nájera et al., 2021a; see chapter 2.2),
d. Summer 2018: 2nd of August for Str-SSBS, and the 3rd for Sok-SSBS and Bzr-SSBS (**Appendix 2:** Font Nájera et al., 2021a, see chapter 2.2; **Appendix 3:** Font Nájera et al., 2021b, see page 9),
e. Autumn 2018: 6th of November for Str-SSBS, and the 7th for Sok-SSBS and Bzr-SSBS (**Appendix 2:** Font Nájera et al., 2021a; see chapter 2.2).

Sediment and surface water samples were stored in cold (4°C) and dark conditions during transportation to the laboratory.

Further activities, including determination of *in situ* environmental parameters, chemical and biological analyses were done for each of the five sampling seasons as summarized in **Fig 4**.

3. Environmental parameters, nutrient concentrations, and nutrient removal efficiencies

Physico-chemical parameters [Temperature (T), Dissolved Oxygen (DO) and pH] were measured *in situ* for surface water using a YSI multiparametric probe.

Chemical analysis of surface water and interstitial water samples was performed with ion chromatography for NO_3^-, NO_2^-, NH_4^+, and PO_4^{3-}. Removal efficiencies of SSBSs were estimated with the average differences between the nutrient concentrations at INFs and OTFs (see chapter 2.3 in **Appendix 1:** Font Nájera et al., 2020; and **Appendix 2:** Font Nájera et al., 2021a).

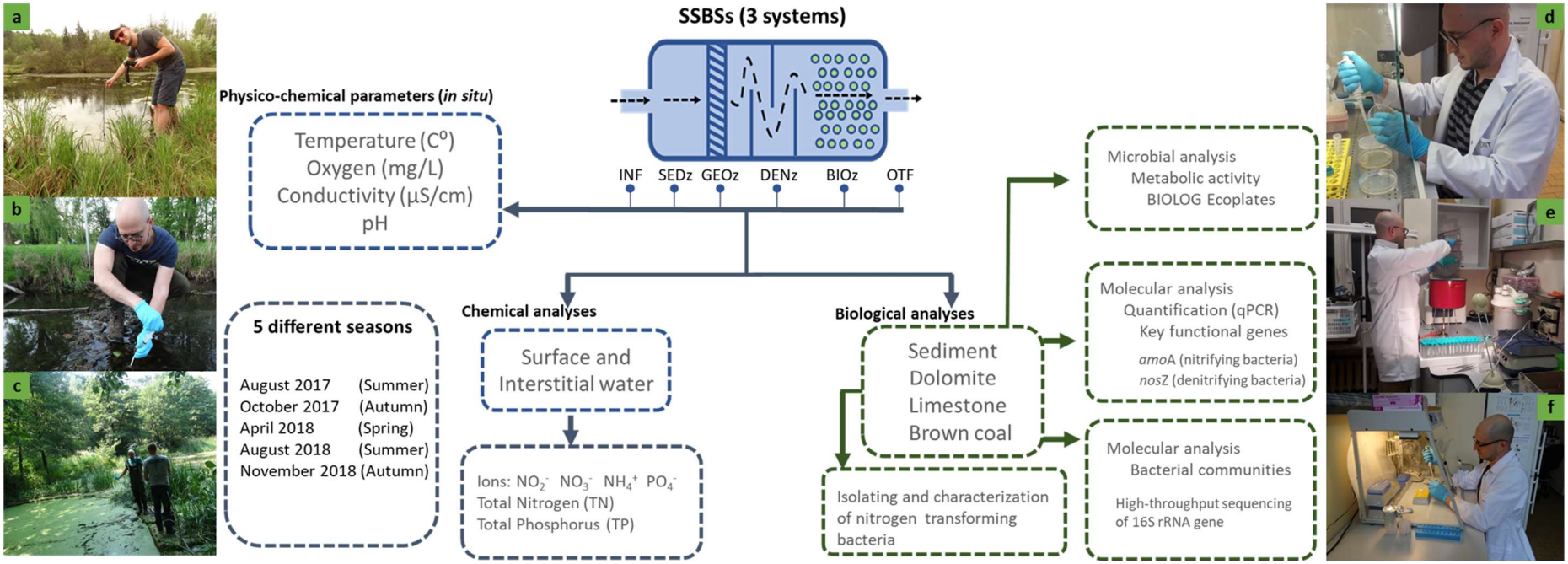

Fig 4. Summary of field and laboratory activities performed in the present study. Measurement of physico-chemical parameters in surface water (a), collection and preparation of samples (b and c), isolation of bacteria from sediments (d), screening assays to identify nitrogen transforming bacteria, and (e) preparation of PCR for quantification of key functional genes of nitrifying and denitrifying bacteria.

4. Microbial metabolic activity using the community level physiological profile (CLPP) approach

CLPP was performed for sediment samples (INF, SEDz, BIOz, and OTF) and biofilm in limestone / dolomite barriers (GEOz), at different time intervals through the specifications of BIOLOG Ecoplates^{TM} for environmental testing. Dynamics on seasonal and spatial variations in microbial communities was assessed through the calculation of average well colour development (AWCD), the functional diversity R (Richness), and the homogeneity H (Shannon-Weiver index). The Ecoplates, containing 96 wells with 31 different carbon sources in triplicate, were measured with a Biolog Microstation ELX808BLG reader analyser. The microbial metabolic profile was measured as the intensity of colour appearance from the reduction of tetrazodium dye for particular carbon sources. This method allowed to estimate the catabolic abilities of total microbial communities and their functional diversity in environmental samples (see chapter 2.4 in **Appendix 1:** Font Nájera et al., 2020; and **Appendix 2:** Font Nájera et al., 2021a).

5. Molecular analysis for the description of nutrient transforming bacterial communities

DNA was isolated from SSBS's sediment, limestone, dolomite, and brown coal samples using the fast DNA spin kit for soil (BIO 101/Q-Biogene), following the manufacturer´s instructions (see section 2.5 in **Appendix 1:** Font Nájera et al., 2020; and **Appendix 2:** Font Nájera et al., 2021a). **Quantitative real time PCR (qPCR)** was performed for two bacterial functional genes involved in nitrification and denitrification processes (*amo*A and *nos*Z, respectively), and the universal 16S rRNA marker for eubacteria. Different standard curves were prepared for each of the selected genes, which were used to calculate the copy number of each gene in the DNA environmental samples (number of gene copies g^{-1} in dry sediment). For the specific case of the genes *amo*A and *nos*Z, the standard curves were prepared with linearized plasmids (digested) obtained from clones containing a purified PCR environmental product (**Appendix 1:** Font Nájera et al., 2020, see section 2.6; **Appendix 2:** Font Nájera et al., 2021a, see section 2.5). Quantification of total eubacteria community was performed through the 16S rRNA gene. Ratios of abundance from both key functional genes were measured according to the total gene abundance calculated for the 16S rRNA gene. Moreover, the relationship between the copy number of key functional genes, environmental parameters, and nutrient concentrations were

assessed through the construction of principal component analyses (PCAs). Significant differences between SSBSs samples in the PCA were assessed through one way ANOVA and the Tukey's honesty significant difference (HSD) tests (**Appendix 2:** Font Nájera et al., 2021a; see section 2.5 and 2.7).

A **bacterial community analysis** was performed to describe microbial biofilm formations on limestone (GEOz) and brown coal (DENz), using high-throughput sequencing for the 16S rRNA (region V3-V4) as a molecular marker. The analysis was focused on biofilm formations, since these zones usually obtained the highest abundances of key functional genes (*amo*A and *nos*Z), and total eubacteria (16S rRNA). Sequencing was performed in "BionanoPark" (Lodz, Poland), with the specifications from the BaseSPace app 16S Metagenomics (Illumina, San Diego California, USA). Relationships between the relative abundance of bacterial taxa (OTUs), the environmental factors, and nutrient concentrations were evaluated in the construction of a PCA (**Appendix 2:** Font Nájera et al., 2021a; see section 2.6 and 2.7).

6. Isolation and screening of nitrogen-transforming bacteria

Up to 50 strains were isolated from sediments collected at each of the three SSBSs (August 2018), using Soil Extract Agar (SEA) solid medium. Each strain was tested for its ability to transform nitrogen compounds using selective media with high concentrations of NO_3^- or NH_4^+. The Giltay medium (GiDM), containing 277 mg L^{-1} of NO_3^-, was used to select potential denitrifying strains. Only 10 strains were able to completely or partially transform NO_3^- in GiDM, and therefore were selected to test their ability for the transformation of NH_4^+ (212 mg L^{-1}) in glucose nitrifying medium (GNM). The strains were properly named after each SSBS where they were isolated: six strains for Sok-SSBS (Sok01, Sok03, Sok05, Sok06, Sok20, and Sok41), two for Bzr-SSBS (Bzr02 and Bzr07), and two for Str-SSBS (Str01 and Str21). The effect of different carbon sources was also tested replacing glucose from the original GNM medium with sodium succinate (SNM), sodium acetate (ANM), or sodium citrate (CNM). The C:N ratio (10) was always kept constant for all above described media. Additionally, the selected strains were tested for the transformation of NH_4^+ under the presence of hydroxylamine (NH_2OH), a toxic intermediary normally occurring in the nitrification process. Strains Bzr02 and Str21 were selected for further experiments (**Appendix 3:** Font Nájera et al., 2021b; see page 9).

7. Molecular characterization of selected bacteria

Genomic DNA was isolated from the 10 selected nitrogen-transforming bacteria using the specifications in the Wizard Genomic DNA purification kit (Promega, Madison, Wisconsin). Bacteria were first identified using the BLAST nucleotide software after amplification and sequencing of the gene 16S rRNA. A neighbour-joining tree was constructed for the 16S rRNA gene using the MEGA7 software to visualize phylogenetic distance between selected strains. Bacteria were also tested for the presence of the genes involved in assimilation of nitrogen – conventional PCR - (*nas*A, assimilatory nitrate reductase), nitrification (*hao*, hydroxylamine oxidoreductase), and denitrification (*nap*A, *nar*G, *nir*S, *nor*B, and *nos*Z), in order to corroborate their biochemical processes in nitrogen transformation. The 16S rRNA gene was sequenced for all 10 strains, and the functional genes for Bzr02 and Str21 using Sanger sequencing method at the "Genomed S.A." laboratories in Warsaw, Poland (**Appendix 3:** Font Nájera et al., 2021b; see page 9).

8. Kinetic dynamics of strains Bzr02 and Str21 in selected nitrogen-transforming media

Both strains were inoculated (at OD_{690} 0.1) and incubated independently in flasks containing nitrifying (NM), denitrifying (DN), and simultaneous nitrifying-denitrifying medium (SNDM). The content of $N\text{-}NH_4$ in NM, and $N\text{-}NO_3$ in DM, were adjusted to 100 mg L^{-1} for each medium. The contents of $N\text{-}NH_4$ and $N\text{-}NO_3$ were adjusted to 50 mg L^{-1} each, to obtain a total content of nitrogen at 100 mg L^{-1} in SNDM. Glucose was added as carbon source in all media with a total content of carbon at 1000 mg L^{-1} (C:N = 10 in all media). All experiments were conducted over a period of 36 hours, collecting samples every 2-4 hours to measure the concentration of nitrogen sources according to the specifications in the standard methods: $N\text{-}NH_4$ by the Nessler's colorimetric assay, $N\text{-}NO_3$ by the ultraviolet spectrophotometric method, and $N\text{-}NO_2$ by the Griess colorimetric assay (**Appendix 3:** Font Nájera et al., 2021b; see page 10).

The Bzr02 was the only strain able to utilize NH_4^+ in the presence of hydroxylamine during the screening tests, and therefore the kinetic dynamics were followed in a controlled experiment similarly as explained for the above described media. Four different flasks were inoculated with Bzr02 in NM media and after 4 hours of incubation each flask was spiked with different concentrations of NH_2OH (0, 10, 20, and 50 mg L^{-1} as final concentration, respectively).

Samples were collected every 4-6 hours, and hydroxylamine concentration was measured by indirect spectrophotometry method (**Appendix 3:** Font Nájera et al., 2021b; see page 10).

For each bacterial assay, nitrogen balance (assimilated and lost into gaseous forms) and removal nitrogen rates (average and maximal) were estimated in triplicate (**Appendix 3:** Font Nájera et al., 2021b; see page 10).

VI. Discussion of most important results

1. Monitoring nutrient removal efficiencies in SSBSs

All three SSBSs were efficient in nutrient removal, however some differences were possible to observe according to their design and time of operation (see **Table 1**).

Table 1. Summary of nutrient removal efficiencies in SSBSs

System	Year	$N\text{-}NO_2$	$N\text{-}NO_3$	$N\text{-}NH_4$	TN	$P\text{-}PO_4$	TP
Sok-SSBS	2017	55.0%	46.0%	**NR**	**NR**	NM	93.0%
	2018	NM	42.9%	**NR**	47.0%	59.0%	64.7%
Bzr-SSBS	2017	NM	NM	NM	NM	NM	NM
	2018	NM	62.5%	71.4%	33.7%	63.0%	65.2%
Str-SSBS	2017	27.0%	30.0%	48.0%	32.0%	NM	36.0%
	2018	NM	63.30%	73.80%	32.90%	84.90%	72.50%

The values represent the average percentual efficiency calculated from two monitoring periods for 2017 (August and October; **Appendix 1**: Font Nájera et al., 2020; see chapter 3.1 in results), and three for 2018 (April, August, and November; **Appendix 2**: Font Nájera et al., 2021a; see chapter 3.1 in results); NM - not measured, NR - no reduction.

The above results indicated that the youngest system - Str-SSBS - presented the best removal efficiencies ($N\text{-}NO_3$, $N\text{-}NH_4$ and $P\text{-}PO_4$) which may be caused, among other things, by the construction of the DENz, a zone devoted to increase the microbial metabolic activity in the transformation of nutrients. Only the Sok-SSBS presented a problem to reduce the concentration of $N\text{-}NH_4$ for both monitoring years. Due to these observations, the implementation of DENz was suggested as an important zone to increase SSBS efficiencies in the overall removal of nitrogen compounds (**Appendix 1**: Font Nájera et al., 2020, see chapter 4.1 in discussion**; Appendix 2**: Font Nájera et al., 2021a, see chapter 3.1 in results).

2. Monitoring microbial metabolic activity in SSBSs

According to the season, microbial metabolic activity in SSBSs was found to be significantly higher in spring and summer for Sok-SSBS (up to 1.86 and 2.07, respectively), Bzr-SSBS (up to 1.65 and 1.81, respectively), and Str-SSBS (up to 1.99 and 2.05, respectively) when compared to autumn (up to 1.28, 1.27, and 1.14, respectively). These results suggested that microbial communities in sediments are more active during warmer periods in spring (14.2 ± 2.5 °C) and summer (21.9 ± 1.9 °C), when compared to colder periods in autumn (11.2 ± 2.2 °C). Similar results have also been reported in other seasonal studies performed in sediments from the river continuum profile (Freixa et al., 2016; Oest et al., 2018) and CWs (Chazarenc et al., 2010) (**Appendix 2**: Font Nájera et al., 2021a, see chapter 4.1 in discussion). However, the significant lower AWCD values observed for Bzr-SSBS in spring (1.65) and summer (1.81), when compared to the other two SSBSs, suggested that the size of the system was an important factor affecting the metabolic activity of heterotrophic microbial communities in sediments. The Bzr-SSBS – with 2600 m^2 of surface area – was observed to have a decreasing content of organic matter in sediments along the horizontal profile, from inflow to outflow direction, while in Sok-SSBS (1040 m^2) and Str-SSBS (119 m^2) the organic matter increased in the same direction. These results showed that the Bzr-SSBS was more efficient in sedimentation process, therefore affecting (decreasing) the metabolic activity of these microbial communities along its horizontal profile (**Fig 5**). A study performed in sediments from CWs also confirmed that the decrease of microbial metabolic activity was related to the progressive decrease of total organic carbon from inflow to outflow direction (Button et al., 2015) (**Appendix 2**: Font Nájera et al., 2021a, see chapter 4.2 in discussion).

According to the horizontal structure of SSBSs, the GEOz – containing limestone as rock barrier - presented the lowest microbial metabolic activity for Sok-SSBS and Str-SSBS (both with AWCD below 1.0) during October 2017, when compared to SEDz and BIOz (both between 1.6 – 2.0) (**Fig 5**). These results indicated that communities in limestone biofilms were not particularly heterotrophic, and therefore it was suggested the future implementation of biopolymers carrying additional carbon substrates, in order to promote their attachment and increase their activity (**Appendix 1**: Font Nájera et al., 2020; see chapter 5). Moreover, in the seasonal monitoring of 2018, the Bzr-SSBS was included in the analysis and it was observed that GEOz containing dolomite presented significantly lower microbial metabolic activity (0.10 or less) when compared to systems containing limestone (between 0.16 to 1.08) (**Fig 5**). These observations suggested that the surface of limestone rocks was less uniform and more

porous than dolomite (Weyl, 1960), allowing the better attachment and development of microbial biofilm. The above results recommend future adaptation or construction of GEOz containing limestone rock, in order to increase the attachment of mentioned communities (**Appendix 2**: Font Nájera et al., 2021a; see chapter 4.2.1 in discussion).

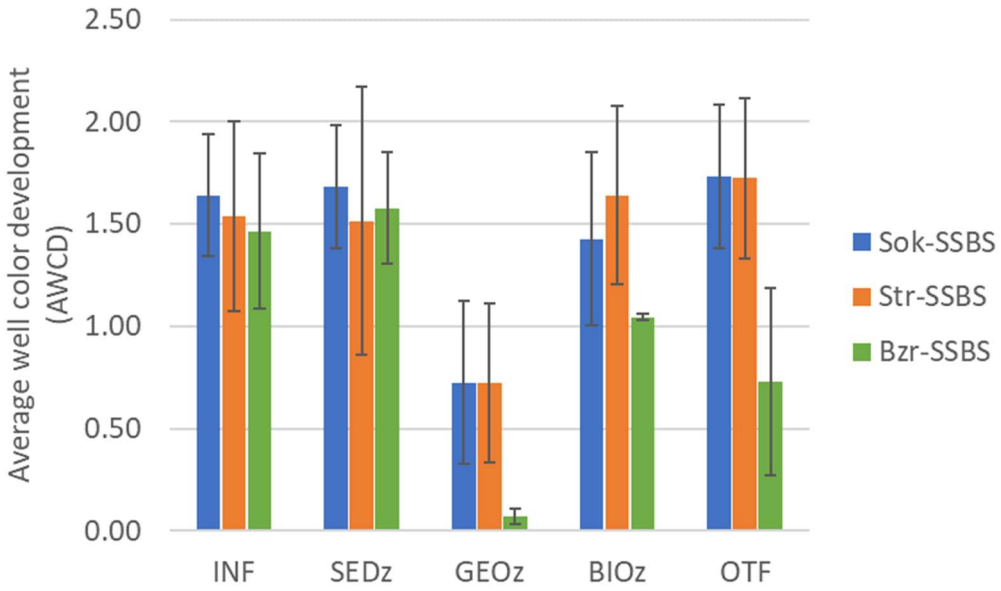

Fig 5. Microbial metabolic activity in SSBSs. AWCD values represent the average from different seasons of collection, and the error bars represent the standard deviation. Sok-SSBS (n=4), Bzr-SSBS (n=3), and Str-SSBS (n=4).

3. Monitoring nutrient-transforming bacterial communities in SSBS horizontal profiles

Nitrifying bacterial communities, described with the abundance of the gene *amo*A, were observed to be more abundant in spring for Sok-SSBS, Bzr-SSBS, and Str-SSBS (up to 8.95 x 10^7, 2.09 x 10^6, and 5.73 x 10^7 gene copy number per gram of dry sediment [g^{-1}], respectively), when compared to summer (up to 1.50 x 10^5, 6.54 x 10^5, and 1.56 x 10^5 gene copy numbers per g^{-1}, respectively) and autumn (up to 9.12 x 10^5, 8.63 x 10^5, 1.64 x 10^5 gene copy numbers per g^{-1}, respectively). A significant negative correlation was obtained between the *amo*A gene copy numbers and the temperature (r^2 = -0.49, p = 0.072 for Sok-SSBS, and r^2 = -0.48, p = 0.069 for Str-SSBS, both with $\alpha = 0.90$), indicating that these communities are influenced by temporal season trends. The above described seasonal trends of nitrifying bacterial communities were similar to studies performed in sediment of rivers (Repert et al., 2014) and lakes (Wang et al., 2019) (**Appendix 2**: Font Nájera et al., 2021a; see chapters 3.3 and 3.4 in results, and 4.1 in discussion). The highest abundances of nitrifiers were observed in GEOz

containing biofilm formation over the limestone (gene copy numbers per g^{-1} up to 8.95 x 10^7 for Sok-SSBS and 5.73 x 10^7 for Str-SSBS), which was also significantly higher than barriers containing dolomite (4.51 x 10^2 for Bzr-SSBS) (**Fig 6**). The ratio *amo*A/16S rRNA (up to 31.1%) allowed to identify a significant portion of these communities developed in biofilms formed over the limestone rocks during spring, indicating that nitrifying bacteria developed better in the porous structure of limestone and favoured conditions with temperate temperature (14.2 ± 2.5 °C) (**Appendix 2**: Font Nájera et al., 2021a; see chapters 3.3 in results, and 4.2.1 in discussion). Moreover, positive significant correlations were observed between the concentration of N-NH_4 and the gene copy numbers of *amo*A, suggesting that these communities were active during spring (**Appendix 2**: Font Nájera et al., 2021a; see chapters 3.4 in results, and 4.1 in discussion). The above results allowed to suggest the future construction of SSBSs with rock barriers containing limestone, rather than dolomite, in order to increase the biofilm formation and the activity of nitrifying communities.

Denitrifying bacterial communities, described with the abundance of the gene *nos*Z, were observed to be more abundant in summer for Sok-SSBS, Bzr-SSBs, and Str-SSBS (up to 2.42 x 10^9, 7.65 x 10^7, and 2.02 x 10^{10} gene copy numbers per g^{-1}, respectively) when compared to spring (2.84 x 10^6, 6.62 x 10^5, and 1.34 x 10^6 gene copy numbers per g^{-1}, respectively) and autumn (5.29 x 10^8, 3.97 x 10^7, and 1.49 x 10^8, respectively). A significant positive correlation was obtained between the *nos*Z gene copy numbers and the temperature (r^2 = 0.48, p = 0.071 for Sok-SSBS, and r^2 = 0.52, p = 0.056 for Str-SSBS, both with α = 0.90), indicating that these communities are influenced by temporal season trends. The similar results have been observed for studies performed in sediment of rivers (Huang et al., 2018) and estuaries (Chon et al., 2011) (**Appendix 2**: Font Nájera et al., 2021a; see chapter 3.3 and 3.4 in results, and 4.1 in discussion). The highest abundance of denitrifiers was observed in biofilm developed over brown coal added in the Str-SSBS DENz during summer in 2017 and 2018 (1.16 x 10^8 and 2.02 x 10^{10} gene copy numbers per g^{-1}, respectively), when compared to the highest abundances found in sediments for Sok-SSBS (4.81 x 10^7 and 2.42 x 10^9 gene copies per g^{-1}, respectively) and Bzr-SSBS only in the summer of 2018 (7.65 x 107 gene copies per g^{-1}) (see **Fig 6**; **Appendix 1**: Font Nájera et al., 2020; see chapter 3.3 in results, and 4.5 in discussion) **Appendix 2**: Font Nájera et a., 2021a; see chapter 3.3 in results, and 4.2.2 in discussion). The ratio *nos*Z/16S rRNA (up to 10.4%) allowed to identify a significant portion of these communities developed in biofilms formed over the brown coal during summer, indicating that denitrifying bacteria developed better in the presence of additional carbon substrate and

favoured conditions with warm temperature (21.9 ± 1.9 °C). Positive significant correlations were observed between the concentration of N-NO_3 and the gene copy numbers of *nos*Z, suggesting that these communities were active during summer (**Appendix 2**: Font Nájera et al., 2021a; see chapters 3.4 in results, and 4.1 in discussion). The above results suggested to include the DENz in future construction of SSBS, in order to promote the formation of biofilms with these communities to increase the microbial metabolic activity in the transformation of nitrogen compounds..

Fig 6. Dynamic abundance of bacterial key functional genes (*amo*A and *nos*Z) and the 16S rRNA of total bacteria along the horizontal profile of SSBSs. Gene copy numbers represent the average of different seasons of collection, and the error bars represent their standard deviation. Sok-SSBS (n=5), Bzr-SSBS (n=3), and Str-SSBS (n=5)

Bacterial community analysis, according to the relative abundance of the gene 16S rRNA, was performed in biofilm formations from the GEOz (limestone barriers) in Sok-SSBS and Str-SSBS, and DENz (brown coal) in Str-SSBS. These samples were collected during the spring and summer seasons, due to the higher relative abundances observed from nitrifying and denitrifying communities when compared to the sediments. The results indicated that *β-Proteobacteria* was the most abundant class in both types of biofilm formations (up to 27.6%; **Fig 7**). This group of bacteria is well known to contain several taxa involved in a broad variety of metabolic processes that include the cycling of nutrients, and their occurrence in such environments suggests their biotechnological potential. Bacterial communities in limestone biofilm (GEOz) were characterized with higher relative abundances of the families *Comamonadaceae* and *Rhodocyclaceae* (up to 10.5% and 9.3%, respectively; see **Fig 7**), and the genera *Rhodobacter*, *Dechloromonas*, and *Crenotrhix* (up to 4.1%, 4.0%, and 3.9%, respectively) (**Appendix 2**: Font Nájera et al., 2021a; see chapters 3.5 in results, and 4.2.1 in discussion), while in brown coal biofilm (DENz) by the families *Comamonadaceae*, *Flavobacteriaceae*, and *Crenotrichaceae* (up to 18.5%, 10.2%, and 6.9%, respectively; see **Fig 7**), and the genera *Limnohabitants*, *Crenothrix*, and *Rhodobacter* (10.8%, 7.5%, and 4.2%, respectively) (**Appendix 2**: Font Nájera et al., 2021a; see chapters 3.5 in results, and 4.2.2 in discussion). PCA analysis indicated that the relative abundances of these bacterial families were closely associated to the increasing concentration of nutrients: i) *Comamonadaceae* with the concentration of N-NH_4, *Flavobacteriaceae* with N-NO_3, and *Crenotrichaceae* with TN, therefore suggesting that they were closely associated to the transformation of nitrogen compounds (**Appendix 2**: Font Nájera et al., 2021a; see chapters 3.5 in results). Other studies have also found significant association of the above mentioned bacterial taxa with the nitrogen cycling processes in a river (Repert et al., 2014), different lakes (Palacing-Lizarbe et al., 2019), a CW (Wang et al., 2016), and a WWTP (Ji et al., 2019). In the case of the family *Rhodocyclaceae*, with special consideration of the genus *Dechloromonas*, it was more close associated to the increasing concentration of TP and P-PO_4 in GEOz. These results suggested that this family could represent an important community of polyphosphate accumulating bacteria, within the diverse group of PAOs, in biofilm formations over limestone barriers. Both above mentioned bacterial taxa have been previously linked to the accumulation of phosphorus in WWTP (McMahon et al., 2002; Coats et al., 2017); however, this is the first time that PAOs are associated to microbial biofilms in SSBSs, thus, suggesting that future research could be focused on the elucidation of their role in phosphorus removal from water in the horizontal profile of SSBSs (**Appendix 2**: Font Nájera et al., 2021a; see chapter 4.2.1 in discussion).

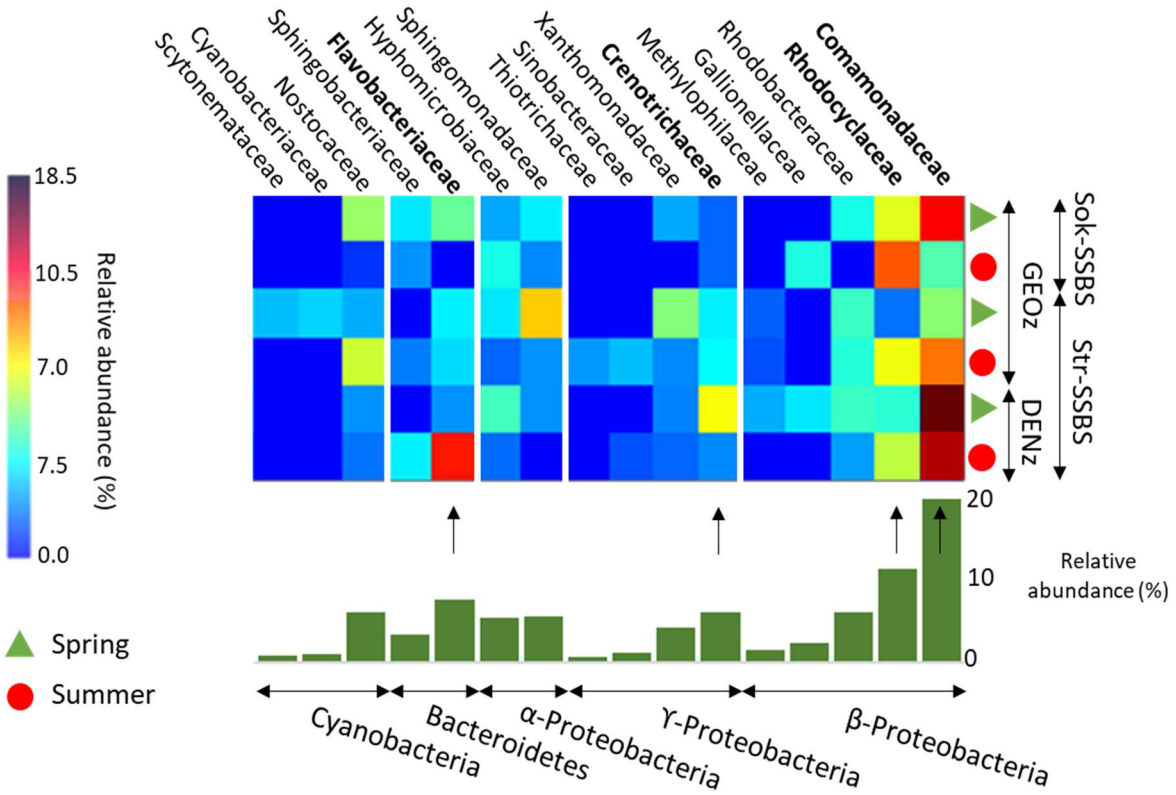

Fig 7. Heat map representing bacterial community families in biofilm formations (GEOz and DENz). The gene 16S rRNA was used to estimate the relative abundance of different taxa (OTUs).

4. Isolation and characterization of nitrogen-transforming bacteria from SSBSs sediments

From a total of 150 heterotrophic bacterial strains isolated from SSBSs sediments, only ten were able to perform partial or total removal of nitrogen sources when inoculated in selective nutrient media. The strains Str01 and Sok05 - identified as *Bacillus simplex* (98.36%) and *Kocuria rosea* (99.08%) according to the gene 16S rRNA – were observed to reduce NO_3^- to NO_2^- in GiDM in anaerobic conditions. The presence of the *nar*G gene for both bacteria allowed to confirm the above mentioned process, and therefore were considered to be nitrate reducers. The strains Sok01 (*Hydrogenophaga taeniospiralis*, 99.32%), Sok06 (*Bacillus aereus*, 99.63%), and Sok20 (*Janthinobacterium lividum*, 99.34%), were able to continue the process of NO_3^- to gaseous forms in GiDM in anaerobic conditions, and the presence of the *nos*Z gene supported that they were facultative denitrifying bacteria. All above five mentioned strains were not able to grow and transform NH_4^- in NM in aerobic conditions, and therefore were considered not to participate in the nitrifying process. The strains Sok03 (*Pseudomonas*

guineae, 99.45%), Sok41 (*Acidovorax radicis*, 99.29%), and Bzr07 (*Pseudomonas migulae*, 99.83%) were found to completely remove NO_3^- from GiDM and contain the *nos*Z gene, indicating that they were also facultative anaerobic denitrifying bacteria; however, these bacteria, along with Str01 and Sok05, were able to grow and transform NH_4^- in NM in aerobic conditions. Despite the above descriptions, only two strains (Bzr02 – *Citrobacter freundii*, 99.39%, and Str21 – *Pseudomonas mandelii*, 99.55%) were selected for further description of kinetic dynamics in nitrogen transformation controlled assays, since both were able to transform NO_3^- and NH_4^+ in a larger number of selective media, and contained a higher number of genes involved in nutrient cycling processes (**Appendix 3**: Font Nájera et al., 2021b; see page 2 and 3). The **Table 2** compares the average removal rates of Bzr02 and Str21 with other nitrogen transforming bacteria that have been published.

Table 2. Comparing the average removal rate of N-NH_4 and N-NO_3 for the strains Bzr02 and Str21, with the performance from other published nitrogen transforming bacteria

nitrifying medium (NM)	
Strain	average removal of N-NH_4 (mg L^{-1} h^{-1})
Pseudomonas putida Y-9	7.4
***Pseudomonas mandelii* Str21**	**7.21**
Pseudomonas stutzeri T13	7.09
Pseudomonas stutzeri YZN-001	5.53
***Citrobacter freundii* Bzr02**	**5.41**
Klebsiella pneumonae CF-S9	4.3
Pseudomonas stutzeri AD1	3.1
Pseudomonas sp. JQ-H3	2.7
Klebsiella pneumonae EGD-HP19-C	2.29
Enterobacter cloacae CF-S27	2.22
Pseudomonas tolaasii Y-11	2.04
Alcaligenes denitrificans WY200811	0.69

denitrifying medium (DM)	
Strain	average removal of N-NO_3 (mg L^{-1} h^{-1})
***Pseudomonas mandelii* Str21**	3.89
Bacillus cereus GS-5	2.7
Klebsiella pneumonae CF-S9	2.2
Pseudomonas tolaasii Y-11	2.04
Pseudomonas stutzeri AD1	1.98
Pseudomonas sp. JQ-H3	1.78

simultaneous nitrifying-denitrifying medium (SNDM)	
Strain	average removal of N-NH_4 (mg L^{-1} h^{-1})
***Citrobacter freundii* Bzr02**	5.07
***Pseudomonas mandelii* Str21**	3.35
Klebsiella pneumonae CF-S9	3.3
Pseudomonas tolaasii Y-11	2.13
Bacillus cereus GS-5	2.69
Janthinobacterium svalbardensis F19	0.62

Strain	average removal of N-NO_3 (mg L^{-1} h^{-1})
Bacillus cereus GS-5	2.94
Klebsiella pneumonae CF-S9	2.6
***Pseudomonas mandelii* Str21**	2.29
***Citrobacter freundii* Bzr02**	1.44
Janthinobacterium svalbardensis F19	1.19
Pseudomonas tolaasii Y-11	0.52

(**Appendix 3**: Font Nájera et al., 2021b)

The bacterium *C. freundii* Bzr02 was able to remove 99.0 ± 0.2% of N-NH_4 after 22 hours of incubation in NM (initial concentration at 100 mg L^{-1}), with an average rate of 5.41 ± 0.13 mg L^{-1} h^{-1} (**Table 2**), and a maximal rate of 16.17 ± 0.97 mg L^{-1} h^{-1}. Nitrification intermediary products from the oxidation of NH_4^+, such as NO_3^- and NO_2^-, were not observed during the entire assay. Total nitrogen balance analysis at the end of the experiment identified that 94.7 ± 1.4 mg of the N-NH_4 were assimilated by bacteria and only 0.75 mg were lost as nitrogen gas. The above results suggested that Bzr02 was assimilating N-NH_4 rather than performing

nitrification (**Appendix 3**: Font Nájera et al., 2021b; see page 3). Despite the above, Bzr02 was found to utilize N-NH_4 in the presence of hydroxylamine – which is another intermediary product in the nitrification process – however, the transformation of N-NH_4 was not observed until significant amount of hydroxylamine was removed from the media. Again, intermediary products from the oxidation of NH_4^+ and, in this case hydroxylamine - such as NO_3^- and NO_2^-, were not observed in the entire assays (**Appendix 3**: Font Nájera et al., 2021b; see page 6). The presence of the *hao* gene confirmed its ability to remove hydroxylamine from the media, however again the Bzr02 was not performing nitrification. Instead, it was proposed that the enzyme HAO reduced hydroxylamine to nitrogen gas, rather than performing oxidation in the nitrification process (**Appendix 3**: Font Nájera et al., 2021b; see page 7). The strain Bzr02 was not able to utilize N-NO_3 (100 mg L^{-1}) when it was given as a sole nitrogen source in DM, suggesting that high concentrations could be toxic for this bacterium (**Appendix 3**: Font Nájera et al., 2021b; see page 4). However, when N-NO_3 was added in lower concentration in combination with N-NH_4 (50 mg L^{-1}, each) in SNDM for up to 36 hours, the strain Bzr02 was able to utilize both nitrogen sources simultaneously. In the case of N-NO_3, the average rate of removal was 1.44 ± 0.16 mg L^{-1} h^{-1} (**Table 2**), and the maximal was 7.52 ± 0.10 mg L^{-1} h^{-1}, while for N-NH_4 the average rate of removal was 5.07 ± 0.09 mg L^{-1} h^{-1} (**Table 2**), and the maximal was 10.44 ± 0.18 mg L^{-1} h^{-1}. Bzr02 was able to remove up to 94.1 ± 1.3% of N-NH_4 and 70.2 ± 3.6% of N-NO_3. Interestingly, the detection of N-NO_2 was significant (up to 20.02 mg L^{-1}) during the experiment with SNDM, however the results indicated that it was produced from the reduction of NO_3^- rather than the oxidation of NH_4^+ (**Appendix 3**: Font Nájera et al., 2021b; see page 6). The Bzr02 contained the genes *nap*A and *nar*G, both nitrate-reducing enzymes, confirming its ability to reduce NO_3^- to NO_2^- in SNDM. Moreover, Bzr02 was not able to completely remove N-NO_3 and N-NO_2 from the medium, indicating that the bacterium does not perform the complete process of denitrification, which was also confirmed from the lack of genes involved in such process (*nir*S, *nor*B, and *nos*Z). Despite the above, nitrogen balance experiment at the end of the assay indicated that 68.3 ± 1.8 mg of TN was assimilated by bacteria and 29.2 ± 2.1 mg remained in the media as N-NO_2 and N-NO_3 forms, suggesting that the strain Bzr02 was assimilating all forms of nitrogen in SNDM with a preference for N-NH_4 (**Appendix 3**: Font Nájera et al., 2021b; see page 7).

The bacterium *P. mandelii* Str21 was able to remove 98.9 ± 0.6% of N-NH_4 after 16 hours of incubation in NM (initial concentration at 100 mg L^{-1}), with an average rate of 7.21 ± 0.12 mg L^{-1} h^{-1} (**Table 2**), and a maximal rate of 10.20 ± 0.25 mg L^{-1} h^{-1}. Nitrification intermediary

products from the oxidation of NH_4^+, such as NO_3^- and NO_2^-, were not observed during the entire assay (**Appendix 3**: Font Nájera et al., 2021b; see pages 3 and 4). Total nitrogen balance analysis at the end of the experiment identified that 94.3 ± 2.0 mg of the N-NH_4 were assimilated by bacteria and only 1.24 mg were lost as nitrogen gas. The above results suggested that Str21 assimilated N-NH_4 rather than performed nitrification (**Appendix 3**: Font Nájera et al., 2021b; see pages 6 and 7). In contrast to Bzr02, the strain Str21 was able to remove 87.7 ± 2.0% of N-NO_3 (100 mg L^{-1}) in DM during 28 hours, with an average removal rate of 3.89 ± 0.16 mg L^{-1} h^{-1} (**Table 2**), and a maximal rate of 6.66 ± 0.27 mg L^{-1} h^{-1}. The N-NO_3 was reduced to N-NO_2 (detected up to 18.66 ± 1.68 mg L^{-1}), which was furtherly reduced to nitrogen gas (**Appendix 3**: Font Nájera et al., 2021b; see page 4 and 5). Total nitrogen balance experiment identified that 25.4 mg of nitrogen was lost as gas, while 68.2 ± 1.2 mg were assimilated by the bacterium. The above results indicated that Str21 was able to perform denitrification process and assimilation of nitrogen simultaneously in DM. The detection of the genes *nar*G, *nir*S, *nor*B, and *nos*Z, corroborated the process of denitrification, while the detection of the gene *nas*A corroborated the process of nitrogen assimilation (**Appendix 3**: Font Nájera et al., 2021b; see page 7). Moreover, the strain Str21 was also able to utilize N-NO_3 and N-NH_4 simultaneously in SNDM for up to 36 hours. In the case of N-NO_3, the average rate of removal was 2.29 ± 0.22 mg L^{-1} h^{-1} (**Table 2**), and the maximal was 2.61 ± 0.17 mg L^{-1} h^{-1}, while for N-NH_4 the average rate of removal was 3.35 ± 0.04 mg L^{-1} h^{-1} (**Table 2**), and the maximal was 4.52 ± 0.22 mg L^{-1} h^{-1}. Str21 was able to remove up to 94.6 ± 1.5% of N-NH_4 and 75.4 ± 2.6% of N-NO_3. Again, N-NO_2 was detected (up to 12.19 ± 0.77 mg L^{-1}) as a product from the reduction of NO_3^-, rather than the oxidation of NH_4^+. In contrast to Bzr02, the N-NO_2 was completely removed from SNDM, presumably being transformed into nitrogen gas forms in the process of denitrification. Despite the above, the N-NH_4 was removed much faster than N-NO_3, suggesting the preference to utilize N-NH_4, similarly as it was observed for the strain Bzr02 (**Appendix 3**: Font Nájera et al., 2021b; see page 6). Total nitrogen balance experiment estimated that 12.6 mg of nitrogen were lost as gas, while 74.3 ± 1.6 mg were assimilated by bacteria, indicating that this strain favoured nitrogen assimilation rather than denitrification. These results suggested that the performance in denitrification by Str21 - in both DM and SNDM - could have been influenced by the aerobic conditions of the assay, since it is believed to be a facultative anaerobic denitrifying bacterium. (**Appendix 3**: Font Nájera et al., 2021b; see pages 7 and 8).

The above results suggested that both bacterial strains, *C. freundii* Bzr02 and *P. mandelii* Str21, are important members in the N-cycling processes in SSBSs sediments. Moreover, they are also potential candidates with biotechnological applications to be used as microbial activators to enhance the removal efficiency of nitrogen compounds in NBS, such as SSBSs (**Appendix 3**: Font Nájera et al., 2021b; see conclusion in pages 8 and 9).

VII. Conclusions and verification of hypotheses

The first hypothesis was confirmed according to the next main conclusions:

According to the environmental conditions, the temperature was found to be one of the most important factors influencing microbial communities in SSBSs. First, microbial metabolic activity was found to be higher during spring and summer, when compared to autumn. Second, nitrifying bacterial communities (gene copy number of *amo*A) were more abundant in more temperate temperatures during spring (14.2 ± 2.5 °C), while denitrifying communities (gene copy numbers of *nos*Z) were more abundant in warm temperatures in summer (21.9 ± 1.9 °C). Significant correlations between the temperature and the abundance of key functional genes (positive for *nos*Z and negative for *amo*A) indicated that nitrogen transforming bacteria were influenced by temporal seasonal fluctuations. The nutrient concentration was also observed to be an important factor, since there was a significant correlation between the concentration of $N\text{-}NH_4$ and the abundance of nitrifying bacteria (spring), while there was a significant correlation between the concentration of $N\text{-}NO_3$ and the abundance of denitrifying bacteria (summer).

According to the construction design of the systems, the implementation of GEOz containing limestone, and the construction of DENz containing brown coal, were probably the most important factors influencing the metabolic activity, abundance and diversity of microbial communities, but also the efficiency in the removal of nutrients from contaminated water in SSBSs. In the case of GEOz, when the barriers were constructed with limestone, over dolomite, the heterotrophic metabolic activity and the abundance of nitrifying communities were significantly higher in biofilms formed over their surface. Moreover, nitrifying bacterial communities were usually higher in these biofilms when compare to SSBSs sediments. Bacterial community analysis, using the 16S rRNA, suggested that the *Comamonadaceae*, *Rhodocyclaceae* (*Dechloromonas*), *Rhodobacter*, and *Crenotrhix* could comprise important N-transforming communities in limestone biofilms, and more interestingly, the *Rhodocyclaceae*

– with special consideration of *Dechloromonas* – could be linked to the process of phosphorus accumulation in these biofilms. In contrast, the DENz – containing brown coal - were much more abundant with biofilms containing denitrifying bacterial communities, when compared to any other zone in SSBSs. Bacterial community analysis suggested that the *Comamonadaceae* (*Limnohabitants*), *Flavobacteriaceae*, *Crenotrichaceae* (*Crenothrix*), and *Rhodobacter* could comprise important N-transforming communities in brown coal biofilms. The example of Str-SSBS, with the youngest time in operation, and the construction of both of the above mentioned zones, could be the result of the most efficient nutrient removal observed when it was compared to the Sok-SSBS (lacking the DENz), and the Bzr-SSBS (containing a GEOz with dolomite and lacking the DENz).

The second hypothesis was confirmed according to the next main conclusions:

Two heterotrophic strains, identified as *Citrobacter freundii* Bzr02 and *Pseudomonas mandelii* Str21, were found to have significant biotechnological potential for the removal of nitrogen compounds in contaminated water. The strain Bzr02 was observed to contain the genes *nap*A and *nar*G, which were involved in its ability to reduce NO_3^- to NO_2^- (a nitrate reducer). The Bzr02 also contained the gene *hao* which was involved in its ability to remove hydroxylamine from the medium. In the case of the strain Str21, the genes *nar*G, *nir*S, *nor*B, and *nos*Z were detected, confirming its ability to perform complete denitrification. Despite the above, both strains were observed to prefer the pathway of nitrogen assimilation rather than dissimilatory pathways of nitrogen transformation, which were probably influenced from the aerobic conditions of the experiments. Nevertheless, both strains could be considered as potential candidates for the application in SEDz of SSBSs, since their ability of assimilation could be used to sequestrate nitrogen in the sediments. Finally, the sediments as an important sink of nitrogen compounds would require their periodical extraction to avoid resuspension in the water, and therefore, the proper management of SSBSs.

VIII. Attachments

Appendix 1: Font Nájera A., Serwecinska L., Szklarek S., Mankiewicz-Boczek J. 2020. *Characterization and comparison of microbial communities in sequential sedimentation-biofiltration systems for removal of nutrients in urban rivers*. Ecological Engineering, 149, 105796, doi.org/10.1016/j.ecoleng.2020.105796

Lodz May 26th, 2021

Statement of co-authorship

I declare that my contributions to the preparation of the original work were: i) collection of samples, ii) execution of experiments - analysis of microbial metabolic activity and genetic analysis, iv) revision of the existing knowledge about the present topic, v) writing the original draft of manuscript, vi) correction and editing after review, vii) and the final visualization of the work. I assess that my participation represents 40% of the work.

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I declare that my contributions to the preparation of the original work were: i) supervision of microbiological activity analysis, and ii) the edition of the text in the manuscript. I assess that my participation represents 15% of the work.

Dr. Liliana Serwecińska
European Regional Centre for Ecohydrology
of the Polish Academy of Sciences

I declare that my contributions to the preparation of the original work were: i) help in collection of samples, and ii) performance and elaboration of chemical analysis. I assess that my participation represents 10% of the work.

Dr. Sebastian Szklarek
European Regional Centre for Ecohydrology
of the Polish Academy of Sciences

I declare that my contributions to the preparation of the original work were: i) the conceptualization and idea of the research, ii) supervision of whole genetic part of study, iii) revision/edition of whole data obtained, iv) and planning of main chapters and further edition of the text in the manuscript. I assess that my participation represents 35% of the work.

Prof. dr hab. Joanna Mankiewicz-Boczek
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Characterization and comparison of microbial communities in sequential sedimentation-biofiltration systems for removal of nutrients in urban rivers

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ABSTRACT

The present study described the nutrient removal efficiency of two multi-zone biofilters with different working time, eight vs two years, focusing in metabolic and genetic characteristics of microbial communities. They are known as sequential sedimentation-biofiltration systems (SSBSs) for the removal of nitrogen and phosphorus compounds in urban rivers. Nutrient removal performances for both SSBSs were up to 55% for N-NO_2, 46% for N-NO_3 and 96% of TP, however, an increase of N-NH_4 in the outflow was observed for the eight-year SSBS (Sok-SSBS). Analysis of microbial communities in sediments showed similar metabolic activity for all zones in both SSBSs, suggesting no significant difference according to the working time, but the lowest metabolic activity was observed for geochemical zones, which contained a limestone barrier for the removal of phosphorus. The quantity of key functional genes, involved in nitrogen transformation cycle, showed that denitrifying bacteria (up to 1.16×10^8 copies of *nosZ* gene g^{-1}) were more abundant than nitrifying bacteria (up to 5.68×10^5 copies of *amoA* gene g^{-1}) in SSBSs sediments. Maximum abundances of both gene copy numbers were observed in the denitrifying zone presented only in the two-year SSBS (Str-SSBS). The above results suggested that both SSBSs could be recommended as a useful solution to improve water quality in urban areas. However, the implementation of the denitrifying zone, containing brown coal, significantly increased the abundances of denitrifying and nitrifying bacterial communities. In consequence, the efficiency of N-NH_4 removal in Str-SSBS could be enhanced compared to Sok-SSBS. Furthermore, microbial metabolic activity in geochemical zones could be strengthened with the application of biopolymers carrying additional carbon substrates, in order to promote biofilm formation in the limestone barrier.

1. Introduction

Great quantities of nutrients are delivered into aquatic ecosystems by untreated water from domestic, industrial areas and storm-water runoff (Nurk et al., 2005; Adyel et al., 2016). In consequence, nutrient accumulation occurs in lower ponds situated on urban rivers, increasing their susceptibility to eutrophication and the appearance of toxic cyanobacterial blooms (Zanchett and Oliveira-Filho, 2013; Szulc et al., 2015; Jurczak et al., 2018). Due to this, constructed wetlands (CWs) and biofilters have been implemented to treat urban rivers, proving to be low cost and ecological friendly approaches, however, it is still marked that they not always perform sufficient nutrient removal from water (Faulwetter et al., 2009; Truu et al., 2009).

The present research was performed in two multi-zone biofilters, described as sequential sedimentation-biofiltration systems (SSBSs). They are constructed with continuous independent compartments (zones): the sedimentation, geochemical and biofiltration zones, that perform different functions in nutrient removal (nitrogen and phosphorus compounds) from polluted urban rivers directly linked to lakes or pounds (Zalewski et al., 2012; Szulc et al., 2015; Serwecińska et al., 2017; Szklarek et al., 2018). Monitoring conducted in an older SSBS in Sokołówka River (Sok-SSBS, eight years) indicated removal up to 56% of total nitrogen (TN) and 37% of total phosphorus (TP) (Szklarek, 2016). In turn, the newly constructed SSBS in Struga Gnieźnieńska River (Str-SSBS, two years) indicated 70% removal of NO_3^-, and more than 50% removal of TP and PO_4^- (Serwecińska et al., 2017). In other

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studies, multi-zone biofilters have proved to increase the removal of TN (between 54 and 92%) and TP (between 27 and 99%) in contaminated waters from urban rivers, due to their more complex structures (Jia et al., 2014; Zhimiao et al., 2016; Adyel et al., 2016). Until now, nutrient removal efficiency in multi-zone biofilters has been studied using physicochemical parameters, and their hydrodynamics (Truu et al., 2009). In the case of microbial communities, there is lack of information on such operating systems, and therefore, its knowledge is important to understand whether and why the efficiency in nutrient removal varies throughout the working time of multi-zone biofilters, and how their construction affects the processes of nutrient transformation.

Microbial communities play an important role in the transformation and removal of nutrients from water and sediments (Truu et al., 2009, Zhang et al., 2014b; Cheng et al., 2016), thus, knowledge about their metabolic activity is essential to optimize SSBSs. The number of studies analysing microbial communities in constructed wetlands (CW) has increased in the last decade. They include spatial and temporal microbial dynamics (Weber, 2009; Chazarenc et al., 2010), the diversity of plants (Bissegger et al., 2014; Button et al., 2016) and the effects of xenobiotic compounds (Zhang et al., 2019). However, there is lack of studies in the case of multi-zone biofilters treating urban rivers. Therefore, the use of physiological microbial profiles may facilitate to understand their metabolic potential in sediments from different SSBSs zones.

The use of molecular markers that target bacterial key functional genes in nitrifying-denitrifying processes have been widely applied. The nitrifying bacteria have been described with the detection of *amo*A gene, which is involved in the NH_4^+ transformation to hydroxylamine (NH_2OH) (Rotthauwe et al., 1997). They are known as ammonia oxidizing bacteria (AOB), and are predominantly represented by autotrophs (*Nitrosomonas* spp., and *Nitrosospira* spp.), (Kern, 2003; Dong and Reddy, 2010). In contrast, the denitrifying bacteria have been described from a larger array of key functional genes (*nap*A, *nar*G, *nir*S, *nir*K, *cnor*B, *qnor*B and *nos*Z) that are involved in the denitrification pathway (NO_3^- reduction throughout subsequent intermediates until gas N_2) (Faulwetter et al., 2009; Levy-Booth et al., 2014). In general, studies have placed particular interest in the detection of the *nos*Z gene which is involved in the last stage completing the process to the gas N_2 (Ma et al., 2008; Jones et al., 2013). To our knowledge, both genes have not been used to describe nitrifying and denitrifying bacterial communities in SSBSs, and therefore, this knowledge allows to distinguish their preferences in the different zones of SSBSs.

In the present study we compared the operational effectiveness of two SSBSs (Sok-SSBS and Str-SSBS), constructed within urban rivers, for the removal of nutrients carried by untreated water or storm-water runoff. The study of microbial communities in sediments was proposed to discuss their metabolic and nutrient-transforming potential with two different approaches: (1) the use of community level physiological profile (CLPP) to estimate the metabolic profile diversity of heterotrophic microbial communities, and (2) the quantification of key functional genes to measure abundance of bacterial communities involved in transformation of nitrogen compounds. Moreover, DNA sequencing analysis was performed to describe nucleotide similarity between retrieved sequences from SSBSs sediment samples and other published nitrifying and denitrifying bacteria. The results were used to identify preferences of microbial communities in the horizontal profiles of both SSBSs, and to propose future adaptation and/or construction of new SSBSs that would promote higher abundances and metabolic activity of microbial communities to increase their effectiveness in nutrient removal.

2. Materials and methods

2.1. Study site description

Two different SSBSs, constructed in different Polish cities (Lodz and Gniezno), were selected for the present study. The general structure of an SSBS was described by the work of Zalewski et al., (2012), in the urban Sokołówka River at the park Adam Mickiewicz in the city of Lodz (Sok-SSBS, Fig. 1a). The water from the river is diverted to the SSBS and it is exposed to at least three well-differentiated zones that perform different functions in order to improve the water quality: (1) *The intensified hydrodynamic sedimentation zone* (SEDz), which receives the water from the inflow (INF) and reduces velocity and turbulence with the help of obstructing barriers. In this zone, the water stabilizes and inflowing solids and other large organic particles sediment at the bottom. (2) *The intensified biogeochemical processes zone* (GEOz), which is constructed, in both cases, from a limestone barrier that reduces the phosphorus concentration in the water by its adsorption and/or mineralization. (3) *The biofiltration zone* (BIOz) is a constructed wetland (CW) composed of selected plant species (*Phragmites australis, Typha latifolia* and *Acorus calamus*) that uptake high quantities of nutrients from water and sediments. Afterwards, the water retreats through the outflow (OTF) back to the original course of the river. A more detailed physical description of the Sok-SSBS was presented at the work of Szklarek et al., (2018). The Sok-SSBS has a 65 × 16 m^2 dimension comprising a total area of 1.04 × 10^{-3} km^2. It principally treats storm water originating from a 5.72 km^2 draining catchment comprised of high-density residential areas with impermeable soil. It was constructed in 2011 as part of the SWITCH project (6 FP EU, GOCE 018530) in close cooperation with the Municipality of Lodz.

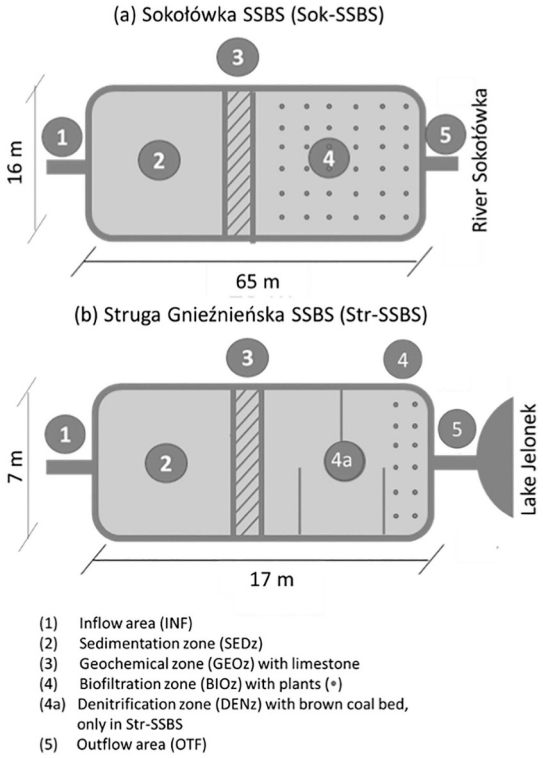

Fig. 1. General description of the structure and construction of SSBSs.

The second SSBS is located within the Struga Gnieźnieńska River at the city of Gniezno (Str-SSBS, Fig. 1b). It was constructed during the year 2016 as part of the project GEKON2/03/267948/21/2016 to treat storm water runoff and control algal blooming in the urban Lake Jelonek located downstream. The Str-SSBS contains the SEDz, GEOz, and BIOz, which operate very similar to the Sok-SSBS, however some modifications were added: the construction of a *denitrifying zone* (DENz) between the GEOz and the BIOz, which contains a bed of brown coal used as carbon source with the purpose to enhance microbial activity in the transformation of nutrients. The Str-SSBS has a 17 × 7 m^2 dimension comprising a total area of 0.12 × 10^{-3} km^2. Finally, it is

important to mention that the Str-SSBS is not only smaller in dimension when compared to the Sok-SSBS, but also younger in operation (two years vs eight years approximately, respectively).

2.2. Sample collection and handling

Samples for chemical, microbiological and molecular analysis were collected in different areas from the horizontal profile of both SSBSs, starting from the OTF, progressively in the SSBS zones, and finalizing at the INF (see Fig. 1a, b). Water and sediment samples were collected at the INF, SEDz, BIOz and OTF of both SSBSs. Limestone was collected at the GEOz in both SSBSs, and brown coal at the DENz in the Str-SSBS. Samples were collected, for the Sokołówka and Struga Gnieźnieńska SSBSs, in two different periods: (1) the 2nd and 3rd of August 2017, and (2) the 19th and 24th of October 2017, respectively.

Water samples were initially collected in 50 mL sterile plastic containers. Additionally, water in situ parameters were measured in each sampling area using a YSI multimetric probe [temperature (T°), dissolved oxygen (DO) and pH]. Sediment samples were collected from the surface of the bottom (0–5 cm in depth) using a sterile spatula, mixed and deposited in a sterile plastic container (150 mL). Five different limestones were extracted from the GEOz using sterile tweezers and deposited in sterile plastic containers with enough river water to prevent biofilm drying on the surfaces (Lear et al., 2010). A similar process was performed to collect brown coal in the DENz from the Str-SSBS. All samples were preserved and transported to the laboratory in dark and cold conditions (4 °C). Samples for microbiological and molecular analysis were kept cold (4 °C) in the laboratory and processed within 72 h after collection. Samples for chemical analysis were frozen (−18 °C) and processed after one week of collection.

2.3. Chemical analysis of nutrients

The $N\text{-}NH_4$, $N\text{-}NO_2$ and $N\text{-}NO_3$ were determined for water samples after ion analysis using Dionex ® ion chromatograph as specified by Szklarek et al. (2018). Total nitrogen (TN) was analysed according to Kjeldahl method (Hach, 1997), and for total phosphorus (TP) the sample was first digested with Oxysolv® (Merk), and then measured by the ascorbic acid colorimetric method (Golterman et al., 1978). The analysis was performed for water samples collected at the INF, SEDz, BIOz and OTF from both SSBSs, the DENz in Str-SSBS, at both periods of collection. Operational performance of both SSBSs was analysed according to the differences between the nutrient concentrations at the INF and the OTF.

2.4. Community level physiological profile (CLPP)

Catabolic activity of microbial communities in sediments (INF, SEDz, BIOz and OTF) and limestone (GEOz) was analysed for both SSBSs in samples collected in October 2017, using the specification of BIOLOG EcoPlates™ dedicated for environmental studies (Insam and Goberna, 2004; Button et al., 2016). Under sterile conditions, 10.0 g of sediments were added to 90 mL of deionized water in 300 mL sterile Erlenmeyers, shacked at 120 rpm for 30 min in room temperature and left to settle for 15 min. Tenfold diluted sediment extracts were prepared with deionized sterile water and 200 µL were inoculated into each EcoPlate well. For the limestone samples, a 2.0 cm^2 biofilm formation was scratched from the surfaces of the rocks using a sterile blade and processed similarly as with sediment samples (Lear et al., 2010). Negative control was prepared using 200 µL of deionized sterile water. Plates were incubated in dark at 27 °C for 10 days and measurements were made every 24 h of incubation using a Biolog Microstation ELX808BLG reader analyser at 590 nm. CLPP and diversity of heterotrophic microbial communities of each SSBSs zone were assessed through the AWCD (average well colour development, which expresses microbiological activity), Shannon-Weaver index (Index H representing functional diversity) and Richness (index R referring to the number of utilized carbon substrates) according to Zak et al. (1994). The CLPP analysis is limited to describe specific groups of microorganisms that are involved in processes for the removal of nutrients, and therefore, the detection of key functional genes involved in nitrification and denitrification process was proposed (see section 2.6).

2.5. DNA extraction

DNA was isolated and purified from 500 mg (wet mass) of sediments (INF, SEDz, BIOz and OTF from both SSBSs) and brown coal (DENz in Str-SSBS) collected in August and October 2017, using the specifications from FastDNA™ SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA). For brown coal samples, a 2.0 cm^2 area of biofilm formation at the surface was scratched with a sterile blade and processed similarly as with sediment samples. Parallelly, 1.0 g of sediments and brown coal were dried (105 C°, 24 h) in order to estimate qPCR results as per gram of dry sediment (Johansson et al., 2016; Buxton et al., 2018).

2.6. Quantitative analysis of functional genes involved in nitrifying and denitrifying processes

The key bacterial functional genes *amo*A (nitrification) and *nos*Z (denitrification) were analysed from sediments (INF, SEDz, BIOz and OTF from both SSBSs) and brown coal (DENz in Str-SSBS) collected in August and October 2017, by quantitative PCR (qPCR) using Maxima SYBR green/ROX qPCR master mix (Thermo Scientific) and a Rotor Gene Q thermocycler (Qiagen). The primer set amoA-1F/amoA-2R (491 bp) (Rotthauwe et al., 1997) and nosZF/nosZR (380 bp) (Chon et al., 2011) were used for qPCR assay. The reaction (25 µL) contained 12.5 µL of ready to use master mix, 30 pmol of primers and 1 µL DNA template (~75 ng). All reactions were prepared in triplicate. The thermal cycling program for both genes included: initial denaturation at 95 °C for 5 min, 45 cycles of denaturation at 95 °C for 15 s; annealing at 62 °C (*amo*A gene) or 61 °C (*nos*Z gene) for 30 s; and extension at 72 °C for 60 s. Followed by a melting curve from 65 to 95 °C. Gene copy number was read at phase of extension. Standard curves for both genes were prepared using plasmids carrying a purified PCR product from environmental samples, according to the specifications in Ma et al. (2018). PCR assays contained eight serially diluted standards (2×10^1–2×10^8 gene copies μL^{-1}), prepared using the plasmids, and a non-template standard. For both genes, the efficiency of the reactions based on the standard curves were always over 90% and the r^2 were over 0.98 (see supplementary material Fig. S1a, S1b). Melting curve analysis showed a defined sharp peak for both genes with low appearance of shoulder peaks (Supplementary material Fig. S2a, S2b).

2.7. Sequencing

PCR amplicons from sediment samples at the SEDz in both SSBSs (selected due to good quality of DNA extracted material) and plasmids for the preparation of qPCR standard curves were purified with QIAEX II gel extraction kit (Qiagen) according to manufacturer's instruction. The purified PCR products were sequenced by capillary sanger method by Genomed® laboratories in Warsaw, Poland (http://www.genomed.pl/). Nucleotide sequences were aligned and assembled using the software BioEdit sequence alignment editor (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Identity of the edited sequences were verified by BLAST analysis (http://blast.ncbi.nlm.nih.gov/) and deposited in the GeneBank database. Accession numbers for the plasmids are: MT129646 (*amo*A gene) and MT129649 (*nos*Z gene), and accession numbers for sediment samples in the SSBSs (SEDz) are: MT129644 and MT129645 (*amo*A gene for Sok-SSBS and Str-SSBS respectively), MT129647 and MT129648 (*nos*Z gene for Sok-SSBS and Str-SSBS respectively).

2.8. Data analysis

For the CLPP analysis, higher differences between AWCD values were observed after 120 h of BIOLOG EcoPlate incubation, and therefore were used to describe the microbial metabolic activity, the Shannon-Weaver diversity and the richness indexes for sediment samples in both SSBSs (see supplementary material Fig. S3). Furthermore, the above indexes were used to describe differences along the areas of each SSBS. Significant differences were assessed through the one-way ANOVA and Tukey's honesty significant difference (HSD) tests, while the *t*-test of independent samples was used to estimate differences in analogous areas between both SSBSs, using the software Statistica 13.1 (Dell™). Finally, the AWCD index was used to perform a principal component analysis (PCA), in order to observe differences between the zones of both SSBSs according to the 31 metabolized carbon sources in the test. To facilitate the spatial distribution of the samples, all 31 substrates were organized in five different chemical groups: (1) carbohydrates, (2) polymers, (3) carboxylic and ketonic acids, (4) amino acids, or (5) amines and amides according to Weber and Legge (2009).

For the quantitative analysis of functional genes (*amo*A and *nos*Z), the copy numbers were estimated as per gram of dry sediment and compared between the different areas of both SSBSs (INF, SEDz, BIOz and OTFz for both SSBSs and the DENz for the Str-SSBS). Significant differences between gene copy numbers were described using the two-way ANOVA test and the Tukey's honesty significant difference test (HSD). The Pearson correlation test was used to describe the relationship between the abundance of bacteria (dictated by the gene copy number), the physicochemical parameters and nutrient concentration in the water. Analysis of data was performed using the software Statitisca 13.1 (Dell™).

3. Results

3.1. Physicochemical parameters and nutrient reduction in SSBSs

A summary of chemical data for nutrient concentrations and physicochemical parameters of water was presented in Table 1. The results showed removal of N-NO_2 (55% for Sok-SSBS and 27% for Str-SSBS, respectively), N-NO_3 (46% and 30%, respectively) and TP (93% and 33%, respectively) in both SSBSs (Table 1). The concentration of N-NO_2 was low at the INF in both SSBSs (between 0.003 and 0.104 mg L^{-1}) (Table 1). In turn, the concentration of N-NO_3 at INF was higher at Str-SSBS (2.027 mg L^{-1} and 6.280 mg L^{-1}) than at Sok-SSBS (1.186 mg L^{-1} and 3.171 mg L^{-1}) (Table 1). No reduction was observed for N-NH_4 at the Sok-SSBS due to an increase in the concentration at the OTF in both dates, which was also reflected in the concentration of TN (Table 1). On the contrary, the Str-SSBS showed positive removal of N-NH_4 (48%), however the INF concentrations were lower (0,007 mg L^{-1} in August and 0.202 mg L^{-1} in October) than those observed for Sok-SSBS (0.288 mg L^{-1} in August and 0,621 mg L^{-1} in October) (Table 1).

The physicochemical parameters showed lower temperature range for the Sok-SSBS (11.0 °C - 21.1 °C) when compared to Str-SSBS (15.7 °C - 24.0 °C). On the contrary, lower oxygen concentration range was observed in Str-SSBS (6.5–6.6 mg L^{-1} O_2) than in Sok-SSBS (4.0–9.9 mg L^{-1} O_2). Finally, the range of pH for both SSBS remained neutral (6.83–7.89) (Table 1).

3.2. Community level physiological profile of microbial population

The microbial metabolic activity, Shannon-Weaver diversity and Richness indexes from sediment samples in both SSBSs were summarized in Fig. 2 (see also supplementary material Table S1). Higher metabolic activity was observed at the INF, SEDz, BIOz and OTF, indicated by their AWCD values ranging between 1.5 and 2.0 approximately (Fig. 2a). In contrast, the limestone in the GEOz presented the lower metabolic activity profile for both SSBSs, where their AWCD values remained below 1.0 and were significantly different ($p < .05$) from the other areas in SSBSs (Fig. 2a, see also supplementary material Table S2 and S3).

Moreover, when the analogous areas between SBSSs were compared, significant differences were observed for the diversity and richness indexes between the SEDz, GEOz and BIOz inside the structure of both SSBSs (Fig. 2b, c, see also supplementary material Table S4). The SEDz and BIOz presented the highest diversity at the Sok-SSBS (H: 3.35 and 3.34 respectively) when compared to Str-SSBS (H: 3.31 and 3.29 respectively) (Fig. 2b). Similar results were observed for the richness at the Sok-SSBS (R: 29.7 and 30.3 respectively) when compared to the Str-SSBS (R: 29 and 28.9 respectively) (Fig. 2c). In the case for the GEOz in both SSBSs, significant higher diversity and richness index was observed for Str-SSBS (H: 3.18 and R: 26.3) when compared to Sok-SSBS (H: 3.0 and R: 21.7) (Fig. 2b, c).

The principal component analysis (PCA) revealed differentiation in catabolism for the five different substrate groups between the sampling areas of both SSBSs (Fig. 3, see also supplementary material Table S5). A summary of the AWCD index categorized in the five different chemical groups is also shown in the supplementary material Table S6. For the Sok-SSBS, the principal component 1 (PC1) accounted for 78% of

Table 1
Physicochemical parameters and nutrient reduction (%) in water for the Sokołówka and Struga Gnieźnieńska SSBSs.

Date	Area	N-NO_2 mg L^{-1}	N-NO_3 mg L^{-1}	N-NH_4 mg L^{-1}	TN mg L^{-1}	TP mg L^{-1}	pH	Temperature C^0	Oxygen mg L^{-1}
Sokołówka SSBS (Sok-SSBS)									
August	Inflow	0.104	1.186	0.288	2.00	0.090	7.18	21.1	5.6
	Outflow	0.014	0.005	2.393[a]	3.00[a]	0.023	6.83	18.7	3.2
October	Inflow	0.088	3.171	0.621	4.20	0.464	7.63	11.0	14.2
	Outflow	0.073	2.348	0.676[a]	3.33	0.014	7.34	11.5	4.7
Media	Inflow	0.096	2.178	0.455	3.10	0.277	7.41	16.1	9.9
	Outflow	0.043	1.176	1.534[a]	3.17[a]	0.019	7.09	15.1	4.0
% removal		55	46	NR[a]	NR[a]	93	NA	NA	NA
Struga Gnieźnieńska SSBS (Str-SSBS)									
August	Inflow	0.003	2.027	0.007	5.50	0.093	7.55	24.0	6.0
	Outflow	0.003	2.156[a]	0.008[a]	3.10	0.088	7.54	23.4	6.1
October	Inflow	0.027	6.280	0.202	6.60	0.151	7.95	16.3	7.0
	Outflow	0.019	3.646	0.101	5.10	0.068	7.89	15.7	7.2
Media	Inflow	0.015	4.153	0.105	6.05	0.122	7.75	20.2	6.5
	Outflow	0.011	2.901	0.054	4.10	0.078	7.72	19.6	6.6
% removal		27	30	48	32	36	NA	NA	NA

[a] Values representing an increase of concentration in the water, meaning a no reduction in nutrient (NR). NA: Not applicable.

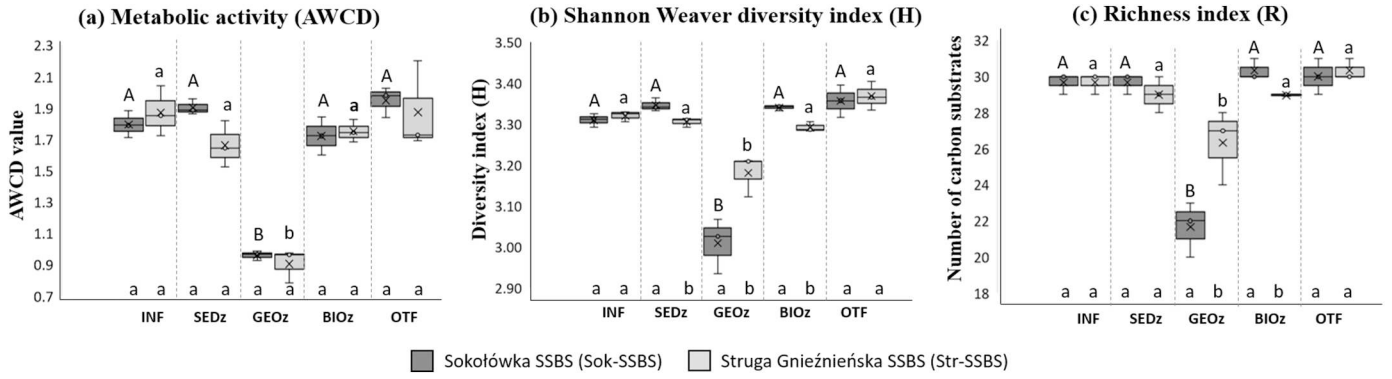

Fig. 2. Summary profile of heterotrophic microbiota activity at 120 h of incubation.
AWCD: average well colour development index. INF: Inflow; SEDz: Sedimentation zone; GEOz: Geochemical zone; BIOz: Biofiltration zone; OTF: Outflow. Significant different values ($p < .05$) between Sok-SSBS zones are marked by capital letters (A to B), and between Str-SSBS zones are marked by lower case letters (a to b), both over the boxplots. Significant different values ($p < .05$) between analogue zones of both SSBSs are marked by lower case letters (a to b) under the boxplots. Each boxplot represents one sample from the respective SSBS zone measured in triplicate, and the error bars the standard error of the mean ($n = 3$).

the variability which was significantly correlated to all five groups of carbon substrates (Table S5). The PC2 accounted for 10% of the variability and was positively correlated to amines and amides and negatively correlated to carboxylic and ketonic acids, however no statistically significant correlations were observed (Table S5). For the Str-SSBS, the PC1 accounted for 80.5% of the total variably, which was also associated to significant correlations with the same substrates as explained for the PC1 in Sok-SSBSs (Table S5). In turn, the PC2 accounted for 8% of the total variability, showed a weak positive correlation with amines and amides, and a negative correlation with amino acids, both being no statistically significant (Table S5). The horizontal component (PC1) showed how the INF, SEDz, BIOz and OTF positively correlated with the increment in substrate utilization, for both SSBSs (Fig. 3). Moreover, it was observed how the GEOz negatively correlated with the utilization of substrates in both SSBSs (Fig. 3). The previous analysis corresponded with the lower metabolic activity for the GEOz described by the AWCD, diversity and richness indexes (Fig. 2). In the case of the Sok-SSBS, the vertical component (PC2) clearly differentiated between the sampling areas according to the substrate of utilization. The INF, SEDz and OTF differentiated from one another, while the BIOz showed a more broad preference in carbon source utilization (Fig. 3a). In contrast, differentiation within the Str-SSBS samples was rather difficult due to the overlap between the sample areas (Fig. 3b).

Carbon substrate utilization of microbial communities in the samples is shown in Fig. 4 (see also supplementary material, Table S6). Generally, polymers were more catabolized at the INF, SEDz and BIOz from both SSBSs and also at the GEOz in Str-SSBS (Fig. 4). Additionally, carbohydrates seem to be highly catabolized by microbial communities in the above mentioned areas from both SSBSs. In contrast, the GEOz in Sok-SSBS showed different profiles of substrate utilization, where carboxylic/ketonic acids were more catabolized (Fig. 4a). A similar profile of carbon utilization was observed for the OTF area in both SSBSs (Fig. 4). When the substrates were analysed independently, glycogen was the most important catabolized substrate in the group of polymers, D-mannitol in carbohydrates and L-arginine in amino acids (see supplementary material, Fig. S4 and Fig. S5). In the case of GEOz in both SSBSs, they showed considerably lower metabolic values (AWCD < 1.0) for carbohydrates (β-methyl-D-glucoside, D-xylose, *N*-acetyl-D-

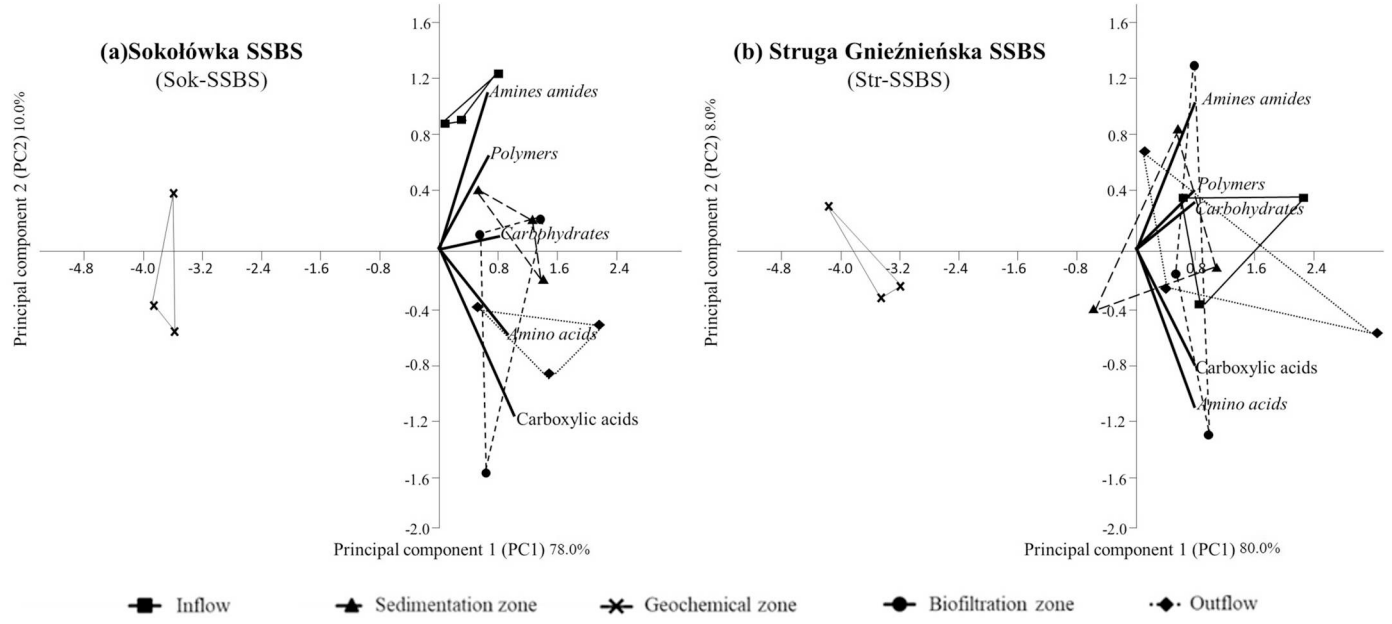

Fig. 3. Principal component analysis (PCA loadings) on carbon sources microbial activity from Sok-SSBS and Str-SSBS.
Percentage of total variance is explained for each component. All calculations are based on the AWCD index. Each SSBS has 5 samples with three replicates, identified with the same symbol and are joint together to observe the distance between them.

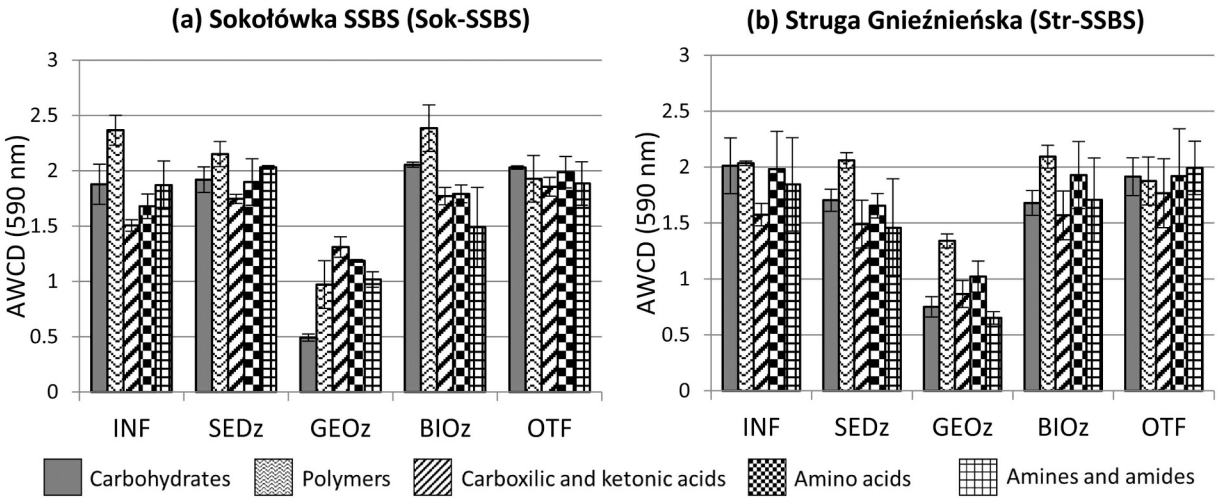

Fig. 4. Carbon substrate utilization by heterotrophic microbial communities along the horizontal profile of both SSBSs.
INF: Inflow; SEDz: Sedimentation zone; GEOz: Geochemical zone; BIOz: Biofiltration zone; OTF: Outflow. Error bars represent the standard error of the mean (n = 3).

glucosamine, D-cellobiose and α-D-lactose), polymers (α-cyclodextrin and glycogen), carboxylic and ketonic acids (4-hydroxy bezoic acid and T-hydroxybutyric acid), amino acids (L-phenylalanine and glycil-L-glutamic acid) and amines (phenylethylamine), when compared to the SEDz and BIOz from both SSBSs (see supplementary material Fig. S4 and S5).

3.3. Dynamics of bacterial key functional genes involved in nitrifying and denitrifying processes

A summary containing the averages of gene copy numbers of both genes (g^{-1} dry sediment) and their significant differences in sediments from both SSBSs is presented in Fig. 5 (see also supplementary material Tables S7 and S8, respectively). Generally, both SSBSs presented significantly higher copy numbers of *nos*Z gene (maximum: 4.81 × 10^7 copies g^{-1} in Sok-SSBS and 1.16 × 10^8 in Str-SSBS) than that of *amo*A gene (maximum: 5.10 × 10^5 copies g^{-1} in Sok-SSBS and 5.68 × 10^5 in Str-SSBS) for both dates of collection (Fig. 5). The copy numbers of *nos*Z gene decreased significantly in both SSBSs samples from August (4.81 × 10^7 copies g^{-1} for Sok-SSBS and 1.16 × 10^8 for Str-SSBS) to October (1.86 × 10^6 copies g^{-1} for Sok-SSBS and 7.41 × 10^6 for Str-SSBS). In turn, in the case for the *amo*A gene, copy numbers were different between both SSBSs: Sok-SSBS slightly decreased from August to October (5.10 × 10^5 and 3.63 × 10^5 gene copies g^{-1}, respectively), while an increase was observed for the Str-SSBS (2.58 × 10^5 and 5.68 × 10^5 gene copies g^{-1}, respectively) (Fig. 5). The ratio between gene copy number of *nos*Z over *amo*A remained significantly higher for both SSBSs, especially in samples from August in Str-SSBS (ratios from 242 up to 2603) than in Sok-SSBS (ratios from 36 up to 94) (Table 2).

Considering the different zones in SSBS it was found that the SEDz showed higher significant *nos*Z gene copy numbers for both SSBSs in August (4.81 × 10^7 copies g^{-1} in Sok-SSBS and 9.97 × 10^7 in Str-SSBS) (Fig. 5a, c), however such results were not easily observed for the same zone during October (Fig. 5b, d). In the case of the *amo*A gene, the SEDz presented higher copy numbers for the Sok-SSBS in both periods (5.10 × 10^5 copies g^{-1} in August and 2.80 × 10^5 in October) (Fig. 5a, b). However, the highest *nos*Z gene copy numbers were determined in the Str-SSBS at zone with brown coal (additional DENz) in both dates of collection (1.16 × 10^8 copies g^{-1} in August and 7.41 × 10^6 in October) (Fig. 5c, d). Moreover, the copy numbers of the *amo*A gene also increased significantly for the zone with brown coal from August to October period (4.45 × 10^4 and 5.68 × 1 0^5 copies g^{-1}, respectively) for the same biofilter (Fig. 5d).

Pearson correlations (r) between the gene copy numbers in SSBSs with the nutrient concentrations were described in Table 3. The *nos*Z gene showed negative significant correlation with N-NO_2, N-NO_3 and N-NH_4 in water samples from the Str-SSBS (Table 3). The Sok-SSBS also showed a negative significant correlation to N-NO_3 in water, however no other significant correlation for the same gene was observed. On the contrary, the *amo*A gene was found only to have a negative significant correlation with TN in water from the Sok-SSBS, and lack of significant correlation with other nitrogen forms for the Str-SSBS (Table 3).

Pearson correlations between the gene copy numbers and the environmental factors (temperature, oxygen concentration and pH) were also described in Table 3. The copy numbers of the *nos*Z gene was found to significantly correlate for the temperature and pH in both SSBSs (Table 3). In contrast, the *amo*A gene showed no significant correlation to any of the measured environmental factors (Table 3).

3.4. DNA sequence similarity of sediment samples

Using BLAST analysis, the *nos*Z gene purified from sediment samples was found with similarity to *Thiobacillus thioparus* (88%), *T. denitrificans* (87%) and *Paracoccus sphaerophysae* (80%), from the α-Proteobacteria (see supplementary material, Table S9). In contrast, sequences for the *amo*A gene showed similarity to *Nitrosopira* spp. (93% - 97%), and *Nitrosomonas* spp. (81%), from the β-Proteobacteria (Table S9).

4. Discussion

4.1. Nutrient reduction efficiency in SSBSs

The present results suggested that both, the Sok-SSBS and the Str-SSBS, performed removal of N-NO_2 (55% and 27%, respectively), N-NO_3 (46% and 30%, respectively) and TP (93% and 36%, respectively) during the two dates of sample collection representing summer and autumn seasons (Table 1). Higher removal of the above mentioned nutrients in the Sok-SSBS suggested a higher performance in the system with longer operational time. Unfortunately, N-NH_4 increased from inflow (INF = 0.455 mg L^{-1}) to outflow (OTF = 1.534 mg L^{-1}) in the Sok-SSBS and suggested a problem for its removal (Table 1). In turn, the Str-SSBS with two years of operational time, showed 48% removal of N-NH_4 when compared to Sok-SSBS in the present study (Table 1). However, the concentration at INF in Str-SSBSs was lower than at Sok-SSSBs (0.105 mg $L^{-1}$and 0.455 mg L^{-1}, respectively). In an earlier study conducted for the first two hydrological years of the Sok-SSBS, the N-NH_4 removal efficiency has already been reported to be low (2.8%) and the concentration was not significantly different between

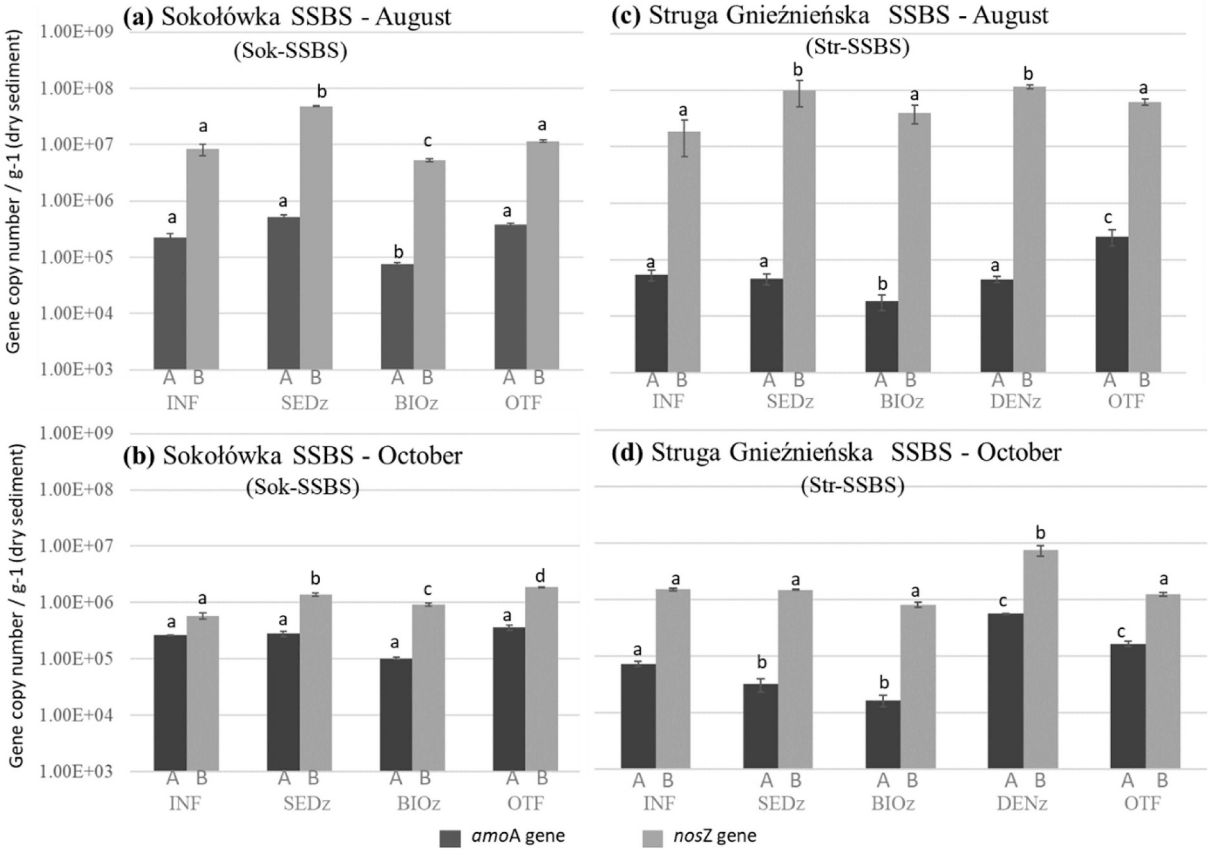

Fig. 5. Dynamics of *amo*A (491 bp) and *nos*Z (380 bp) gene copy numbers through the horizontal profile of the SSBSs.
INF: Inflow; SEDz: Sedimentation zone; BIOz: Biofiltration zone; DENz: Denitrification zone; OTF: Outflow. Significant differences ($p < .05$) between different genes in the same SSBS zone are marked by capital letters (A to B) under the columns, and significant differences ($p < .05$) between different SSBS zones for the same gene are marked by lowercase letters (a to d) above the columns. Significant differences are also well described in supplementary material Table S8. Error bars show standard errors in triplicate of each PCR sample ($n = 3$).

Table 2
Difference ratio between the copy numbers of *nos*Z/*amo*A genes in the horizontal profile of the SSBS.

SSBS sampling area	Sokołówka SSBS (Sok-SSBS)		Struga Gnieźnieńska SSBS (Str-SSBS)	
	August	October	August	October
INF	36.4	2.2	342.6	20.5
SEDz	94.4	5.0	2165.1	46.3
BIOz	70.6	9.0	2197.1	49.5
DENz	ND	ND	2602.9	13.0
OTF	30.9	5.1	241.9	7.4

ND: No data. The *nos*Z/*amo*A ratio represents how many times the denitrifying community is over the nitrifying community. It was calculated according to the gene copy number of each key functional gene. When the ratio > 1 the abundance of gene *nos*Z is higher than gene *amo*A. INF: Inflow; SEDz: Sedimentation zone; BIOz: Biofiltration zone; DENz: Denitrification zone; OTF: Outflow.

INF (0.85 mg L^{-1}) to OTF (0.83 mg L^{-1}) (Szklarek et al., 2018). The above observations suggested that the removal of N-NH_4 has been insufficient since the beginning of Sok-SSBSs operation and its performance has not improved to the present time. On the other hand, removal of N-NH_4 at Str-SSBS could be related to the addition of the specialized denitrification zone (DENz) containing brown coal, which is not found in Sok-SSBS. Moreover, similar level of N-NO_3 removal between the previous (45%, Szklarek et al., 2018) and present study (46%) suggested that longer operational time does not enhance this process for the Sok-SSBS. On the contrary, the longer operational time seemed to have an impact on the efficiency of TP removal, since it was as high as 93% for the present study when compared to the 37% average TP removal in an earlier study conducted between the years 2011 to 2013 (Szklarek et al., 2018). The above observation suggested that the lower removal efficiency for TP in an earlier study could be related to a younger operational time and insufficiently developed geochemical zone.

4.2. Metabolic activity of heterotrophic microbial communities

The metabolic activity registered for the INF, sedimentation zone (SEDz), biofiltration zone (BIOz) and OTF [average well colour development (AWCD) between 1.5 and 2; Fig. 1a, b, 2a] suggested similarity of microbial activity through the horizontal profile of both SSBSs, with the exception of the geochemical zones (GEOz) that showed significantly lower metabolic activity (AWCD below 1.5, $p < .05$, Fig. 2a and Table S3). This results could be associated the better habitat provided by the abundant organic matter that is deposited in the matrix of biofilters that are connected to rivers (Taylor and Owens, 2009; Truu et al., 2009). In contrast, biofilm formations over the limestone surface in rivers are usually composed of more selected epilithic microbial communities adapted to specific organic and inorganic compounds through accumulation at the solid-liquid interface (Konhauser et al., 1994).

Diversity (Shannon Weaver, H) and richness (R) indexes were significantly different between the SEDz, GEOz and BIOz in both SSBSs

Table 3
Pearson correlations between *amo*A and *nos*Z gene copy numbers, environmental parameters and nutrient concentrations for both SSBSs.

Parameter	Sokołówka SSBS (Sok-SSBS)			Struga Gnieźnieńska SSBS (Str-SSBS)		
	Means	Log_{10} *amo*A	Log_{10} *nos*Z	Means	Log_{10} *amo*A	Log_{10} *nos*Z
*amo*A	2.74E+05	0.96*	0.54	7.26E+04	0.91*	0.35
*nos*Z	9.76E+06	0.50	0.84*	2.82E+07	0.24	0.90*
pH	7.22	0.24	−0.66**	7.72	−0.14	−0.95*
Temperature (C°)	15.89	0.17	0.95*	19.69	0.20	0.95*
Oxygen (mg L^{-1})	4.88	0.03	−0.63**	5.35	0.13	−0.38
N-NO_2 (mg/l)	0.06	0.03	−0.53	0.02	−0.34	−0.92*
N-NO_3 (mg/l)	1.26	0.07	−0.83*	4.37	−0.41	−0.84*
N-NH_4 (mg/l)	1.08	−0.25	0.39	0.11	−0.35	−0.87*
TN (mg/l)	3.11	−0.66**	−0.31	4.45	−0.01	−0.49
TP (mg/l)	0.07	0.05	−0.55	0.17	−0.48	−0.39

* Is significant correlation at ($p < .05$)
** Is significant correlation at ($p < .1$).

(Fig. 2b, c; Fig. S4), which suggested that although metabolic potential could be similar in the SSBSs zones, they were performed by different microbial populations. These results could be associated to differences in the INF chemical composition for both SSBS and variations in the construction and design of the SSBSs zones, e.g.: (1) Sok-SSBS covered larger areas than Str-SSBS and therefore presented higher water retention times, (2) Sok-SSBS usually received colder water than Str-SSBS, (3) the BIOz at Sok-SSBS is characterized by higher plant density for the uptake of nutrients, and (4) the addition of a DENz at the Str-SSBS to enhance denitrification, therefore affected the microbial diversity and its activity. In the case of the GEOz in both SSBSs, biofilm formation on the limestone showed higher diversity and richness at the Str-SSBS (Fig. 2b, c), which suggested a more diverse microbial community at the two-year SSBS. The previous result could be associated to the higher water flow observed for the GEOz in Str-SSBS, in contrast to the limestone barrier that was covered in sediments at the Sok-SSBS. Higher attachment and growth of microorganisms on submerged limestone rocks is more common for rivers with flowing water, as in the case of the limestone barrier in Str-SSBS (Mills and Maubrey, 1981). However, the removal efficiency of TP in Sok-SSBS was higher than in Str-SSBS (93% and 36%, respectively; Table 1), which may be related to the higher TP concentration at the INF of Sok-SSBS (0.277 mg L^{-1}) when compared to Str-SSBS (0.122 mg L^{-1}). In a previous study, phosphorus binding activity in limestone was found positively correlated with the increase of phosphorus concentration in the water (Arias and Brix, 2005).

The SSBSs operational time (eight vs two years) was also a factor modifying functional microbial communities within both SSBSs. The principal component analysis (PCA), taking into consideration the different substrates metabolized by microbial communities in the samples (carbohydrates, polymers, carboxylic and ketonic acids, amino acids, and amines/amides), showed a clear differentiation of the INF, SEDz and OTF at the Sok-SSBS with longer operational time (Fig. 3a). In contrast, the same results were not observed for the Str-SSBS with the younger time of operation (Fig. 3b). In the study from Chazarenc et al. (2010) functional microbial communities showed higher metabolic activity, diversity and richness in constructed wetlands (CWs) with longer time of operation. They also argued, that sediment stabilization in older CWs was responsible for the differentiation of microbial communities in the samples. Moreover, the AWCD for the utilization of polymers and carbohydrates at the eight-year Sok-SSBS suggested a higher metabolic activity of the microbial communities, however no statistical significance was observed for the AWCD (Fig. S4). Therefore, the above discussion suggested that longer time of operation in SSBSs helps in the differentiation of microbial communities in their horizontal profile, however metabolic activity remains similar despite the time of operation. Such observations suggested that both SSBSs present similar biodegradation potential, in terms of carbon substrate utilization, despite differences in operational time. Additionally, polymers (glycogen) and elementary sugars (such as D-mannitol) have been reported to be significantly metabolize in sediments from constructed wetlands, probably because of the great amount of energy that they provide for the Krebs cycle process (Chazarenc et al., 2010).

4.3. Dynamics of key functional genes in nitrification and denitrification processes

In the present study, the denitrifying bacterial community (dictated by the *nos*Z gene copy numbers g^{-1} of dry sediment) was more abundant (4.81×10^7 copies g^{-1} in Sok-SSBS and 1.16×10^8 in Str-SSBS) than the nitrifying community (dictated by the *amo*A gene copy numbers g^{-1} of dry sediment; 5.10×10^5 copies g^{-1} in Sok-SSBS and 5.68×10^5 in Str-SSBS) in both periods of collection (Fig. 5). A significant positive correlation was observed between the temperature and the abundance of denitrifying bacteria for both SSBSs (r^2: 0.95, $p < .05$, Table 3), which suggested their communities grow better in warm temperatures (18–24 °C). Such results allowed to explain the high abundance of denitrifying bacteria at the warmer period of August samples (4.81×10^7 copies g^{-1} for Sok-SSBS and 1.16×10^8 in Str-SSBS, Fig. 5a and c), and the progressive decrease in their abundance for the colder period (7–11 °C) in October samples (1.86×10^6 copies g^{-1} for Sok-SSBS and 7.41×10^6 in Str-SSBS, Fig. 5b and d). In a study from Chon et al. (2011) the *nos*Z gene at sediment samples from estuarine and waste water effluent CWs was also found to have higher abundances in warmer (average 20 °C) than in colder seasons (average 2 °C) (up to 1.00×10^9 copies g^{-1} and 1.00×10^6, respectively). Similar results were expected for the nitrifying bacteria harbouring the *amo*A gene, however low correlation with temperature was observed for both SSBSs (r^2: 0.17–0.20, $p > .05$, Table 3). Despite the above observation, the lower abundance of nitrifying bacteria when compared to the abundance of denitrifying bacteria has also been described in other studies, e.g.: in Wang et al. (2014), waste water treatment plant (WWTP) sludge samples presented a range of $45.8–7.33 \times 10^3$ copies of *amo*A gene per ng/DNA while *nos*Z gene copy numbers were in a range of $2.63 \times 10^4–4.66 \times 10^5$ (63 to 574 higher than the abundance of nitrifying bacteria). In Zhang et al. (2014a), estuarine sediments also presented lower abundance of nitrifying bacterial communities (up to 5.0×10^4 copy numbers of *amo*A gene g^{-1} wet sediment) than denitrifying bacteria (up to 1×10^6 copy numbers of *nos*Z gene g^{-1} wet sediment).

Low correlation between the *amo*A gene and N-NH_4 concentration could be related to the low inflow of ammonium in both systems (average of 0.455 mg L^{-1} in Sok-SSBS and 0.105 mg L^{-1} in Str-SSBS). Previous studies have described that autotrophic nitrifying bacteria were not significantly affected when N-NH_4 concentration was below 10 mg L^{-1} in water and sediments (Prinčič et al., 1998; Le et al., 2019).

Moreover, a significant negative correlation between the *nos*Z gene and the concentration of N-NO_3 was observed for both SSBSs, which could be associated with an increase in the amount of denitrifying bacteria and the decreasing amount of nitrate used to obtain energy during bacterial respiration (Kraft et al., 2011).

4.4. DNA sequence analysis of sediment samples

Sequences for the *nos*Z gene showed close similarity with three heterotrophic bacterial strains (Table S9), in which only *Thiobacillus denitrificans* has been described to perform full denitrification in anaerobic condition. The strain *T. thioparus* does not grow well in anaerobic conditions despite the presence of nar, nir, nor and nos operons encoding the synthesis of denitrifying proteins (Hutt et al., 2017). The above observation suggested a possible denitrification pathway in more aerobic conditions for the strain. Finally, the strain *Paracoccus sphareophysae* has also been described to perform denitrification and gas formation (Deng et al., 2011). In the case of sequences for the *amo*A gene, close similarity was observed with autotrophic strains from *Nitrosospira* spp., and *Nitrosomonas* spp. (Table S9), which have been described as ammonia oxidizing bacteria (AOB) with dominant roles in the process of nitrification in the environment (Rotthauwe et al., 1997).

4.5. Denitrifying and nitrifying bacterial community preferences in SSBSs

High abundances of denitrifying bacterial community at the DENz were associated to the addition of brown coal as a substrate. The high content of organic matter in brown coal (60–70% of compressed fossil peat) facilitates the donning of free electrons for the reduction of nitrogen forms in the process of biological denitrification, therefore improving the development of surface biofilm and the efficiency of nutrient removal from water (Zumft, 1997; Kuypers et al., 2011). In a previous study, the brown coal was proved to actively reduce above the 82% from effluents with high initial concentrations of NO_2^- (2145.55 mg L^{-1}), NO_3^- (2284.81 mg L^{-1}), and NH_4^+ (675.89 mg L^{-1}) after proper activation with the addition of microbiota from denitrifying barriers used to treat farming wastewaters (Mankiewicz-Boczek et al., 2017). Similar for nitrifying bacterial communities, preferences for brown coal in DENz was described by the higher abundances of *amo*A gene for the sampling period in October (5.68×10^5 copies g^{-1}). However, it is important to mention that nitrifying communities dictated by the abundance of *amo*A gene are strictly autotrophic (Rotthauwe et al., 1997), and therefore their high abundance associated to a carbon substrate is rather unexpected. To our knowledge, there are no studies describing abundances of autotrophic nitrifying bacteria in brown coal, and we can only speculate that active nitrification processes may be accomplished between the interaction of heterotrophic and autotrophic nitrifying bacteria. Therefore in future studies there is a great interest to investigate the relationship between bacteria harbouring the *amo*A gene and the addition of brown coal in the above mentioned SSBSs.

5. Future optimization of SSBS zones to improve the overall nutrient removal

The efficiency of nutrient removal changes through the extended operational time in any type of constructed biofilters, usually decreasing until they become inefficient if no periodical maintenance is applied. Periodical monitoring of environmental and biological parameters that are indicators to identify drops in their efficiency, and such observations may be used as feedback strategies to improve and optimize the design in old or newly constructed systems (Beharrel, 2004).

For the case in the Sok-SSBS, inefficiency in N-NH_4 removal may be associated to periodical events like the water draining in autumn and the removal of sediments from the SEDz in spring, which in turn may also affect abundance of bacterial communities. Such problem may be solved with the experience obtained from the newly designed DENz at the Str-SSSBS, which has demonstrated that denitrifying and nitrifying bacterial communities have been significantly higher when brown coal was added. The brown coal could be added and fixed to the bottom of the SEDz so it is not easily removed when the system is cleaned. Another solution includes its addition to the bottom in the BIOz, where it could be added to a designated plant-free area or mixed within the plant sediments.

In the case of the GEOz, its microbial metabolic potential, diversity and richness could be enhanced while providing more surface area for the attachment and development of microbial biofilms on limestone. Pore size in limestone plays an important role in the attachment of microbial biofilm at their surface, and it can increment in moderate to fast flowing water in rivers (Konhauser et al., 1994). Such observation helped to explain significant higher functional microbial diversity and richness for the GEOz in Str-SSBS when compared to the Sok-SSBS where the limestone barrier was cover by sediment (Fig. 2b, c). Additionally, in a study from Suantika et al. (2016) biofilm microbial communities in limestone also showed to include nitrifying bacterial communities that actively removed 33% of N-NH_4 (70 ppm as initial concentration) in one day, and therefore can considerably help in removal of nitrogen compounds. Other idea becomes from the product “BioKer” which could be added to the limestone in the GEOz. This product contains specialized biopolymers that carry additional carbon sources to stimulate growth of microorganisms over the surface of a small round ceramic structure (Ministerstwo Nauki i Szkolnictwa Wyższego, 2018).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoleng.2020.105796.

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Appendix 2: Font Nájera A., Serwecinska L., Szklarek S., Mankiewicz-Boczek J. 2021a. *Seasonal and spatial changes of N-transforming microbial communities in sequential sedimentation-biofiltration systems - Influence of system design and environmental conditions.* International Biodeterioration & Biodegradation, 159, 105203, doi.org/10.1016/j.ibiod.2021.105203

Lodz May 26th, 2021

Statement of co-authorship

I declare that my contributions to the preparation of the original work were: i) the conceptualization and idea of the work, ii) collection of samples, iii) execution of experiments - analysis of microbial metabolic activity and genetic analysis,, iv) formal analysis of results, v) revision of the existing knowledge about the present topic, vi) writing the original draft of manuscript, vii) correction and editing after review, viii) and the final visualization of the work. I assess that my participation represents 50% of the work.

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I declare that my contributions to the preparation of the original work were: i) revision of microbiological data analysis, iii) and the edition of the text in the manuscript. I assess that my participation represents 10% of the work.

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I declare that my contributions to the preparation of the original work were: i) help in collection of samples, and ii) performance and elaboration of chemical analysis. I assess that my participation represents 10% of the work.

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I declare that my contributions to the preparation of the original work were: i) the general conceptualization of the idea of publication, ii) supervision of genetic experiments, iii) revision/edition of genetic data analysis, iv) and the edition of the text in the manuscript. I assess that my participation represents 30% of the work.

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Seasonal and spatial changes of N-transforming microbial communities in sequential sedimentation-biofiltration systems - Influence of system design and environmental conditions

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ABSTRACT

The microbial activity in nitrogen (N) and phosphorus (P) transformation is an important parameter ensuring proper functioning of innovative biotechnological solutions involved in urban water treatment - sequential sedimentation-biofiltration systems (SSBSs). The study focused on metabolic activity, seasonal variability, and construction differences of microbial communities in three SSBSs. Carbon source utilization indicated that microbial metabolic potential was higher in spring and summer as compared to autumn. Bacterial gene abundances in nitrification (*amo*A) and denitrification (*nos*Z) indicated that nitrifiers were higher in spring (14.2 ± 2.5 °C) and denitrifiers in summer (21.9 ± 1.9 °C). Brown coal biofilm (denitrification zone) presented the highest *nos*Z (2.02×10^{10} copies g^{-1}). Bacterial community analysis using 16S rRNA and correlations with TN, NO_3-N, and NH_4-N concentration, suggested that the *Comamonadaceae* (*Limnohabitants*), *Flavobacteriaceae* (*Flavobacterium*), *Crenotrichaceae* (*Crenothrix*), and *Rhodobacter* were important communities involved in N transformations. Limestone biofilm (geochemical zone) presented the highest *amo*A (8.95×10^7 copies g^{-1}), and the *Comamonadaceae*, *Rhodocyclaceae* (*Dechloromonas*), *Rhodobacter*, and *Crenothrix* were suggested to be involved in nutrient transformation processes. Metabolic activity and abundance of microbial communities increased when the temperature was higher than 10 °C, and it consequently helped to improve the removal efficiency of nutrients in SSBSs.

1. Introduction

Sequential sedimentation-biofiltration systems (SSBSs) are proposed as new, easy operating and low cost nature-based solutions for the treatment of urban contaminated rivers (Jurczak et al., 2019). According to the ecohydrological principles, three SSBSs were designed to increase the resilience by enhancing the removal capacity of environmental pollutants in urban areas (Zalewski, 2014; Jurczak et al., 2018; Font Nájera et al., 2020). They are currently being developed as an applied model for urban nutrient removal. SSBSs differ from conventional constructed wetlands (CWs) with the introduction of complex multizone structures: a sedimentation, geochemical, biofiltration (Szklarek et al., 2018), and denitrification zone (Font Nájera et al., 2020), while CWs usually only present a biofiltration zone (Truu et al., 2009). The structure in SSBSs is proposed to ensure the active removal of nutrients regardless of the season and periods of increased rainfall or drought. Previous studies on SSBSs in urban rivers have shown to reduce up to 93% of total phosphorus (TP), 71.5% of total nitrogen (TN), 91.3% of NO_3^-, 55% of NO_2^- and 48% of NH_4^+ (Serwecinska et al., 2017; Szklarek et al., 2018; Font Nájera et al., 2020). One of these systems, located in the city of Lodz in Poland, was included in the innovative hybrid system for the purification of street stormwater runoff supplying urban recreation reservoirs (Jurczak et al., 2019), and was implemented in the LIFE + project entitled: "Ecohydrologic rehabilitation of recreational reservoirs "Arturówek" Lodz as a model approach to rehabilitation of urban reservoirs". Based on the design, construction and monitoring results, this hybrid system was awarded by the European Commission in 2018 as the best of the best environment project for the year 2016.

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SSBSs are designed to be an innovative biotechnological solution, using the comprehensive knowledge of natural processes to treat contaminated water, including sedimentation of particles, adsorption and mineralization of phosphorus (P) in rocks and the uptake of nutrients by plants (Zalewski et al., 2012; Szklarek et al., 2018; Jurczak et al., 2019; Font Nájera et al., 2020). The knowledge of microorganisms, with the above-mentioned processes, is also a crucial element to improve and optimize the efficiency and operation of SSBSs. The microbial community can carry out numerous nutrient removal processes in water and sediments (Zhang et al., 2013; Zhi et al., 2015). In the case of nutrient cycling, bacterial nitrification and denitrification have been described as important ecological processes for nitrogen (N) transformation in aquatic ecosystems (Freese et al., 2006; Canfield et al., 2010). The process of bacterial nitrification starts with the oxidation of NH_4^+ to form NO_2^- and NO_3^-, which are subsequently reduced to N_2 in the denitrification process (Kraft et al., 2011). The most widespread nitrifying bacteria in the environment include the ammonia oxidizing bacteria (AOB), nitrite oxidizing bacteria (NOB) and the complete ammonia oxidizing bacteria (COMAMMOX), while denitrifying bacteria are represented by more numerous and varied taxonomic bacterial groups (Burgin and Hamilton, 2007; Canfield et al., 2010; Holmes et al., 2009). Bacteria can also remove P from water by accumulating it into complex polymers (polyphosphates). These bacteria belong to a broad taxonomic group of microorganisms known as the polyphosphate accumulating organisms (PAOs), however, they have been studied mostly for waste water treatment plants (Tarayre et al., 2016; Coats et al., 2017).

Our previous study represented the first research focusing on the preliminary characterization of microbial communities in two SSBSs with similar construction (Font Nájera et al., 2020). The geochemical zones containing limestone barriers presented the lowest metabolic activity, and the application of a denitrification zone, containing brown coal, was found to considerably increase the abundance of denitrifying and nitrifying bacteria. Further knowledge is needed to understand the role of microbial communities on the transformation of nutrients in SSBSs with different construction, including their seasonal variability in abundance and metabolic activity. The knowledge about diversity of bacterial communities involved in the process of nutrient transformation could also aid in the better design of new SSBSs. Moreover, we hypothesized that the efficiency of nutrient reduction in SSBSs could be improved, regarding the limits establish by the Polish legislation, with the application of geochemical zones containing barriers with limestone or dolomite, and denitrifying zones containing brown coal.

The aim of the present study was to describe microbial communities in SSBSs involved in nutrient transformations, with a special focus on their sensitivity in two factors: the seasonality and the differences in the system construction. The microbial communities were characterised with two different approaches: (i) the community level physiological profile (CLPP) to describe their metabolic activity and carbon source utilization, and (ii) the quantification of N-transforming bacterial functional genes in the processes of nitrification (*amo*A) and denitrification (*nos*Z). Both approaches were applied for three periods representing different seasons (spring, summer, and autumn of 2018). The SSBS from Bzura River (Łódź city) was included in the present study, which is different in construction and design from the first analysed systems on the Sokołówka and Struga Gnieźnieńska Rivers. Therefore, the present study includes novelty with a comparison of the above mentioned microbial communities between three different systems, which has not been addressed in previous research. The study monitored changes of oxygen concentration, pH, temperature and the concentrations of NH_4-N, NO_3-N, PO_4-P, TP and TN in the water across seasons, and were analysed to investigate the relationship between the nitrification/denitrification gene abundances and chemical processes. Additionally, bacterial community structure was described for SSBSs zones where microbial biofilm developed using 16S rRNA high-throughput sequencing. The above analysis allowed to identify bacterial taxa that could be linked to the N and P-transforming processes in SSBSs. All the knowledge obtained in the present study will help to improve the planning and construction of SSBSs to ensure optimal conditions for the growth and activity of microorganisms involved in nutrients transformation.

2. Materials and methods

2.1. Studied sites and SSBSs construction

The study was performed in three different SSBSs: the Sokołówka-SSBS (Sok-SSBS) with 9 years of operation, the Bzura-SSBS (Bzr-SSBS) with 5 years and the Struga Gnieźnieńska-SSBS (Str-SSBS) with 3 years. The Bzr-SSBS (2600 m^2 of surface area) is the biggest system followed by Sok-SSBS (1040 m^2) and the Str-SSBS (119 m^2). The structure and size of the systems are presented in Fig. 1. The systems include three or four internal zones performing different processes for water treatment: (i) the sedimentation zone (SEDz) to retain organic and coarse material, (ii) the geochemical zone (GEOz) for P adsorption using barriers with limestone (Sok-SSBS and Str-SSBS) or dolomite (Bzr-SSBS), (iii) the denitrification zone (DENz) using brown coal to scale up microbial metabolic activity (only Str-SSBS), and (iv) the biofiltration zone (BIOz) with macrophytes for the uptake of nutrients (N and P species). The systems were constructed to treat urban contaminated water and stormwater runoff as well as to control harmful toxic cyanobacterial blooms in lowland recreational reservoirs, which are located below the systems (Szklarek et al., 2018; Font Nájera et al., 2020). More information for each SSBS is included in Table S1.

2.2. Sample collection and processing

Water and sediment samples for chemical, molecular and microbiological analysis were collected from the horizontal profiles at the inflow (INF), SEDz, BIOz and outflow (OTF) in all SSBSs. For GEOz, limestone or dolomite was collected in the barriers. For DENz, brown coal was collected at the bottom. Conditions for sample collection and transportation to the laboratory are well described in Font Nájera et al. (2020). *In situ* physico-chemical parameters [temperature (T°), dissolved oxygen (DO) and pH] were measured using a YSI multimetric probe, directly above the sediment layer. Samples were collected in three different seasons of the year 2018: (1) 25th of April for Str-SSBS, and the 26th of April for Sok-SSBS and Bzr-SSBS (spring); (2) 2nd and 3rd of August, respectively (summer); and (3) 6th and 7th of November, respectively (autumn). The frozen water limited the operation of SSBSs during the winter season, and therefore was not included in the present study. Samples for microbiological and molecular analysis were processed within 48 h after collection. Samples for chemical analysis were categorized in: (1) surface water, (2) interstitial water and (3) sediments. Samples for interstitial water (aqueous solution in the pore space between the sediment particles) were obtained after slow speed centrifugation of sediments (2500 g) (Ankley et al., 1994). Interstitial water was analysed in the present study because it represents the intermediary aqueous solution where microbial communities dwell and obtain dissolved nutrients and carbon sources provided by the sediments.

2.3. Chemical analysis of nutrients

Nutrient concentrations (NH_4-N, NO_3-N, PO_4-P) in surface and interstitial water samples were measured using the Dionex® ion chromatograph (Szklarek et al., 2018). TN was measured with the Kjeldahl method (Hach, 1997). For TP, the sample was digested with Oxysolv® (Merk) and measured by the ascorbic acid colorimetric method (Golterman et al., 1978). Organic matter content was measured for sediment samples using the loss on ignition standard method (LOI; Santisteban et al., 2004).

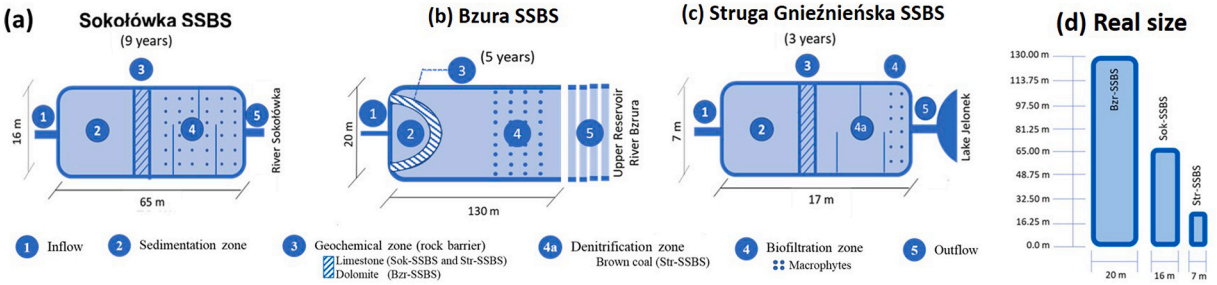

Fig. 1. Description of SSBSs construction, the horizontal profile of (a) Sok-SSBS, (b) Bzr-SSBS and (c) Str-SSBS, and the real size of the systems. The graphic description of each SSBS was adapted from other studies: (a) Szklarek et al. (2018); (b) Szulc et al. (2015) and Jurczak et al. (2019); and (c) Font Nájera et al. (2020).

2.4. Community level physiological profile (CLPP)

Catabolic activity of heterotrophic bacteria was assessed using the specifications from BIOLOG™ Ecoplates (Biolog Inc., Hayward CA, USA). Each plate contained 31 different carbon sources and a blank control (no carbon source) in triplicate, mixed with the dye tetrazolium violet (Button et al., 2015b). CLPP analysis was performed for sediment samples collected at the INF, SEDz, BIOz and OTF, and microbial biofilm formed over the limestone or dolomite at the GEOz barriers in all SSBSs. All above mentioned zones were constructed for the three SSBSs, and therefore were included in the CLPP analysis. Sterile conditions, handling, and preparation of the suspension samples from the sediments and biofilm were previously described (Font Nájera et al., 2020). All inoculated plates were incubated in dark at 27 °C for 10 days and measured every 24 h using a Biolog MicroStation ELX808BLG reader analyser at 590 nm.

2.5. DNA isolation and quantitative analysis of selected genes in N transformation

DNA was isolated from 500 mg (wet mass) of sediments (INF, SEDz, BIOz and OTF), biofilm from limestone/dolomite (GEOz) and brown coal (DENz) samples, according to the FastDNA™ SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA). Handling and processing of biofilm samples were previously described (Font Nájera et al., 2020). Abundance of nitrifying and denitrifying bacterial communities were described using the genes *amo*A and *nos*Z, respectively. In general, other studies have placed particular interest in the detection of both genes in environmental samples, since *amo*A harbouring communities of the *Nitrosomonadaceae* family are known to perform an important role in nitrification and are usually dominant in sediments from freshwater ecosystems (Lukumbuzya et al., 2020), and the *nos*Z gene has been used as a marker targeting communities that are able to complete the process of denitrification (Jones et al., 2013; Ma et al., 2008). Gene abundances were assessed with quantitative PCR (qPCR) using Maxima SYBR green/ROX master mix (Thermo Scientific) and the Rotor Gene Q thermocycler (Qiagen). The primer sets amoA-1F/amoA2R (491 bp; Rotthauwe et al., 1997) and nosZF/nosZR (380 bp; Chon et al., 2011) were used for the qPCR assays as described in Font Nájera et al. (2020). For comparison purposes, the total abundance of bacteria was estimated with the 16S rRNA gene using the primer set 341f/525r (174 bp) according to López-Gutiérrez et al. (2004). Standard curves were prepared using plasmids carrying a purified PCR product from environmental samples (Ma et al., 2008). Eight serial diluted standard curves (2×10^1–2×10^8 gene copies uL^{-1}) and one negative control were used for qPCR. The efficiency (%) and the r^2 of the reactions were high for the N-transforming genes (>90% and >0.98, respectively; Font Nájera et al., 2020), and the 16S rRNA gene (98.7% and 0.97, respectively; Fig. S1). Bacterial key functional gene abundances were analysed as g^{-1} of dry sediments (Buxton et al., 2018).

2.6. Bacterial community structure analysis

The structure of bacterial communities was described for biofilm samples from limestone (GEOz at Sok-SSBS and Str-SSBS) and brown coal (DENz at Str-SSBS) collected during summer and spring of 2018. Samples were selected due to the high differences of key functional gene abundances observed between sampling seasons. Autumn samples were not included in the analysis due to the low significant difference observed with the summer season. High throughput new generation sequencing was used by targeting the region V3-V4 of the gene 16S rRNA using the primer ser S-D-Bact-0341-b-S-17/S-D-Bact-0785-a-A-21 (494 bp; Klindworth et al., 2012) with a High Illumina Mini Seq platform (San Diego, CA, USA). Preparation of DNA libraries and sequencing were performed by the Science and Technology Park "BionanoPark" (Lodz, Poland). The fastq files containing reads were classified using the RDP Naïve Bayesian classifier, which allows to identify the taxonomic level for paired-end reads, and the Greengenes 13_5 database was used to assign an operational taxonomic unit (OTU) to each read. Original data sets in FASTQ files were uploaded at the NCBI Sequence Read Archive under the project PRJNA645459 with six bio samples: SAMN15505344, SAMN15505345, SAMN15505346, SAMN15505347, SAMN15505348 and SAMN15505349.

2.7. Data analysis

Nutrient removal efficiencies (%) were estimated with the difference in nutrient concentration between the INF and the OTF (the media from three different sampling periods). The nutrient concentrations were also compared with the limits established by the Polish legislation (Dz.U, 2019 poz. 2149).

For CLPP analysis, average well colour development (AWCD), Shannon-Weaver diversity (H) and Richness (R) were calculated (Zak et al., 1994). The 120 h of Ecoplate incubation time was used to describe catabolic activity of microbial communities based on the greater variance observed between responses in the samples (Button et al., 2015a). Significant differences ($p < .05$) were assessed through the one-way ANOVA and Tukey's honesty significant difference (HSD) test. Carbon substrate utilization was organized in five chemical groups: (1) carbohydrates, (2) polymers, (3) carboxylic and ketonic acids, (4) amino acids and (5) amines and amides (Weber et al., 2009).

The gene abundances representing nitrifying and denitrifying bacterial communities in different seasons (*amo*A and *nos*Z, respectively) were compared for the three SSBSs. The average of abundance was calculated for both genes in each season of collection and for all three systems. The averages were used to evaluate significant differences ($p < .05$) between bacterial communities and the season of collection with the non-parametric Kruskal Wallis and Mann Whitney pairwise tests. Moreover, the abundance of the 16S rRNA gene was compared with the abundance of N-transforming genes, in order to describe the percentual composition of nitrifiers and denitrifiers in relation to the total bacterial community.

A principal component analysis (PCA) was used to identify patterns

Table 1
Comparing the average physico-chemical parameters, nutrient concentrations, organic matter and removal efficiencies estimated for SSBSs in three different seasons (spring, summer and autumn), 2018, with the limits according to the Polish legislation.

SSBS	Area	pH	Temp. C°	Oxygen mg L^{-1}	NO_3-N mg L^{-1}	NH_4-N mg L^{-1}	TN mg L^{-1}	PO_4-P mg L^{-1}	TP mg L^{-1}	Organic Matter mg g^{-1}
Sok	INF	7.81 ± 0.19	13.63 ± 4.62	10.10 ± 2.79	0.94 ± 0.55	**0.59 ± 0.34**	4.20 ± 1.20	0.20 ± 0.15	0.34 ± 0.17	**63.26 ± 14.16**
	OTF	7.43 ± 0.02	13.53 ± 5.00	3.96 ± 1.39	0.54 ± 0.39	**0.67 ± 0.24**	2.23 ± 0.21	0.08 ± 0.06	0.12 ± 0.04	**79.71 ± 39.03**
Nutrient removal efficiency (%)					42.9	**NR**	47	59	64.7	**NR**
Bzr	INF	7.71 ± 0.09	14.90 ± 4.04	10.05 ± 0.31	0.24 ± 0.09	0.28 ± 0.30	2.16 ± 0.92	0.09 ± 0.09	0.23 ± 0.11	83.50 ± 23.54
	OTF	7.72 ± 0.40	16.37 ± 5.85	6.81 ± 1.44	0.09 ± 0.09	0.08 ± 0.06	1.43 ± 1.32	0.03 ± 0.03	0.08 ± 0.06	26.32 ± 3.24
Nutrient removal efficiency (%)					62.5	71.4	33.7	63	65.2	68.5
Str	INF	7.99 ± 0.29	17.47 ± 3.01	6.37 ± 3.84	0.92 ± 0.91	0.47 ± 0.22	3.48 ± 1.07	0.42 ± 0.12	0.44 ± 0.11	**21.06 ± 4.43**
	OTF	7.84 ± 0.23	18.27 ± 4.02	7.13 ± 4.26	0.34 ± 0.43	0.12 ± 0.10	2.33 ± 0.65	0.06 ± 0.05	0.12 ± 0.03	**24.97 ± 7.49**
Nutrient removal efficiency (%)					63.3	73.8	32.9	84.9	72.5	**NR**
Limits according to the Polish legislation (Dz.U, 2019 poz. 2149)										
Sok (17)	NA	7.0–7.9	≤24	≥6.8	≤3.4	≤0.738	≤4.9	≤0.101	≤0.3	NE
Bzr (17)										
Str (24)	NA	7.0–8.1	≤24	≥7.2	≤1.7	≤0.35	≤2.8	≤0.101	≤0.21	NE

Temp.: temperature; physico-chemical parameters were measured above the sediment layer; nutrient concentrations were calculated from surface water; organic matter was calculated from dry sediments. Values represent the media and the standard deviation of three sampling seasons (n = 3). Nutrient removal efficiency (%) was estimated with the difference in nutrient concentration between INF: inflow and OTF: outflow (media from three different sampling periods). Bold numbers indicate no nutrient reduction or increment in the nutrient concentration, from INF to OTF direction. NR: removal efficiency was not calculated, due to an overall nutrient increment in all three sampling seasons. Full seasonal description of chemical analysis was described in Table S2. (17) and (24): classification established by the Polish legislation.

that could explain the relationship between the abundance of N-transforming genes, physico-chemical parameters and nutrient concentration from the interstitial water. The Pearson correlation test (*r*) was used to estimate the significance. The one-way ANOVA and the Tukey's HSD test were used to assess differences between seasonal sampling periods in the PCA.

For the bacterial community structure analysis, the 16S rRNA sequences with assigned OTUs were assessed through the relative abundance (%) between samples, to identify key bacterial taxa that were abundant according to (a) the season of collection and (b) the type of sample (limestone in Sok-SSBS and Str-SSBS, or brown coal in Str-SSBS). The PCA was performed to identify key physico-chemical parameters and N and P species from the surface water that could link the taxonomic composition (bacterial family) with the functional potential (nutrient transformation) of the sample in GEOz and DENz.

3. Results

3.1. Nutrient removal efficiency

A summary of environmental parameters and the nutrient removal efficiency for SSBSs is presented in Table 1. Complete seasonal description of physico-chemical parameters and nutrient concentrations in surface and interstitial water was described in Table S2. The Str-SSBS showed the highest removal efficiencies for PO_4-P and TP in surface water (84.9% and 72.5%, respectively), due to the higher INF (0.42 and 0.44 mg L^{-1}, respectively) when compared to the Sok-SSBS and the Bzr-SSBS (Table 1). The removal efficiencies of NO_3-N, NH_4-N and TN in surface water were similar between Str-SSBS (63.3, 73.8 and 32.9%, respectively) and Bzr-SSBS (62.5, 71.4 and 33.7%, respectively), however a higher INF of N species were observed for Str-SSBS when compared to Bzr-SSBS (Table 1). The Sok-SSBS presented no removal of NH_4-N from surface water (Table 1), which was also reflected in the removal efficiency of NH_4-N and TN for interstitial water (Table S2). For the organic matter in sediments, only the Bzr-SSBS showed effective removal (68.5%) from INF (83.50 mg g^{-1}) to OTF (26.32 mg g^{-1}) direction (Table 1), while no reduction was observed at the Sok-SSBS and Str-SSBS (Table 1).

The physico-chemical parameters and nutrient concentrations of SSBSs were also compared with the limits according to the Polish legislation (Table 1). All SSBSs were generally within an acceptable range according to the measured physico-chemical parameters, with an exception only observed for oxygen concentration at the OTF in Sok-SSBS (Table 1).

3.2. Microbial metabolic activity

The CLPP analysis, revealed the microbial metabolic activity of heterotrophic bacteria in SSBSs, and is summarized in Fig. 2 and Table S3. The dynamic of microbial metabolic activity was similar for both, the Sok-SSBS and the Str-SSBS, where the AWCD in summer (up to 2.07 and 2.05, respectively) and spring (up to 1.86 and 1.99, respectively) were significantly higher than in autumn (up to 1.28 and 1.14, respectively) (Fig. 2a, c). Considering individual zones, for GEOz and BIOz, the metabolic activity in summer was found significantly higher in respect to spring for the Sok-SSBS and the Str-SSBS (Fig. 2a, c and Table S3). For Bzr-SSBS, the metabolic activity was significantly lower (regardless of the season) when compared to the other two systems (Fig. 2). There was no seasonal significant difference for the GEOz and the BIOz in Bzr-SSBS (Fig. 2b). The lowest metabolic activity was observed within the GEOz at the Bzr-SSBS regardless of the season (0.10 or less, Fig. 2b). The Shannon-Weaver diversity and richness indexes showed similar patterns to the metabolic activity for all SSBSs (Table S3).

The microbial carbon sources utilization pattern is presented in Fig. 3. The results suggested that microbial communities performed the

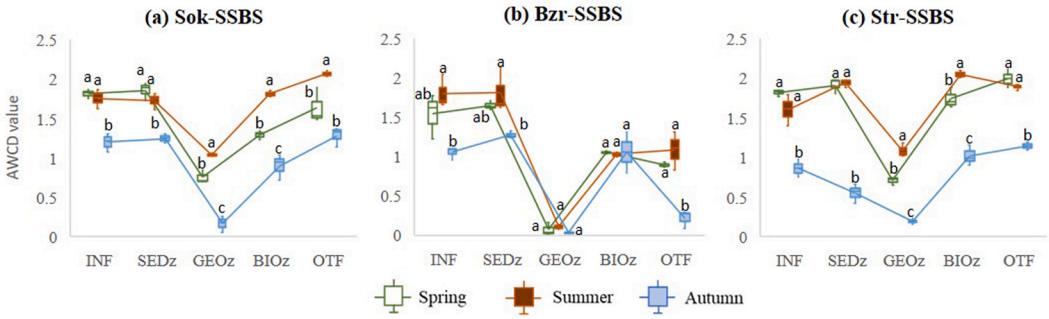

Fig. 2. Seasonal dynamics of the microbial metabolic activity in SSBSs sediments. High AWCD values represent high metabolic activity. Significant differences ($p < .05$), between seasonal AWCD values for individual zones, were marked by lower case letters (a to c). The statistical analysis was described in Table S4. Each boxplot represents one sample from the respective SSBS zone measured in triplicate, and the error bars the standard error of the mean (n = 3).

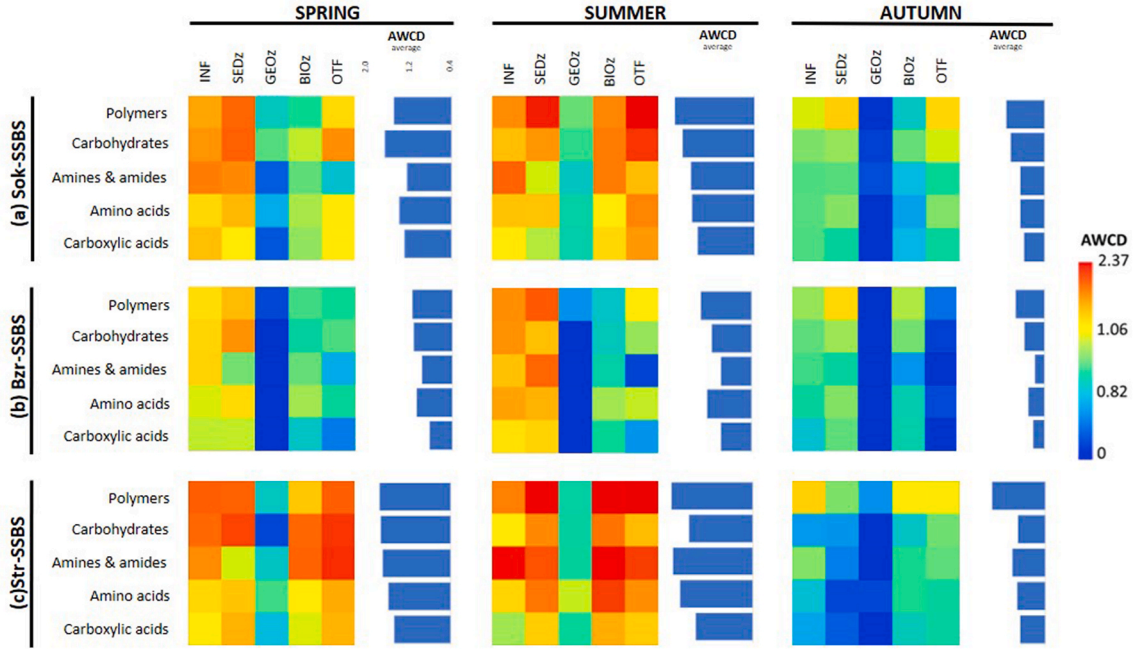

Fig. 3. Seasonal and spatial variability of carbon substrate utilization by microbial communities in (a) Sok-SSBS (b) Bzr-SSBS and (c) Str-SSBS. AWCD: average well colour development. High AWCD values represent high profiles of carbon source utilization. Blue bars, on the left of each matrix, represent the cumulative average of specific carbon source utilization. The AWCD values for independent carbon sources were detailed in Table S5 for Sok-SSBS, Table S6 for Bzr-SSBS and Table S7 for Str-SSBS.

highest overall carbon sources utilization during summer (Fig. 3). The level of carbon source utilization dropped significantly during autumn in all systems (Fig. 3). The Sok-SSBS and Str-SSBS showed higher profiles of carbon utilization by microorganisms in summer and spring (Fig. 3a, c). The GEOz, in all three seasons and systems, was characterized by the lowest carbon source utilization, as it was in the case of metabolic activity (Figs. 2 and 3). According to the carbon substrate chemical groups, glycerol, which represents polymers, and D-mannitol, N-acetyl-D-glucosamine and D-cellobiose, among carbohydrates, were the most catabolized substrates (Fig. 3). In the case of Str-SSBS and Sok-SSBS, the carbohydrates, amine/amides and amino acids were also highly catabolized, however their utilization was different between seasons (Fig. 3a, b). In the case of Bzr-SSBS, carbohydrates and amino acids were also utilized, with the exception of amine/amides, which showed a lesser profile of utilization (Fig. 3c).

3.3. Dynamics of functional genes in N transformation

The seasonal and spatial averages of key functional gene abundances (copy numbers g^{-1} of dry sediment) for bacterial nitrification (*amo*A) and denitrification (*nos*Z), and their comparison with the total abundance of bacteria (16S rRNA), were presented in Fig. 4 and Table S8. The 16S rRNA abundances were higher in summer for Sok-SSBS, Bzr-SSBS, and Str-SSBS (up to 3.89×10^{10}, 4.02×10^{10}, and 1.94×10^{11} gene copies g^{-1}, respectively), followed by autumn (up to 5.11×10^{9}, 5.07×10^{9}, and 1.32×10^{10} gene copies g^{-1}, respectively), and the lowest abundances were observed for the spring (up to 8.10×10^{8}, 5.31×10^{8}, and 3.65×10^{8} gene copies g^{-1}, respectively). For Sok-SSBS and Str-SSBS, the *amo*A was significantly higher during spring (up to 8.95×10^{7} and 5.73×10^{7} gene copies g^{-1}, respectively), when compared to summer and autumn (Fig. 4a, c). The opposite relationship was observed for *nos*Z at Sok-SSBS and Str-SSBS, which was significantly higher during summer (up to 2.42×10^{9} and 2.02×10^{10} gene copies g^{-1}, respectively) and autumn (up to 2.39×10^{8} and 1.49×10^{8} gene copies g^{-1}, respectively), when compared to spring (Fig. 4a, c). For Bzr-SSBS, the *amo*A showed no significant seasonal differences (Fig. 4b). The gene *nos*Z in Bzr-SSBS was significantly higher during summer (up to 4.84×10^{6} copies g^{-1}) and autumn (up to 2.12×10^{6} copies g^{-1}), when

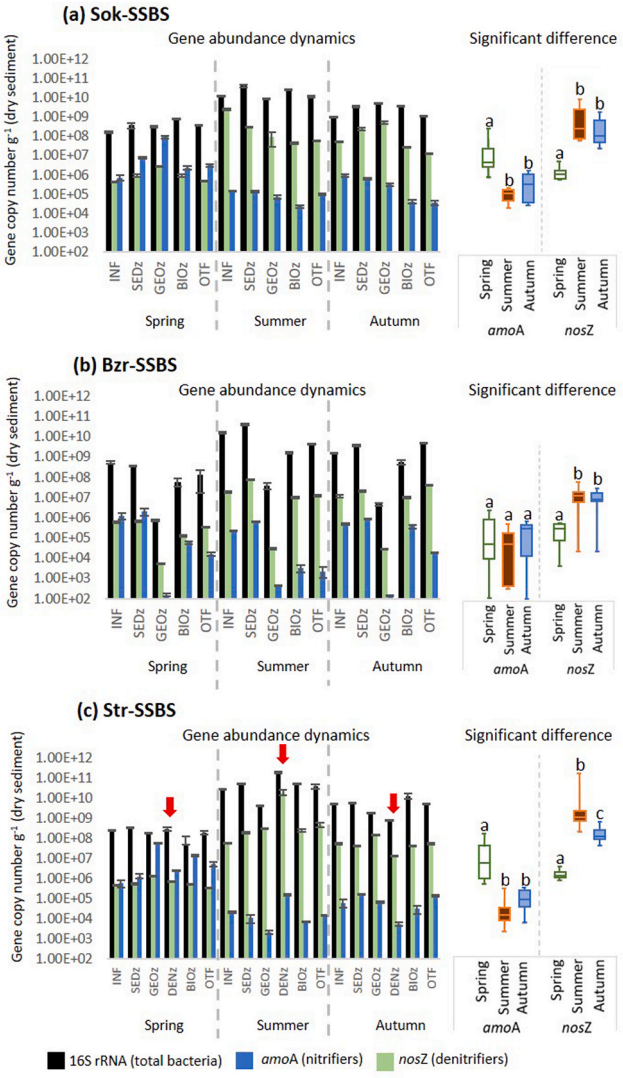

Fig. 4. Seasonal and spatial dynamics of total bacteria and N-transforming gene abundances in (a) Sok-SSBS, (b) Bzr-SSBS and (c) Str-SSBS. The denitrifying zone (DENz) was marked with an arrow to annotate the difference in the horizontal profile for the Str-SSBS. Box plots represent all samples in the horizontal profile of SSBSs: INF, SEDz, GEOz, BIOz and OTF (n = 5); the DENz in Str-SSBS was also added (n = 6). Significant differences ($p < .05$), between seasonal abundances for each gene, were marked by lower case letters (a to c). The statistical analysis was described in Table S10.

compared to spring (Fig. 4b). According to the ratio between the copy numbers of N-transforming genes and the 16S rRNA (Table S9), the *amo*A showed the highest proportions in spring (up to 31.1%), when compared to summer and autumn (Table S9). On the contrary, the *nos*Z showed higher ratios during summer and autumn (up to 20 and 10.3%, respectively), when compared to the spring (Table S9). Considering individual zones, the GEOz in Sok-SSBS and Str-SSBS (containing limestone) showed higher gene copy numbers g^{-1} of *amo*A (8.95×10^7 and 5.73×10^7, respectively) and *nos*Z (5.29×10^8 and 3.09×10^8, respectively), when compared to the GEOz in Bzr-SSBS (containing dolomite) (Fig. 4). For Str-SSBS, the DENz (containing brown coal) showed the highest gene copy numbers g^{-1} for *nos*Z during summer (2.02×10^{10}; Fig. 4c).

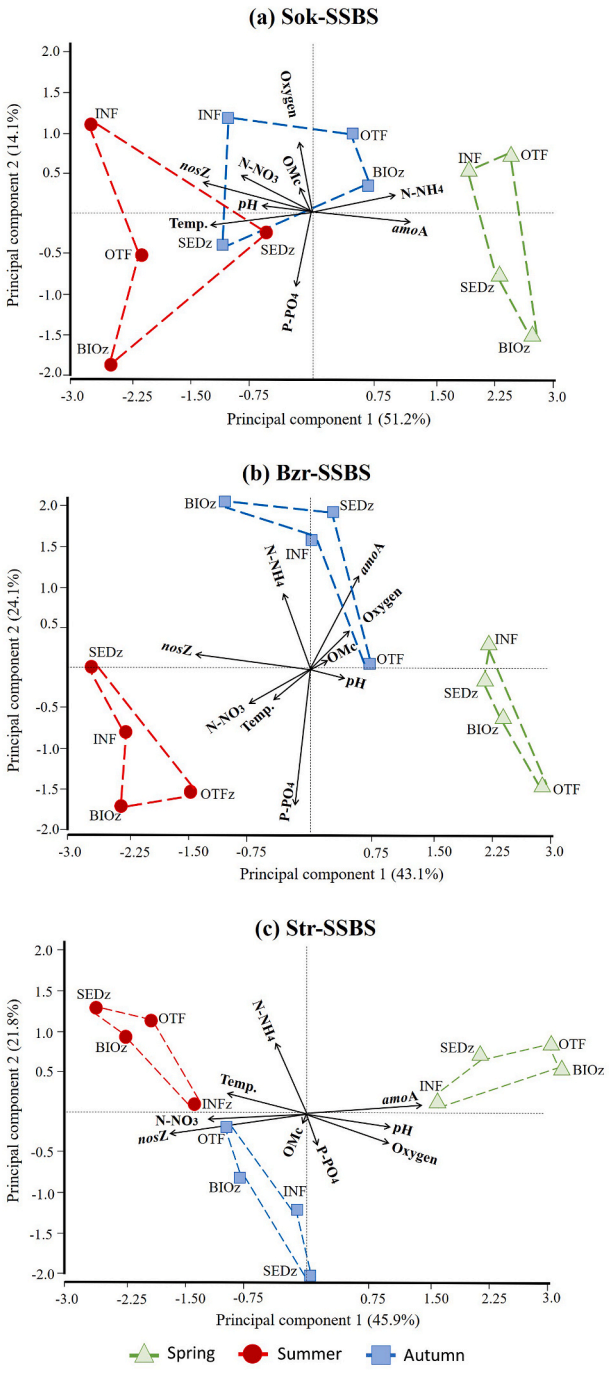

Fig. 5. Principal component analysis (PCA) explaining the seasonal and spatial relationship between sediment samples collected in (a) Sok-SSBS, (b) Bzr-SSBS and (c) Str-SSBS. The environmental parameters and N-transforming gene abundances were used to explain the data variation. The diagrams were prepared with the PC1 and PC2, which both explained the highest variability (%) for all three SSBSs (65–67%, approximately). The PCA scores and loadings were described in Tables S11 and S12. The samples were grouped according to the season of collection, and the groups were found significantly different when the PC1 scores were considered for the statistical analysis (one-way ANOVA test and Tukey HSD, $p < .05$), see Table S13.

3.4. Relationship between the environmental parameters and the abundance of N-transforming genes

The seasonal and spatial variability of sediment samples, according to the relationship between the environmental parameters and the

Table 2
Pearson correlation (r) between the environmental factors and the N-transforming gene copy numbers.

	Sok-SSBS				Bzr-SSBS				Str-SSBS			
	*amo*A		*nos*Z		*amo*A		*nos*Z		*amo*A		*nos*Z	
Parameter	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*
*amo*A	NA	NA	−0.58*	0.055	NA	NA	−0.04	0.986	NA	NA	0.92*	0.001
*nos*Z	−0.58*	0.055	NA	NA	−0.04	0.986	NA	NA	0.92*	0.001	NA	NA
pH	−0.25	0.542	0.67*	0.032	0.24	0.793	−0.52*	0.078	0.60*	0.025	−0.67*	0.023
Temperature (C°)	−0.49*	0.072	0.48*	0.071	−0.50*	0.079	−0.02	0.975	−0.48*	0.069	0.52*	0.056
Oxygen (mg L^{-1})	0.12	0.655	0.05	0.925	0.48*	0.063	−0.06	0.983	0.77*	0.011	−0.70*	0.019
NO_3-N (mg L^{-1})	−0.18	0.532	0.68*	0.021	−0.60*	0.045	0.19	0.838	−0.77*	0.015	0.70*	0.018
NH_4-N (mg L^{-1})	0.58*	0.044	−0.64*	0.029	0.56*	0.061	−0.46	0.582	0.14	0.834	−0.26	0.777
PO_4-P (mg L^{-1})	0.01	0.932	0.29	0.811	−0.28	0.832	−0.12	0.899	−0.32	0.872	0.22	0.734
Organic matter (mg g^{-1})	−0.21	0.734	0.18	0.865	0.32	0.684	−0.23	0.783	−0.04	0.987	0.12	0.845

Copy numbers of key functional genes were transformed to Log_{10} values; NA: Not applicable; *r** is significant at $p < .1$.

abundance of the genes *amo*A and *nos*Z, are presented in Fig. 5. The correlation between the environmental parameters and the abundance of key functional genes is described with the Pearson correlation index in Table 2. For Sok-SSBS and Str-SSBS, the majority of the parameters were better explained by the horizontal PC1 (variability: 51.2% for Sok-SSBS, Fig. 5a; and 45.9% for Str-SSBS, Fig. 5c). Both N-transforming genes were correlated to the PC1 in opposite directions (Fig. 5a, c), meaning a significant negative correlation between the copy numbers of both genes in Sok-SSBS (*r*: −0.58, *p*: 0.055) and Str-SSBS (*r*: −0.92, *p*: 0.001) (Table 2). The *nos*Z was positively correlated to the temperature and the NO_3-N concentration in Sok-SSBS (*r*: 0.67, *p*: 0.032; and r: 0.68, *p*:0.021, respectively) and Str-SSBS (*r*: −0.67, *p*: 0.023; and *r*: 0.70, *p*: 0.018, respectively) (Fig. 5a, c and Table 2). The *amo*A was negatively correlated to the temperature in Sok-SSBS (*r*: −0.49, *p*: 0.072) and Str-SSBS (*r*: −0.48, *p*: 0.069) (Fig. 5a, c and Table 2), and positively correlated with the NH_4-N concentration in Sok-SSBS (*r*: 0.58, *p*: 0.044; Fig. 5a and Table 2). For Bzr-SSBS, the *amo*A negatively correlated with the temperature (*r*: −0.50, *p*: 0.079) and positively correlated with the NH_4-N (*r*: 0.56, *p*: 0.051) (Table 2).

3.5. Bacterial community structures in geochemical and denitrifying zones

The bacterial communities composition is presented according to the season and the most abundant taxa in Fig. 6 and Table S14. Regarding the class, the *β-Proteobacteria* presented higher relative abundances, followed by the *α-Proteobacteria,* and *ϒ-Proteobacteria* (up to 27.6%, 20.2%, and 15.3%, respectively; Fig. 6a). Regarding the family, the *Comamonadaceae* showed the highest relative abundance for DENz (up

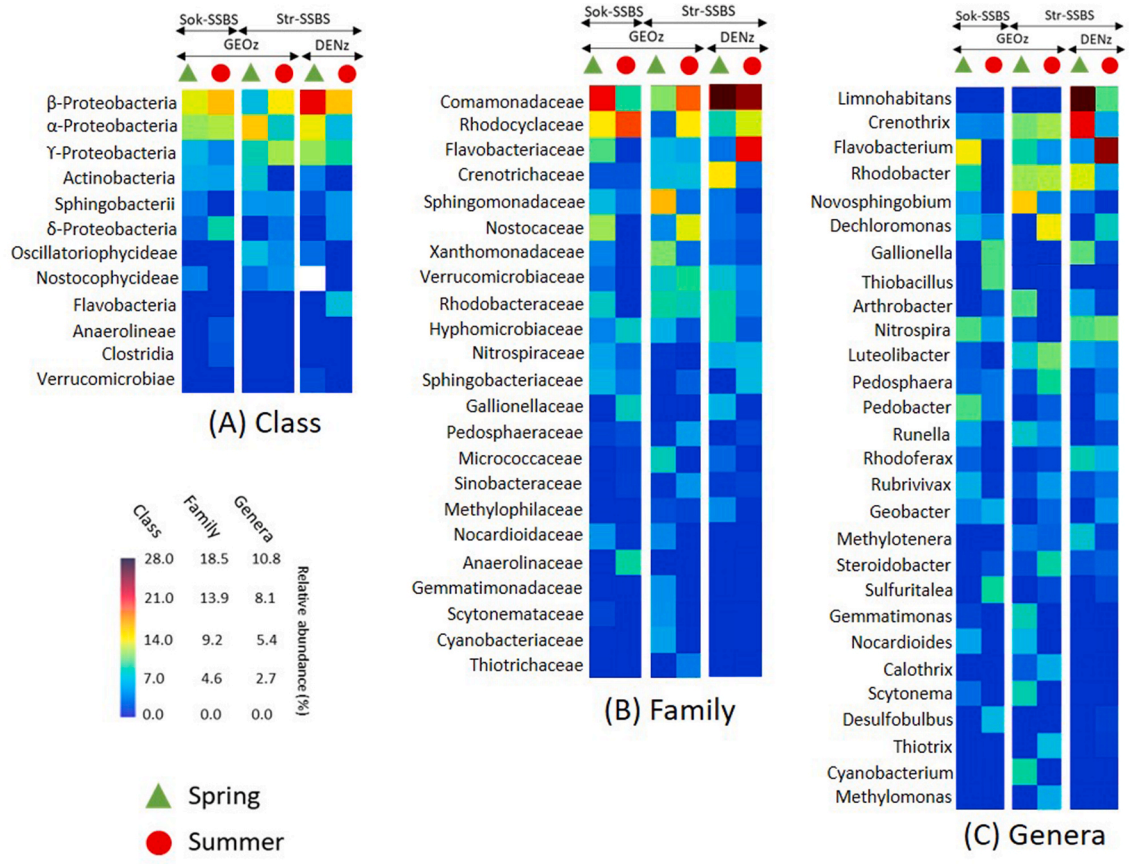

Fig. 6. Relative abundance (%) of bacterial communities in biofilm formations from geochemical (GEOz) and denitrifying (DENz) zones, spring and summer seasons, year 2018, (A) Class, (B) Family, and (C) Genera. Relative abundances (%) are also described in Table S14.

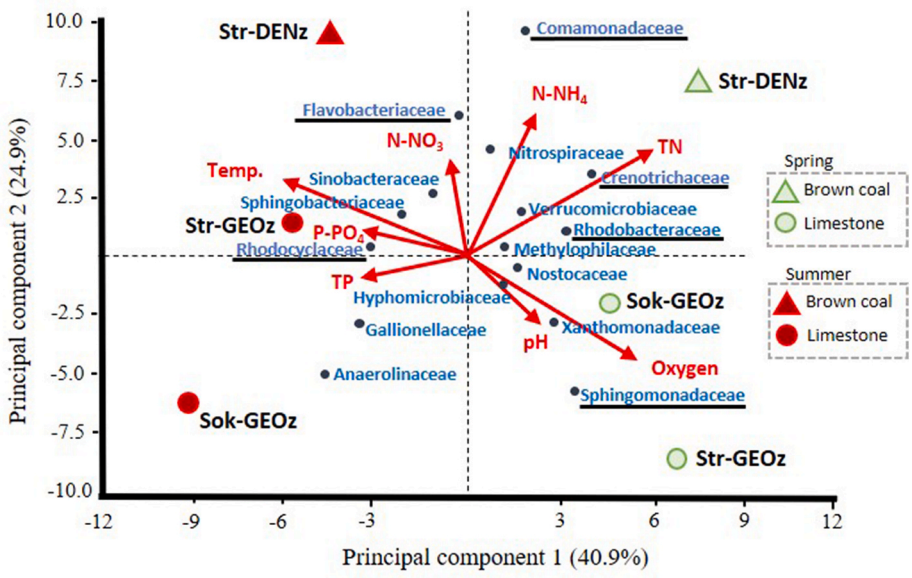

Fig. 7. Principal component analysis (PCA) presenting the spatial and seasonal differentiation of bacterial taxa in biofilms based on the correlation of the environmental parameters and the relative abundance of bacterial families. The diagram was prepared with the PC1 and PC2 (65% variability). The PCA scores were described in Table S15. The PCA loadings for environmental and most abundant bacterial families were described in Table S16. Underlined bacterial families were described in the text.

to 18.5%), when compared to the GEOz (up to 10.5%) (Fig. 6b). The *Comamonadaceae* in DENz shared an important relative abundance with the *Crenotrichaceae* in spring, and the *Flavobacteriaceae* in summer (Fig. 6b). The *Rhodocyclaceae* was higher in GEOz and DENz during summer, when compared to spring (Fig. 6b). The *Sphingomonadaceae* was also high in GEOz at Str-SSBS during spring (Fig. 6b). Regarding the genera *Limnohabitants*, *Crenothrix,* and *Rhodobacter* presented higher relative abundances in DENz during spring (10.8%, 7.5%, and 4.2%, respectively), while *Flavobacterium* in summer (9.4%) (Fig. 6c). In the case of GEOz, *Novosphingobium*, *Rhodobacter, Dechloromonas*, and *Crenothrix* were higher for Str-SSBS (up to 5.1%, 4.1%, 4.0%, and 3.9%, respectively), while *Flavobacterium* for Sok-SSBS (4.5%) (Fig. 6c).

The relationship between bacterial family abundances and the environmental parameters, according to seasons and SSBSs zones, is presented with a PCA in Fig. 7. The PC1 segregated the DENz and GEOz samples according to the season of collection (spring and summer), while the PC2 by the biofilm substrate (brown coal and limestone) (Fig. 7). The summer samples were positively correlated with a higher temperature, while the reverse trend was observed for the spring samples (Fig. 7). The most abundant families for DENz showed a close association with the concentration of a N specie: (i) the *Comamonadaceae* with NH_4-N, (ii) the *Crenotrichaceae* with the TN, and (iii) the *Flavobacteriaceae* with NO_3-N (Fig. 7). For GEOz in Str-SSBS, the *Rhodocyclaceae* was closely associated to the temperature and the concentration of PO_4-P and TP in summer (Fig. 7).

4. Discussion

4.1. The impact of the season

The microbial metabolic activity and carbon source utilization profiles suggested that heterotrophic microbial communities were more active in SSBSs during spring and summer, temperate (14.2 ± 2.5 °C) and warm (21.9 ± 1.9 °C) temperatures respectively, than in lower temperatures in autumn (11.2 ± 2.1 °C; Table S2) (Figs. 2 and 3). Similar results were observed for seasonal studies conducted in sediments from CWs (Chazarenc et al., 2010) and the river continuum profile (Freixa et al., 2016; Oest et al., 2018). For carbon source utilization, the polymers and carbohydrates were reported with the highest profiles of microbial catabolic activity during temperate and warm periods (Fig. 3). The above was associated with microbial community preferences to utilize essential sugars and complex carbohydrate polymers, since they could provide an easier and high energetic source (Chazarenc et al., 2010).

The abundance of nitrifying and denitrifying bacteria (represented by *amo*A and *nos*Z genes, respectively) was also significantly affected by the season of collection. Initially, the nitrifiers were more abundant than denitrifiers during spring (Fig. 4). In the subsequent summer, there was an evident shift of the community structure where denitrifiers were significantly higher than nitrifiers, which was also observed to persist during autumn (Fig. 4). Moreover, the ratio between N-transforming bacteria with the total bacterial community also reflected similar results, indicating that nitrifiers were enriched during spring, while denitrifiers during the summer and autumn (Table S9).

Bacterial community abundances (gene copy numbers g^{-1} of dry sediment) were also corroborated by the positive correlation between the *nos*Z gene and the temperature, while in contrast, the opposite relationship was found for the *amoA* gene (Fig. 5 and Table 2). In other publications, the gene *nos*Z was also higher in warm periods (22 ± 2 °C) *vs* cold periods (7 ± 5 °C) for sediments in rivers (2×10^7 and 4×10^6 copies g^{-1}, respectively; Huang et al., 2018) and estuaries (1×10^7 and 1×10^6 copies g^{-1}, respectively; Chon et al., 2011). In turn, the gene *amo*A was higher in spring (9 ± 7 °C) *vs* the summer (18 ± 7 °C) for river sediments (1.5×10^4 and 8×10^3 copies g^{-1}, respectively; Repert et al., 2014) and lakes (4×10^6 and 1×10^5 copies g^{-1}, respectively; Wang et al., 2019). Other studies have shown that optimum temperatures for growth and activity of denitrifiers are between 20 and 35 °C (Fischer and Whalen, 2005), and nitrifiers between 15 and 35 °C (Tourna et al., 2008). Warm temperatures allow the activation of enzymes involved in denitrification and nitrification processes (Wei et al., 2017; Li et al., 2018a). However, for nitrifiers, the higher inflow of NH_4-N during spring as compared to the summer (6.5 ± 0.3 and 2.4 ± 2.7 mg L^{-1} in interstitial water, respectively; Table S2) was probably also an influencing factor affecting their abundance, since nitrifying bacteria are known to grow well in NH_4-N rich environments (Di et al., 2009).

Bacterial communities involved in N transformations were affected by the concentration of nutrients in interstitial water. The denitrifiers in Sok-SSBS and Str-SSBS, were positively correlated in summer to the concentration of NO_3-N (Table 2), which suggests that they could have played an important role in the N transformation in summer. Additionally, the above mentioned statement was also supported by the high microbial metabolic activity registered by CLPP (Fig. 2), considering

that denitrifying bacteria are mainly composed of heterotrophic strains (Burgin and Hamilton, 2007; Canfield et al., 2010). In contrast, nitrifying bacteria in Sok-SSBS and Bzr-SSBS were positively correlated in spring to the concentration of NH_4-N (Table 2), suggesting that nitrifiers likely played an important role in N transformation in spring.

4.2. The impact of the system design and construction

Proper reduction of nutrients below the limit established by the Polish legislation (Dz.U, 2019 poz. 2149) was noticed for the three SSBSs (Table 1), which indicated on a good water quality at the OTF of the systems.

Microbial communities differed significantly for the Bzr-SSBS, when compared to the Sok-SSBS and Str-SSBS. In Bzr-SSBS, the relationship between the organic matter content and the microbial metabolic activity in sediments was found statistically significant ($r^2 = 0.79$; $p = .066$; $\alpha = 0.1$), since they both were observed to decrease from INF to OTF (Table 1 and Fig. 2b). The study conducted in CWs also revealed the progressive decrease of metabolic activity from the inflow to the outflow direction (AWCD up to 1.2 and 0.55, respectively), due to a decrease in the total organic carbon in the same direction (up to 102 and 41 mg L^{-1} in interstitial water, respectively; Button et al., 2015a). For Sok-SSBS and Str-SSBS, the high metabolic activities in sediments were probably a result of the increasing organic matter content through their horizontal profile (Table 1). These results suggested that the sedimentation process was more effective in Bzr-SSBS, which could be associated to the biggest size (including width and length) of the system (Fig. 1). The retention time and the intensity of water flow probably were other important factors affecting microorganisms in SSBSs, however these hydrological parameters were not monitored.

4.2.1. Geochemical zones (GEOz)

In the present study, particular interest was placed in biofilm formed over the rocks, to investigate if the associated microbial communities had a metabolic potential for nutrient transforming processes. The results indicated that the microbial metabolic activity increased for the barriers constructed with limestone rock (the Sok-SSBS and Str-SSBS) during spring and summer (Fig. 2a, c), in comparison with the Bzr-SSBS (containing dolomite) (Fig. 2b). One possible reason may be associated to the lower porosity of dolomite rocks (Weyl, 1960), and therefore could have hindered the bacterial cells attachment and forming of biofilm.

The abundance of limestone bacterial communities involved in nitrification and denitrification processes were six to four orders higher when compared to dolomite (Fig. 4). It is interesting that other studies have reported the co-existence of nitrifying and denitrifying bacterial communities in biofilms cultured without any external carbon supply (Kindaichi et al., 2004; Okabe et al., 2005). In these biofilms the cross feeding of microbial waste from nitrifying bacteria was available for heterotrophic denitrifying communities.

The Proteobacteria – especially *β-Proteobacteria* – are known to occupy a broad variety of metabolic strategies that include the cycling of nutrients (Cheng et al., 2016; Fu et al., 2019). Therefore, high abundances of these communities in GEOz suggested that Proteobacteria could have a potential role in N and P transformation processes in limestone barriers (Fig. 6a). The highest ratio between the genes *nos*Z/16SrRNA for GEOz was observed in Str-SSBS during summer (7.0%), when compared to Sok-SSBS (1.1%) (Table S9), suggesting that the most abundant families: *Comamonadaceae* and *Rhodocyclaceae* (Fig. 6b), could have comprised potential denitrifying communities in limestone barriers. In other studies, both families have also been associated with N-cycling processes (Repert et al., 2014; Palacin-Lizarbe et al., 2019; Ji et al., 2019). Regarding the genera *Rhodobacter*, *Crenothrix*, and *Dechloromonas* were abundant in Str-SSBS GEOz during summer (Fig. 6c), which are also known to contain many strains that participate in N-cycling processes (Repert et al., 2014; Li et al., 2018b). In the case of the family *Sphingomonadaceae* – especially *Novosphingobium* – seemed not to have been closely associated with the concentration of any N species (Fig. 7), suggesting that they probably not played an important role in N-cycling processes in described conditions, despite that they were observed abundant for Str-SSBS GEOz in spring (Fig. 6b, c). Moreover, potential nitrifying communities carrying the *amo*A gene - *Nitrosomonas* and *Nitrosospira* (which are described as one of the most important nitrifying groups in the natural freshwater environment) - were not strongly detected by the bacterial community analysis, which was contrasting to the ratio between the genes *amo*A/16S rRNA for GEOz during spring (up to 31.1%, Table S9). The above observation was probably associated to a jet undermined taxonomical characterization of the *Nitrosomonadaceae* diversity in microbial databases, due to their difficult isolation and cultivation under laboratory conditions (Lukumbuzya et al., 2020). Similar results have been observed in other studies conducted in a river (Repert et al., 2014), and constructed mangrove wetlands (Fu et al., 2019).

The *Rhodocyclaceae* was closely associated to the concentration of PO_4-P and TP in limestone barriers at Str-SSBS (Fig. 7), which suggested its close link with the accumulation of P species during summer. High abundance of *Dechloromonas* in summer also indicated that it was probably the most important member of this family (Fig. 6c). In other studies, the *Rhodocyclaceae* – especially *Dechloromonas* - has been linked to accumulation of polyphosphates in WWTP (McMahon et al., 2002; Goel et al., 2005; Coats et al., 2017), and to our knowledge, there are no studies describing potential polyphosphate accumulating organisms (PAOs) in SSBSs. Moreover, efficiency of GEOz in SSBSs has only been described with chemical parameters (Negussie et al., 2012; Szulc et al., 2015; Szklarek et al., 2018; Jurczak et al., 2019), and therefore future research could be focused on the biological processes involved in P transformation.

4.2.2. Denitrification zones (DENz)

The DENz was exclusively constructed in the newest system (Str-SSBS), with brown coal implemented as a carbon source for heterotrophic microbial communities. The highest abundance of denitrifiers in DENz (Fig. 4c) corresponded with the best TN, NO_3-N and NH_4-N removal efficiencies in surface water at the Str-SSBS, in comparison to the Sok-SSBS, constructed as the first one (Table 1). Similar removal efficiencies of N species were found between Str-SSBS and Bzr-SSBS (Table 1), however the biggest size and the lower INF of N species for Bzr-SSBS should have influenced the transformation processes (Fig. 1 and Table 1), considering that it is well known that the concentration of N species are quite critical for the biomass, activity and composition of N-transforming bacterial communities.

Bacterial community analysis suggested that the *β-Proteobacteria* could have a potential role for nutrient transformation in DENz. The highest ratio between the genes *nos*Z/16SrRNA for DENz was observed in summer (10.4%), when compared to spring (0.7%) (Table S9), suggesting that the families with higher abundances: *Comamonadaceae* and *Flavobacteriaceae*, could comprise important communities involved in N transformation processes in brown coal (Fig. 6b). Regarding the genera, the highest abundance of *Flavobacterium* indicated that it was probably an important denitrifying community in summer (Fig. 6c), since it is also known to comprise several species harbouring the *nos*Z gene (Repert et al., 2014). Similarly to GEOz, the nitrifying community harbouring the *amo*A gene (*Nitrosomonadaceae*) was also undermined by the 16S rRNA analysis when compared to the total abundance of *amo*A gene. Despite the above, nitrifying communities in spring seem to also have included members of the families *Comamonadaceae* and *Crenotrichaceae* (Fig. 6b), where *Limnohabitants* and *Crenothrix* were probably two of the most important (Fig. 6c). *Limnohabitants* has been described to contain an ammonia monooxygenase gene (Zeng et al., 2012), while *Crenothrix* a particulate methane monooxygenase gene (Stoecker et al., 2006). Both above mentioned genes are homologous to the *Nitrosomonadaceae amo*A gene, suggesting that they were also potential nitrifying communities in

DENz. In contrast, denitrifying communities were probably represented by the higher abundance of *Rhodobacter* during spring (Fig. 6c). Furthermore, the above mentioned three bacterial families: *Comamonadaceae*, *Flavobacteraceae*, and *Crenotrichaceae*, were closely associated to the concentration of N species in the surface water (Fig. 7), and therefore, suggested their close link to the N transformation processes in DENz. In other studies, these families have also been associated with N-cycling processes in a river (Repert et al., 2014), lakes (Palacin-Lizarbe et al., 2019), and in a CW (Wang et al., 2016).

5. Conclusion

All three systems worked properly in the reduction of nutrients, however, maximal efficiency and reduction below the concentration limits established by the Polish legislation were observed in Str-SSBS, after application of: 1) GEOz containing limestone, and 2) DENz containing brown coal. The microbial community of sediments, in the horizontal profile of three studied SSBSs, were more metabolically active during temperate and warm seasons (spring and summer) than in autumn. Nitrifiers preferred temperate temperature in spring (14.2 ± 2.5 °C) and showed the positive correlation with the concentration of NH_4-N. The highest abundances of nitrifiers were noted within GEOz with limestone, where they formed biofilm composed of nitrifying/denitrifying complex. Bacterial community analysis suggested that two of the most important taxa linked to N-transforming processes were the *Commamonadaceae*, *Rhodocyclaceae* (*Dechloromonas*), *Rhodobacter* and *Crenothrix*. Denitrifiers preferred warm temperature in summer (21.9 ± 1.9 °C) and showed the positive correlation with the concentration of NO_3-N. The highest abundance of denitrifiers was observed within DENz with brown coal, and bacterial community analysis suggested that the most important taxa linked to N-transforming processes were the *Commamonadaceae* (*Limnohabitants*), *Flavobacteriaceae* (*Flavobacterium*), *Crenotrichaceae* (*Crenothrix*), and *Rhodobacter*.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibiod.2021.105203.

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Statement of co-authorship

I declare that my contributions to the preparation of the original work were: i) revision of the existing knowledge about the present topic, ii) execution of experiments, iii) formal analysis of results, iv) writing the original draft of manuscript, v) correction and editing after review, vi) and the final visualization of the work. I assess that my participation represents 50% of the work.

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I declare that my contributions to the preparation of the original work were: i) the conceptualization of materials and methods (microbiological isolation methods and nutrient-transforming methods), ii) help in execution of experiments, iii) revision/edition of data analysis, iv) and the edition of the text in the manuscript. I assess that my participation represents 35% of the work.

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I declare that my contributions to the preparation of the original work were: i) the general conceptualization of the idea of publication, ii) supervision of genetic experiments, iii) and the edition of the text in the manuscript. I assess that my participation represents 15% of the work.

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Culturable nitrogen-transforming bacteria from sequential sedimentation biofiltration systems and their potential for nutrient removal in urban polluted rivers

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Novel heterotrophic bacterial strains—Bzr02 and Str21, effective in nitrogen transformation, were isolated from sequential sedimentation-biofiltration systems (SSBSs). Bzr02, identified as *Citrobacter freundii*, removed up to 99.0% of N–NH_4 and 70.2% of N–NO_3, while Str21, identified as *Pseudomonas mandelii*, removed up to 98.9% of N–NH_4 and 87.7% of N–NO_3. The key functional genes *nap*A/*nar*G and *hao* were detected for Bzr02, confirming its ability to reduce nitrate to nitrite and remove hydroxylamine. Str21 was detected with the genes *nar*G, *nir*S, *nor*B and *nos*Z, confirming its potential for complete denitrification process. Nitrogen total balance experiments determined that Bzr02 and Str21 incorporated nitrogen into cell biomass (up to 94.7% and 74.7%, respectively), suggesting that nitrogen assimilation was also an important process occurring simultaneously with denitrification. Based on these results, both strains are suitable candidates for improving nutrient removal efficiencies in nature-based solutions such as SSBSs.

The excessive inflow of nitrogen compounds has been a serious problem for water bodies in urban areas, including rivers and ponds. High concentrations of NH_4^+, NO_3^- and NO_2^- contribute to the occurrence of favourable conditions for the proliferation of phytoplankton, including cyanobacteria, which consequently affect aquatic and human health with the production of toxins, the decrease of light penetration and the depletion of oxygen in the pelagic zone[1–3]. To address the above-mentioned problem in urban polluted rivers, sequential sedimentation-biofiltration systems (SSBSs) have been implemented. These systems are designed according to the principles of ecohydrology to enhance the capacity of natural systems to remove environmental pollutants and are considered as nature-based solutions (NBS)[4,5]. These eco-friendly systems use a combination of natural processes for water treatment, i.e., sedimentation of solids, absorption of phosphorus, reduction of excessive nitrogen compounds by stimulating denitrification and nitrification processes and phytoremediation. SSBSs are constructed upstream of ponds or reservoirs to reduce anthropogenic eutrophication and, among others, the development of harmful algal blooms including toxic cyanobacteria. These systems have been observed to remove nitrogen compounds up to 59.8% of NH_4^+, 55% of NO_2^-, 91.3% of NO_3^- and 56.9% of total nitrogen (TN)[6–9]. The protection of urban ponds is needed because they regulate water flow and soil erosion during storms, increase the water retention, provide humidity, promote plant evapotranspiration and influence the cooling of urban areas. Moreover, urban ponds also offer aesthetic value, environmental education and recreational opportunities[10–12].

The important elements for the effective functioning of SSBSs are the structure and metabolic activity of microorganism inhabiting sediments. Microbial communities, with special consideration on bacteria, have been recently studied in working SSBSs[9]. The significant positive correlations observed between the measured concentration of nutrients (NO_3^- and NH_4^+) and the abundance of bacterial genes involved in nitrification and denitrification processes indicated that bacterial communities have played an important role in nitrogen

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transformations. Therefore, in the present study we focused on the characteristics of isolated bacterial strains capable of nitrogen removal.

The nitrification involves two consecutive reactions ($NH_4^+ \rightarrow NO_2^- \rightarrow NO_3^-$), and it has been studied in different autotrophic strains: (i) the first reaction was described in ammonia oxidizing bacteria (AOB), in the genera *Nitrosomonas*, *Nitrosospira* (β-Proteobacteria) and *Nitrosococcus* (ϒ-Proteobacteria)[13,14]; while (ii) the second reaction in nitrite oxidizing bacteria (NOB), in the genera *Nitrobacter* (α-proteobacteria), *Nitrococcus* (ϒ-Proteobacteria) and *Nitrospina*[15]. Nitrification also occurs in direct oxidation of $NH_4^+ \rightarrow NO_3^-$ (complete ammonium oxidation, COMAMMOX) by autotrophic strains of *Nitrospira* spp. (Class Nitrospirae)[16,17]. Moreover, nitrification via the hydroxylamine (NH_2OH) pathway, which is an intermediary product between the first nitrification reaction (ammonia oxidation to hydroxylamine), has also been described for *Nitrosomonas*[18] and heterotrophic strains of *Acinetobacter*[19], *Janthinobacterium*[20], *Alcaligenes*[21], *Enterobacter*[22], and *Pseudomonas*[23–25]. Denitrification—a dissimilatory nitrate reduction (DNR) pathway—involves four cascade reactions for the transformation of $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$, which was initially described for heterotrophic facultative anaerobic bacterial strains[26,27]. More recently, research has been focused in the identification of aerobic denitrifying strains that can perform parallel nitrification due to their potential utilization in waste water treatment plants (WWTPs) for the complete removal of nitrogen compounds. Several strains have been isolated and reported to perform simultaneous nitrification–denitrification (SNdN), with the most common genera represented by *Acinetobacter, Agrobacterium, Alcaligenes, Bacillus*, *Klebsiella*, *Enterobacter* and *Pseudomonas*[28].

The majority of the above described nitrogen transforming bacteria have been isolated from sewage in WWTPs, constructed wetlands (CWs) or biofilm formations in experimental bioreactors[28]. To our knowledge, the bacteria carrying out nitrogen transformation processes have not yet been isolated and characterized within the SSBSs. Additionally, there is a limited number of studies discussing the nitrogen balance, most of which were in controlled experiments for selected bacterial strains, in order to confirm their preferred metabolic pathways[29–33].

Therefore, the present study aimed to isolate and characterize heterotrophic bacterial strains that naturally occur in SSBSs, which are responsible for nitrogen transformation in nitrification and denitrification processes. To reach the objective, culturable bacteria were isolated from sediments, nitrogen transformation pathways were determined, and nitrogen balance was described. Additionally, the preference of the strains to perform nitrogen assimilatory over dissimilatory transformation processes was also investigated. Our results were compared with the nitrogen removal efficiency of other published isolated bacterial strains and discussed in the context of biotechnological potential of selected strains to improve the nutrient removal efficiency in NBS technologies.

Results and discussion

Selection and identification of potential nitrogen transforming bacteria. *Initial screening of bacteria capable of nitrogen utilization.* Ten bacterial strains were selected for their ability to transform nitrogen compounds and were summarized in Table 1. All mentioned strains were able to utilize NO_3^- in Giltay denitrifying medium (GiDM). Seven strains (Str21, Bzr07, Sok01, Sok03, Sok06, Sok20 and Sok41) presented no accumulation of NO_2^-, suggesting that it was further reduced by bacteria (Table 1). In contrast, three strains (Bzr02, Str01 and Sok05), only transformed NO_3^- to NO_2^-, which was then accumulated in the medium with no further utilization (Table 1).

In turn, seven among 10 selected strains (Str21, Bzr02, Bzr07, Str01, Sok03, Sok05 and Sok41) were able to utilize NH_4^+ on various nitrifying media with different carbon sources (Table 1). The most efficient removal of NH_4^+ was found in nitrifying medium containing glucose—GNM (up to 48 h for the strains Str21, Bzr02, Bzr07, Sok05 and Sok41; Table 1).

Taxonomic and phylogenetic characteristics. Taxonomical characteristics of selected bacterial isolates, based on the 16 s rRNA, were presented in Table 1, and their phylogenetic relationships were described in Fig. 1. The sequence homology revealed that the studied bacteria belong to significantly different taxonomical groups (Supplementary Table S1). Seven of them were clustered within the phylum Proteobacteria but different bacterial families: (i) the strains Str21, Bzr07 and Sok03, within the family Pseudomonadaceae, presented high similarity with *Pseudomonas mandelii* (99.55%), *P. migulae* (99.83%) and *P. guineae* (99.45%), respectively, (ii) the Sok01 was similar to *Hydrogenophaga taeniospiralis* (99.32%) and the Sok41 to *Acidovorax radicis* (99.29%), both strains within the family Comamonadaceae, (iii) the Bzr02 was similar to *Citrobacter freundii* (99.39%), which belongs to the family Enterobacteriaceae, and (iv) the strain Sok20 to *Janthinobacterium lividum* (99.34%), which belongs to the family Oxalobacteriaceae (Fig. 1). Furthermore, the strains Str01 and Sok06, within the phylum Firmicutes, presented high similarity to *Bacillus simplex* (98.36%) and *B. aereus* (99.63%), respectively, and the strain Sok05 to *Kocuria rosea* (99.08%) in the phylum Actinobacteria (Fig. 1).

Proposed metabolic pathways for nitrogen transformation. Possible bacterial metabolic pathways for nitrogen transformation were described based on the amplification of key functional genes involved in the nitrogen cycling process (Table 1 and supplementary Fig S2). The strains Str01 and Sok05 were considered to be nitrate reducers, since NO_2^- was accumulated in GiDM (Table 1). The above suggestion was supported with the detection of the *nar*G gene (respiratory nitrate reductase), which is involved in the reduction of $NO_3^- \rightarrow NO_2^-$ in anaerobic conditions (Table 1 and supplementary Fig S2). The strains Sok01, Sok06, and Sok20 were considered to be facultative anaerobic denitrifiers, since they were able to continue the reduction of NO_3^- to gas in GiDM, but could not utilize NH_4^+ in any of the nitrifying media in aerobic conditions (Table 1 and supplementary Fig S2). In contrast, the strains Sok41, Sok03 and Bzr07 were considered to be facultative anaerobic denitrifiers that could also utilize NH_4^+ in aerobic conditions (Table 1). All six facultative anaerobic denitrifiers (Sok01, Sok41, Sok20, Sok06, Sok03 and Bzr07) presented the *nos*Z gene (Table 1), which is involved in the last step of denitrification, and therefore, suggested that they performed complete reduction of $NO_3^- \rightarrow N_2$.

No	Strain	Microbiological analysis						
		Denitrification medium (DM)			Nitrification medium (NM)			
		GiDM		GNM	SNM	CNM	ANM	GNM + NH_2OH
No	Strain	Glucose + NO_3 −	Gas formation	Glucose + NH_4 +	Succinate + NH_4 +	Citrate + NH_4 +	Acetate + NH_4 +	Glucose + NH_2OH + NH_4 +
1	Str21	− NO_2 − (24 h)	+	− NH_4 + (24 h)	− NH_4 + (72 h)	− NH_4 + (72 h)	NG	NG
2	Bzr02	+ NO_2 − (48 h)	−	− NH_4 + (24 h)	− NH_4 + (72 h)	− NH_4 + (72 h)	− NH_4 + (144 h)	− NH_4 + (72 h)
3	Bzr07	− NO_2 − (24 h)	−	− NH_4 + (24 h)	− NH_4 + (72 h)	+ NH_4 + (72 h)	NG	NG
4	Str01	+ NO_2 − (48 h)	−	NG	− NH_4 + (72 h)	− NH_4 + (72 h)	− NH_4 + (144 h)	NG
5	Sok03	− NO_2 − (24 h)	−	NG	− NH_4 + (72 h)	− NH_4 + (72 h)	NG	NG
6	Sok05	+ NO_2 − (48 h)	−	− NH_4 + (120 h)	NG	NG	− NH_4 + (144 h)	NG
7	Sok41	− NO_2 − (24 h)	+	− NH_4 + (24 h)	− NH_4 + (72 h)	NG	NG	NG
8	Sok01	− NO_2 − (24 h)	+	NG	NG	NG	NG	NG
9	Sok06	− NO_2 − (24 h)	+	NG	NG	NG	NG	NG
10	Sok20	− NO_2 − (24 h)	+	NG	NG	NG	NG	NG

No	Strain	Genetic analysis						
		Taxonomy	Assimilation	Nitrification			Denitrification		
			*nas*A	*hao*	*nap*A/*nar*G		*nir*S	*nor*B	*nos*Z
No	Strain	16S r RNA	NO_3 − → NO_2 −	NH_2OH → NO_2 −	NO_3 − → NO_2 −		NO_2 − → NO	NO → N_2O	N_2O → N_2
1	Str21	*Pseudomonas mandelii*[a]	+[c]	−	−	+[c]	+[c]	+[c]	+[c]
2	Bzr02	*Citrobacter freundii*[b]	−	+[c]	+[c]	+[c]	−	−	−
3	Bzr07	*Pseudomonas migulae*	−	−	+	−	−	−	+
4	Str01	*Bacillus simplex*	−	−	−	+	−	−	−
5	Sok03	*Pseudomonas guineae*	−	−	−	−	−	−	+
6	Sok05	*Kocuria rosea*	−	−	−	+	−	−	−
7	Sok41	*Acidovorax radicis*	−	−	−	+	−	−	+
8	Sok01	*Hydrogenophaga taeniospiralis*	−	−	−	+	−	−	+
9	Sok06	*Bacillus aereus*	−	−	−	+	−	−	+
10	Sok20	*Janthinobacterium lividum*	−	−	−	+	−	−	+

Table 1. Bacterial strains capable of nitrogen transformation in different media. - NO_2^-: no detection or full transformation of nitrite; + NO_2^-: detection or incomplete transformation of nitrite; (#h): incubation hours; +: detection; −: no detection;—NH_4^+: no detection or full transformation of ammonium; + NH_4^+: detection or incomplete transformation of ammonium; NG: no bacterial growth. Taxonomical ID for the strain Str21[a] was also confirmed with the sequence analysis of *rpo*B gene in supplementary **Fig S1**, and the Strain Bzr02[b] with BIOLOG Gen III plates in supplementary **Table S2**. [c] The nucleotide BLAST similarity analysis for the functional genes detected in strains Str21 and Bzr02 was presented in supplementary **Table S3.**

Bzr02 (*Citrobacter freundii*) and Str21 (*Pseudomonas mandelii*), isolated from the Bzr-SSBS and Str-SSBS, respectively, presented the best results during the screening experiments on transformation of nitrogen compounds. Both strains were able to grow and remove NO_3^- and NH_4^+ in a lower time of incubation in different culture media, and were observed with the highest number of studied key functional genes involved in assimilation, nitrification or denitrification processes (Table 1). Moreover, the Bzr02 was the only strain capable to utilize NH_4^+ with the presence of hydroxylamine in GNM, suggesting that hydroxylamine could be an intermediary product in the nitrification process. Therefore, Bz02 and Str21 were selected for further quantitative experiments in nitrogen transformation assays.

Nitrogen transforming processes—strains Bzr02 and Str21. *Ammonium transformation in nitrifying medium.* Bzr02 and Str21 were cultivated in nitrifying medium (NM) under aerobic conditions, and their growth and utilization of N–NH_4 were followed for 24 h (Fig. 2a,b). The average and maximum removal rates of N–NH_4 for both strains were described in Table 2. Both strains were able to utilize N–NH_4 as a sole nitrogen source. Bzr02 presented a 4 h lag phase with minimal growth at the beginning of the assay (Fig. 2a). The log phase was observed after 4 h of incubation (Fig. 2a), which correlated with the maximum removal rate of N–NH_4 (16.17 ± 0.97 mg L^{-1} h^{-1}, Table 2). A stationary phase occurred between 12 and 18 h, however, the strain was able to remove 82.6% of N–NH_4 until 14 h of incubation (Fig. 2a). The maximum removal of N–NH_4 was observed at 22 h of incubation (99.0 ± 0.2%; Table 2). The average removal rate of N–NH_4 was 5.41 ± 0.13 mg L^{-1} h^{-1} (Table 2), which was significantly higher from other published strains: *Alcaligenes denitrificans* WY200811 (0.69 mg L^{-1} h^{-1})[34], *Klebsiella pneumonae* EGD-HP19-C (2.29 mg L^{-1} h^{-1})[35], *K. pneumonae* CF-S9 (4.3 mg L^{-1} h^{-1})[36] and *Enterobacter cloacae* CF-S27 (2.22 mg L^{-1} h^{-1})[22].

Str21 presented a 6 h lag phase, however, utilization of N–NH_4 started after 2 h of incubation (Fig. 2b). The maximum removal rate of N–NH_4 was observed after 8 h (10.2 ± 0.25 mg L^{-1} h^{-1}; Table 2), which continued

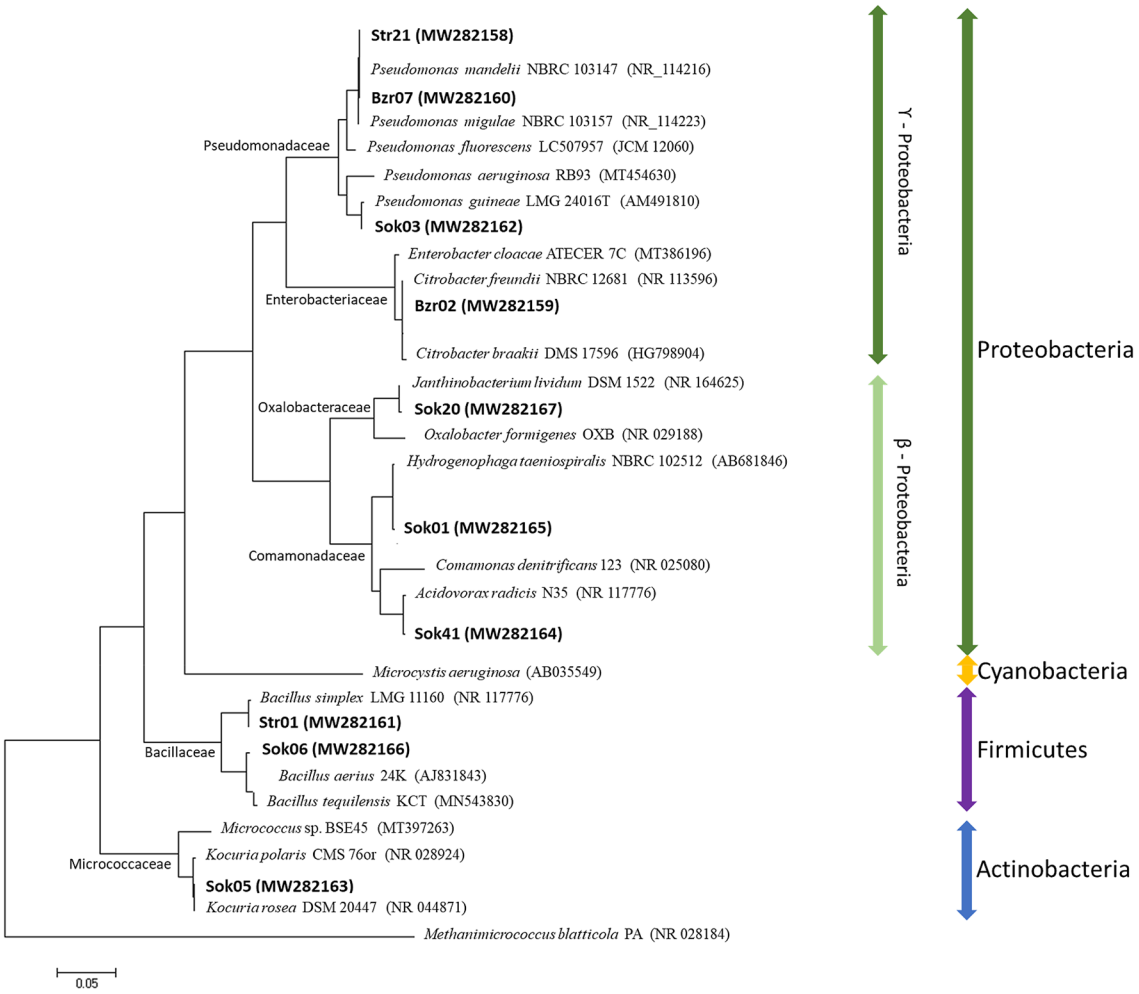

Figure 1. Neighbour-joining phylogenetic tree construction for the nitrogen transforming bacteria isolated in SSBSs. The tree was constructed using the 16S rRNA sequences obtained from GenBank (accession number inside the brackets). The bar under the graph represents the nucleotide substitutions per position. The sequence of *Microcystis aeruginosa* was used as an outgroup to cluster the representative strains in the phylum Proteobacteria, and the sequence of *Methanimicrococcus blatticola* PA (Archaea) as an outgroup to cluster the different bacteria phyla.

until almost complete depletion under 16 h of incubation (98.9 ± 0.6%; Table 2). The average removal rate of $N–NH_4$ was 7.21 ± 0.12 mg L^{-1} h^{-1} (Table 2), which was significantly higher from other strains in the family Pseudomonadaceae: *Pseudomonas* sp. JQ-H3 (2.7 mg L^{-1} h^{-1})[33], *P. stutzeri* YZN-001 (5.53 mg L^{-1} h^{-1})[37], *P. stutzeri* AD1 (3.1 mg L^{-1} h^{-1})[38], *P. tolaasii* Y-11 (2.04 mg L^{-1} h^{-1})[39], and similar to *P. putida* Y-9 (7.4 mg L^{-1} h^{-1})[24] and *P. stutzeri* T13 (7.09 mg L^{-1} h^{-1})[30].

The concentrations of $N–NO_2$ and $N–NO_3$ were insignificant through the complete assays for Br02 and Str21, and therefore no nitrification products were observed to occur (Fig. 2a,b, respectively). Similar results were published for all the above-mentioned strains and other genera, i.e., *Bacillus* SB1[40] and *Acinetobacter* sp. SYF26[41].

Nitrate transformation in denitrifying medium. Bzr02 and Str21 were cultivated in denitrifying medium (DM) under aerobic conditions, and their growth and utilization of $N–NO_3$ were followed for 32 h (Fig. 2c,d). The average and maximum removal rates of $N–NO_3$ for both strains were described in Table 2. Bzr02 was not able to grow and transform $N–NO_3$ when it was added to the medium as the sole nitrogen source. Similar results were reported for *Acinetobacter calcoaceticus* HNR[19], and it was proposed that the strain was sensitive to an initial high concentration of $N–NO_3$ (40 mg L^{-1}) in denitrifying medium. The above observation suggests that Bzr02 was also sensitive to the high initial concentration of $N–NO_3$ (100 mg L^{-1}) in DM.

On the contrary, Str21 was able to utilize $N–NO_3$ as a sole nitrogen source in DM (Fig. 2d). After a 6 h lag phase, the strain began to grow until the log phase was observed from 12 h of incubation (Fig. 2d). The maximum removal rate of $N–NO_3$ was 6.66 ± 0.27 mg L^{-1} h^{-1} (Table 2). Str21 removed $N–NO_3$ to a maximum of 87.7 ± 0.16% during 28 h of incubation. The average removal rate of $N–NO_3$ was 3.89 ± 0.27 mg L^{-1} h^{-1}, which was significantly higher than other strains in the family Pseudomonadaceae: *Pseudomonas* sp. JQ-H3 (1.78 mg L^{-1} h^{-1})[33], *P. tolaasii* Y-11 (2.04 mg L^{-1} h^{-1})[39] and *P. stutzeri* AD1 (1.98 mg L^{-1} h^{-1})[38], and other bacteria: *Klebsiella*

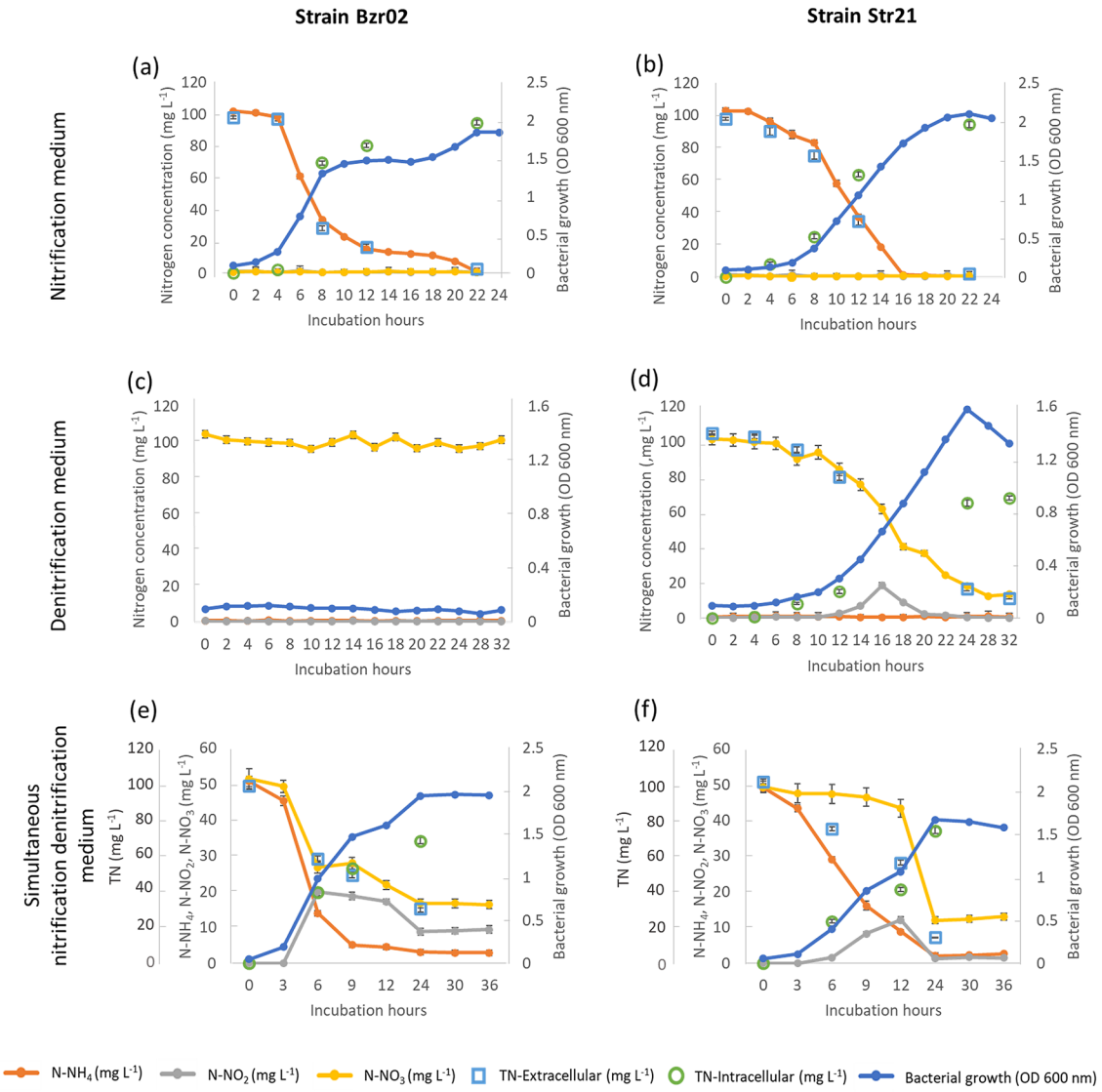

Figure 2. Dynamics of nitrogen transformation for strains Bzr02 and Str21 in nitrifying medium NM (**a**, **b**, respectively), denitrifying medium DM (**c**, **d**, respectively) and simultaneous nitrifying-denitrifying medium SNDM (**e**, **f**, respectively). Values represent the mean and the standard error (n = 3).

Medium	Nitrogen source	Bzr02			Str21		
		Average ($mg\ L^{-1}\ h^{-1}$)	Maximal ($mg\ L^{-1}\ h^{-1}$)	Removal (%)	Average ($mg\ L^{-1}\ h^{-1}$)	Maximal ($mg\ L^{-1}\ h^{-1}$)	Removal (%)
NM	$N\text{-}NH_4$	5.41 ± 0.13	16.17 ± 0.97	99.0 ± 0.2	7.21 ± 0.12	10.20 ± 0.25	98.9 ± 0.6
DM	$N\text{-}NO_3$	NT	NT	NT	3.89 ± 0.16	6.66 ± 0.27	87.7 ± 0.2
SNDM	$N\text{-}NH_4$	5.07 ± 0.09	10.44 ± 0.18	94.1 ± 1.3	3.35 ± 0.04	4.52 ± 0.22	95.6 ± 1.5
	$N\text{-}NO_3$	1.44 ± 0.16	7.52 ± 0.10	70.2 ± 3.6	2.29 ± 0.22	2.61 ± 0.17	75.4 ± 2.6

Table 2. Nitrogen removal rates by strains Bzr02 and Str21 in different nitrogen media. NM: nitrifying medium; DM: denitrifying medium; SNDM: simultaneous nitrifying-denitrifying medium; NT: Not transformed. Values represent the mean and the standard error (n = 3).

pneumonae CF-S9 (2.2 $mg\ L^{-1}\ h^{-1}$)[36] and *Bacillus cereus* GS-5 (2.7 $mg\ L^{-1}\ h^{-1}$)[31]. The formation of $N\text{–}NO_2$ was detected in DM, which was a result from the oxidation of $N\text{–}NO_3$. A maximum concentration of $N\text{–}NO_2$ was observed at 16 h (18.66 ± 1.68 $mg\ L^{-1}\ h^{-1}$) and decreased until it was completely utilized in 20 h of incubation (Fig. 2d). However, $N\text{–}NO_3$ was not completely removed at the end of the assay (13.54 ± 0.60 $mg\ L^{-1}$ in 32 h; Fig. 2d), suggesting that the denitrification process by Str21 was partially inhibited by the aerobic condition.

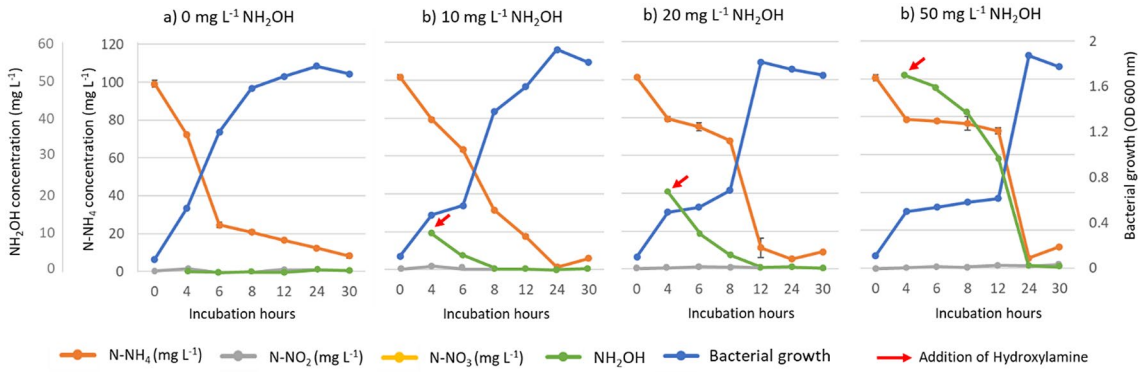

Figure 3. Dynamics of ammonium transformation with addition of hydroxylamine in different concentrations—strain Bzr02, (**a**) 0 mg L^{-1} or control, (**b**) 10 mg L^{-1}, (**c**) 20 mg L^{-1} and (**d**) 50 mg L^{-1}. Values represent the mean and the standard error (n = 3).

Ammonium and nitrate transformation in simultaneous nitrifying-denitrifying medium. Bzr02 and Str21 were cultivated in simultaneous nitrification–denitrification medium (SNDM) under aerobic conditions, and their growth and utilization of N–NH_4 and N–NO_3 were followed for 36 h (Fig. 2e,f). The average and maximum removal rates of N–NH_4 and N–NO_3 for both strains were described in Table 2. Bzr02 was able to remove 94.1 ± 1.3% of N–NH_4 and 70.2 ± 3.6% of N–NO_3 after 36 h of incubation (Table 2). A total of 16.80 ± 1.24 mg L^{-1} of N–NO_3 was accumulated in SNDM after 24 h of incubation, with no further utilization by Bzr02 (Fig. 2e). The formation of N–NO_2 was detected in SNDM, which was a result from the N–NO_3 oxidation. The concentration of N–NO_2 increased to a maximum of 20.02 ± 1.15 mg L^{-1} after 6 h, however, 9.48 ± 0.99 mg L^{-1} of N–NO_2 remained accumulated in SNDM from 24 h of incubation (Fig. 2e). The average removal rate of N–NH_4 (5.07 ± 0.09 mg L^{-1}) was significantly higher than N–NO_3 (1.44 ± 0.16 mg L^{-1}), which suggests that Bzr02 preferred to utilize N–NH_4 in SNDM (Table 2).

Similarly, Str21 was able to remove a higher amount of N–NH_4 (95.6 ± 1.5%) than of N–NO_3 (75.4 ± 2.6%) (Table 2), however, the utilization of N–NO_3 was not significant until after 12 h of incubation (Fig. 2f). The formation of N–NO_2 was detected in SNDM, which was a result from the reduction of N–NO_3, however, some differences were observed when Str21 was compared to Bzr02: (i) the maximum concentration of N–NO_2 was lower (12.19 ± 0.77 mg L^{-1}) and it was observed after 12 h of incubation, and (ii) N–NO_2 was almost completely utilized after 24 h of incubation (Fig. 2f). Moreover, a lower concentration of N–NO_3 (12.07 ± 0.91 mg L^{-1}) was accumulated after 24 h of incubation (Fig. 2f), when compared to Bzr02. The average removal rate of N–NH_4 (3.35 ± 0.04 mg L^{-1}) was higher than of N–NO_3 (2.29 ± 0.22 mg L^{-1}), which also suggested that Str21 preferred to utilize N–NH_4 in SNDM (Table 2). Similar results for other strains, where the removal rate of N–NH_4 was faster than of N–NO_3, have been described for *Klebsiella pneumoniae* CF-S9 (3.3 and 2.6 mg L^{-1}, respectively)[36] and *Pseudomonas tolaasii* Y-11 (2.13 and 0.52 mg L^{-1}, respectively)[39]. However, other strains have been found to remove N–NO_3 faster than of N–NH_4, i.e.: *Bacillus cereus* GS-5 (2.94 and 2.69 mg L^{-1}, respectively)[31] and *Janthinobacterium svalbardensis* F19 (1.19 and 0.62 mg L^{-1}, respectively)[20].

Hydroxylamine influence in the ammonium transformation by the strain Bzr02 in nitrifying medium. Bzr02 was cultivated in NM supplemented with NH_2OH in different concentrations, and the growth and utilization of N–NH_4 and NH_2OH were followed for 30 h (Fig. 3). The experiment was performed to corroborate the nitrification process by Bzr02 since the oxidized products (N–NO_2 and N–NO_3) were not observed during incubation with N–NH_4 as the sole nitrogen source. Bzr02 presented a log phase after 4 h of incubation in the control medium without hydroxylamine, which also corresponded with the maximum removal rate of N–NH_4 (23.80 ± 0.84 mg L^{-1}, Fig. 3a). When NH_2OH was added to 10 mg L^{-1} in NM after 4 h of incubation, the log phase of Bzr02 was observed until after 6 h of incubation (Fig. 3b). The maximum removal of N–NH_4 was 8.03 ± 0.60 mg L^{-1} h^{-1} during the addition of 10 mg L^{-1} NH_2OH, which was significantly lower when compared to the control (Fig. 3a,b). When 20 and 50 mg L^{-1} of NH_2OH were added to NM after 4 h of incubation, the log phase was observed after 8 and 12 h of incubation, respectively (Fig. 3c,d). Moreover, the maximum removal rates of N–NH_4 were 2.05 ± 0.90 and 0.86 ± 0.67 mg L^{-1}, respectively, which were significantly lower when compared to the control (Fig. 3a,c,d). These results suggested that NH_2OH, in high concentrations, significantly inhibited the growth of Bzr02, and in consequence, the removal of N–NH_4. However, the transformation of N–NH_4 was resumed when significant amount of NH_2OH was removed by Bzr02. Furthermore, N–NO_2 was not detected as product from the oxidation of NH_2OH (Fig. 3b,c,d). Similar results in other strains have been reported for: *Enterobacter cloacae* CF-S27[22], *Alcaligenes faecalis*[21], and *Thiosphaera pantotropha* (formerly *Paracoccus denitrificans*)[42].

Confirmation of bacterial nitrogen transforming pathways. The nitrogen balance during the transformation processes for Bzr02 and Str21 was calculated and presented in Table 3. The detection of key functional genes involved in nitrogen cycling was also summarized in Fig. 4, and the results were used to corroborate their nitrogen transforming pathways. For the ammonium transformation assay using NM, Bzr02 and Str21 utilized almost complete nitrogen and incorporated it into their cell biomass (94.7 ± 1.4 and 94.3 ± 2.0 mg L^{-1}, respectively)

Media	Strain	Initial TN (mg L^{-1})	Final TN (mg L^{-1})		Lost N (mg L^{-1})
			Extracellular	Intracellular	
NM	Bzr02	98.4 ± 0.6	2.55 ± 0.85	94.7 ± 1.4	0.75
	Str21	98.0 ± 0.9	2.45 ± 1.06	94.3 ± 2.0	1.25
DM	Bzr02	101.9 ± 2.1	98.3 ± 1.4	1.4 ± 0.55	2.2
	Str21	105.0 ± 1.2	11.40 ± 0.62	68.2 ± 1.2	25.4
SNDM	Bzr02	98.9 ± 1.3	29.2 ± 2.1	68.3 ± 1.8	1.4
	Str21	101.5 ± 1.6	14.6 ± 0.2	74.3 ± 1.6	12.6

Table 3. Nitrogen balance of strains Bzr02 and Str21 during the nitrogen transformation. NM: nitrifying medium; DM: denitrifying medium; SNDM: simultaneous nitrifying-denitrifying medium. Values represent the mean and the standard error (n = 3).

(Table 3). Only a small fraction of nitrogen was lost for Bzr02 and Str21 (0.75 and 1.25 mg L^{-1}, respectively; Table 3), suggesting that it was assimilated when N–NH_4 was given as the sole nitrogen source. The nitrification process seemed not to have occurred, especially because the products from the oxidation of N–NH_4 (N–NO_2 and N–NO_3) were not significantly detected through the entire assays (Fig. 2a,b).

The nitrification process seemed to have occurred for Bzr02 when NH_2OH was added to NM, which is another intermediary product during the first reaction of nitrification ($NH_4^+ \rightarrow [NH_2OH] \rightarrow NO_2^-$). Bzr02 removed NH_2OH from the NM while there was no significant bacterial growth or removal of N–NH_4 (Fig. 3), suggesting that NH_2OH was oxidized (nitrification) rather than assimilated. Additionally, the detection of the gene *hao* (hydroxylamine oxidoreductase, HAO) supports the nitrification process by Bzr02 (Fig. 4a); however, the concentration of N–NO_2 -the product from NH_2OH oxidation- was not significantly detected in all experiments (Fig. 3). These results are different from other strains that produced NO_2^- from the oxidation of NH_2OH, i.e., *Nitrosomonas europaea*[18] and *Pseudomonas* PB16[23]. Other studies suggest that the enzyme HAO also catalyzes a different reaction where NH_2OH is transformed to nitric oxide (NO) in *Alcaligenes faecalis* No.4[43] or reduced to N_2 in *A. facecalis*[44,45] and *Acinetobacter calcoaceticus* HNR[19]. The above results suggests that Bzr02 could have reduced NH_2OH to a nitrogen gas (Fig. 4b), rather than being oxidized to NO_2^- in the process of nitrification.

In the nitrate transformation assay in DM, only Str21 was able to grow and utilize N–NO_3 as the only nitrogen source (Fig. 2d). The initial nitrogen content in DM (105.0 ± 1.2 mg L^{-1}) was utilized by Str21 until 11.40 ± 0.62 mg L^{-1} remained in the medium at the end of the experiment (Table 3). The majority of nitrogen was detected in the cell biomass of Str21 (68.2 ± 1.2 mg L^{-1}) and 25.4 mg L^{-1} was estimated to be lost (Table 3). The above results suggested that Str21 transformed 89.1% of total nitrogen, from which 65.0% was assimilated and the remaining 24.1% was probably lost as a nitrogen gaseous form in the process of denitrification. Str21 was found to contain the gene *nas*A (assimilatory nitrate reductase, NAS; Fig. 4b) that confirmed the process of assimilatory NO_3^- reduction to NO_2^-, and subsequently to NH_4^+. The gene *nas*A is involved in the synthesis of cell biomass[46] (Fig. 4d). Moreover, Str21 was found to contain all studied genes involved in the process of denitrification (*nar*G, *nir*S, *nor*B and *nos*Z; Fig. 4b), suggesting that it is a facultative anaerobic denitrifier (Fig. 4d). The denitrification activity in anaerobic conditions for a similar strain—*Pseudomonas mandelii* strain PD30—has already been described with the gene expression of *nir*S and *nor*B[47,48]. In the above research, it was argued that the gene expression was significantly inhibited in aerobic conditions, and therefore, it was concluded that *P. mandelii* PD30 performed denitrification in exclusive anaerobic conditions. In contrast, for other *Pseudomonas* strains, i.e., *P. stutzeri* YG-24[29], *P.* sp. JQ-H3[33] and *P. mendocina* GL6[49], the removal of nitrogen content as gas was up to 46.0—74.4% in aerobic conditions, suggesting that there was a similar preference for nitrogen denitrification and assimilation, and sometimes, denitrification could be significantly higher. The detection of the gene *nap*A, rather than the gene *nar*G, was probably the most important factor influencing aerobic denitrification in the above three mentioned strains. In the case of Str21, only the gene *nar*G was detected (Fig. 4b), however, the process of denitrification was not completely inhibited when it was incubated in DM, during aerobic conditions (Fig. 2d). We believe that the aerobic conditions in the media could have partially influenced the reduction of N–NO_3 to subsequent forms of nitrogen for Str21, resulting in an evident preference to assimilate nitrogen rather than performing denitrification.

For the N–NH_4 and N–NO_3 transformation assays in SNDM, Bzr02 and Str21 were able to utilize N–NH_4 and N–NO_3 in aerobic conditions. For Bzr02, a total of 68.3 ± 1.2 mg L^{-1} of nitrogen was found in the cell biomass and 29.2 ± 2.1 mg L^{-1} remained in the medium (Table 3). The remaining nitrogen was mostly from N–NO_3 and the accumulation of its reduction to N–NO_2, that were not completely depleted by Bzr02 (Fig. 2e). The above results could be associated from the difficulty of Bzr02 to reduce NO_3^- to NO_2^- in aerobic conditions, as it was explained when it was incubated with higher N–NO_3 concentrations in DM (Fig. 2c). Despite the above, only 1.4 mg L^{-1} of nitrogen was lost (Table 3), suggesting that the dominant metabolic pathway presented by Bzr02 was nitrogen assimilation (Fig. 4c). The gene *nas*A was not detected for Bzr02, indicating that N–NO_3 was rather reduced by a dissimilatory nitrate reductase (NAR or NAP), and then, part of N–NO_2 was incorporated into the cell biomass through the process of assimilatory nitrite reduction[46,50] (Fig. 4c).

Str21 presented 74.3 ± 1.6 mg L^{-1} of nitrogen in the cell biomass and 14.6 ± 0.2 mg L^{-1} remained in the medium with no further utilization (Table 3). A significant concentration of nitrogen (12.6 mg L^{-1}) was lost at the end of incubation for Str21 (Table 3) in comparisson to Bzr02, suggesting that the process of denitrification took place. Moreover, the N–NO_2—produced from the reduction of N–NO_3—was not accumulated in Strs21 as it was observed for Bzr02 (Fig. 2e,f), also supporting that N–NO_2 was further reduced into nitrogen gaseous forms

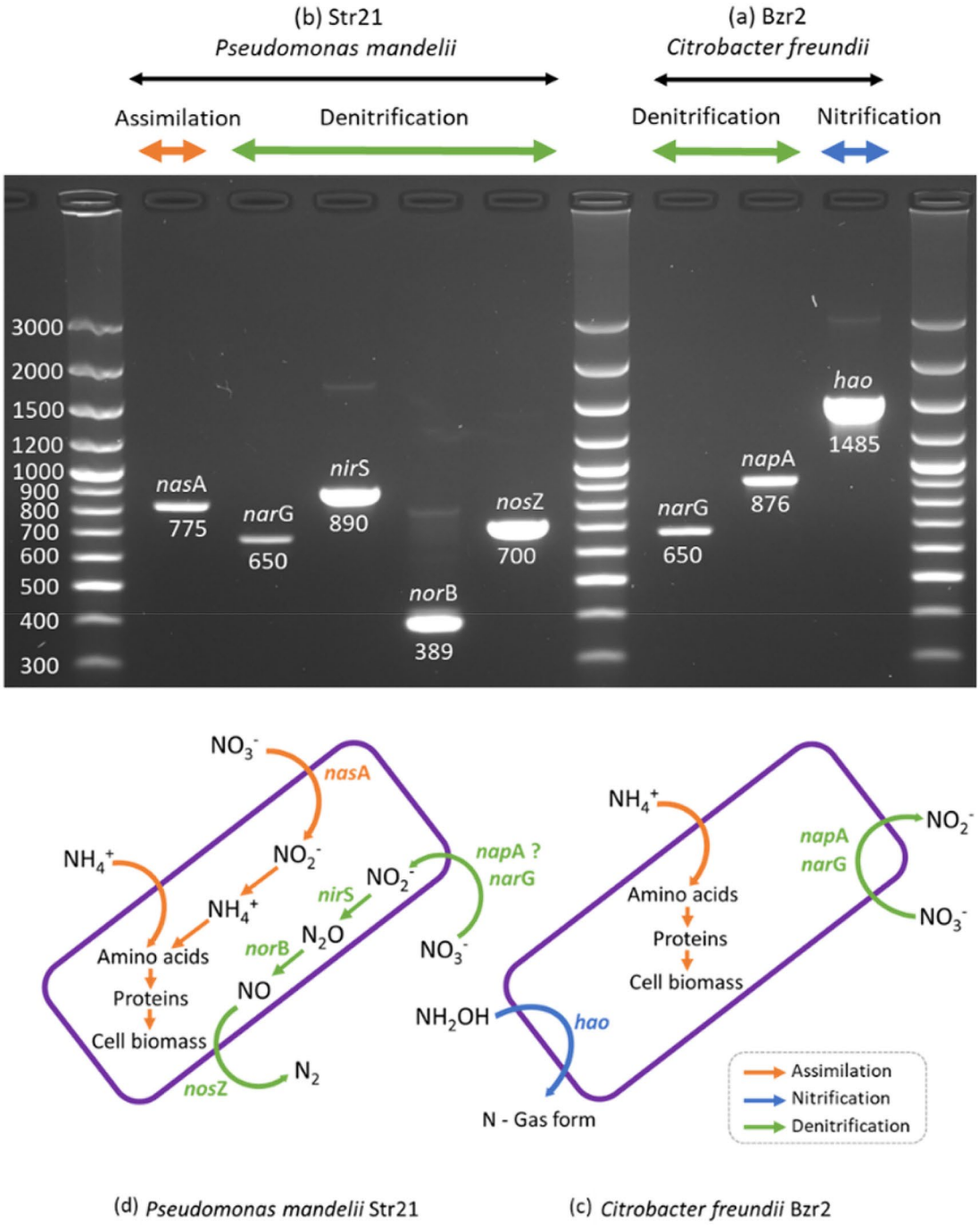

Figure 4. PCR amplification of key functional genes involved in nitrogen transformations for bacterial strains (**a**) Str21 and (**b**) Bzr02, and the predicted nitrogen utilization pathways in (**c**) Str21 and (**d**) Bzr02.

in the process of denitrification. Similarly as it was described during the experiment in DM, the detection of *nas*A suggested that N–NO_3 was incorporated into the cell biomass through the process of assimilatory nitrate reduction, and the detection of all four nitrogen reductase genes (*nar*G, *nir*S, *nor*B and *nos*Z) supported that the lost nitrogen escaped as nitrogen gas during dissimilatory nitrate reduction (denitrification; Fig. 4d). The low denitrification activity by Str21 in SNDM was also the influece of the aerobic conditions, which could be appreciated for the long lag phase were N–NO_3 was not significantly utilized at the first 12 h of incubation (Fig. 2d).

Conclusion

Bzr02 and Str21 (isolated from SSBSs sediments), identified as *Citrobacter freundii* and *Pseudomonas mandelii*, respectively, were found to have potential applications in nature-based solutions to enhance nitrogen compounds removal, such as SSBSs. Nitrate reduction to nitrite in the denitrification process was found for both strains. Str21

seemed to be a facultative anaerobic denitrifier, and therefore, could participate in nitrogen cycling in SSBSs sediments, where oxygen limiting conditions occur. In turn, Bzr02 and Str21 were observed to significantly assimilate N–NH_4 and N–NO_3 into their cell biomass in aerobic conditions, which could subsequently help to improve the efficiency of SSBSs in the nitrogen removal with its sequestration in the sediments. Therefore the application of both strains could be recommended for sedimentation zones, where the release of nitrogen would be controlled by: i) other decomposing microbial communities dwelling in the sediments, and ii) the periodical removal of sediments to maintain the proper operation of SSBSs.

Materials and methods

Samples collection and isolation of bacteria. Sediment samples were collected from the sedimentation zone (August 2018) in three SSBSs constructed for different urban rivers: (i) the River Sokołówka (Sok-SSBS) and (ii) the River Bzura (Bzr-SSBS) in the city of Łódź, and (iii) the River Struga Gnieźnieńska (Str-SSBS) in the city of Gniezno, Poland.[9] Complete description of structure and function for Bzr-SSBS is detailed in Szulc et al.[51] and Jurczak et al.[8], and for Sok-SSBS and Str-SSBS in Font-Nájera et al.[9] Sediment samples were suspended in sterile 0.75% NaCl w/v (10 g of sediment in 90 mL) and shacked for 30 min at 25 °C. Samples were allowed to settle for 15 min and supernatant was used to prepare serial dilutions (1×10^{-1}–1×10^{-6}) according to Mankiewicz-Boczek et al.[52]. 100 µL of each dilution was plated on to Soil Extract Agar (SEA), a solid medium according to Hamaki et al.[53], and incubated for seven days at 25 °C. For each SSBS, 50 heterotrophic bacterial isolates (150 in total) were randomly streaked out and re-plated on to nutrient agar solid medium (NA, Karl Roth).

Screening of nitrogen transforming bacteria. A total of 150 well-separated bacterial colonies were picked from NA and checked for nitrogen transformation abilities in different culturable media (See also media description in supplementary material):

(i) in Giltay denitrifying medium (GiDM) with high content of NO_3^- (N: 277 mg L^{-1}) according to Alexander[54], at 25 °C. Bacterial ability to reduce NO_3^-, under oxygen limited condition (Becton Dickinson Gas Pak System), was qualitatively monitored every 12 h with the semi-quantitative test strips QUANTOFIX nitrate/nitrite (Macherey–Nagel) for 7 d. A total of 10 different bacterial strains were able to completely or partially reduce NO_3^- (denitrification process), and therefore, were selected for further experiments;

(ii) the 10 selected bacterial isolates were incubated in 15 mL glucose nitrifying medium (GNM) described in Pahdi et al.[22], with a small modification—KH_2PO4 was used instead of NaH_2PO4 (0.10 g $MgSO_4\cdot7H_2O$, 3.84 g K_2HPO_4, 1.5 g KH_2PO_4, 0.802 g NH_4Cl [N: 212 mg L^{-1}], 5.3 g glucose $C_6H_{12}O_6$ [C: 2120 mg L^{-1}]), and 2 mL of trace elements were added per 1000 mL of GNM, final pH was 7.2, shacked at 150 rpm and incubated at 25 °C. The trace element solution was prepared according to Pahdi et al.[22]. The effect of different carbon sources was also screened with changes to the nitrifying medium where glucose was replace by: (i) sodium succinate (11.9 g)—succinate nitrifying medium (SNM), (ii) sodium acetate (10.0 g) – acetate nitrifying medium (ANM), and (iii) sodium citrate (8.65 g)—citrate nitrifying medium (CNM). The carbon and nitrogen ratio was kept constant (C:N = 10) in all used media.

Bacteria were also tested for the transformation of NH_4^+ under the presence of hydroxylamine in GNM. Cultures were grown in GNM for 6 h and spiked with high concentration of hydroxylamine (100 mg L^{-1} final concentration) according to Padhi et al.[22]. For the screening purpose, their ability to transform NH_4^+ was qualitatively monitored with the semi-quantitative test strips QUANTOFIX ammonium (Macherey–Nagel), every 12 h during 7 d.

DNA isolation and detection of key functional genes involved in nitrogen transformation processes. DNA was isolated from overnight bacterial cultures (Luria Bertani broth, LB) according to the specification in Wizard Genomic DNA purification kit (Promega, Madison, Wisconsin). The 10 previously selected bacterial strains (Chapter 2.2.) were screened for the presence of key functional genes involved in nitrification (*hao*[22]), denitrification (*nap*A[38], *nar*G[55], *nir*S[56], *nor*B[57] and *nos*Z[58]) and nitrogen assimilation (*nas*A[59]) processes using conventional PCR (Supplementary Table S4). PCR products for the strains Bzr02 and Str21 were purified with the QIAEX II Gel Extraction Kit (Promega, Madison, Wisconsin) and sequenced by Genomed laboratories in Warsaw, Poland (http://www.genomed.pl/). DNA sequences were edited using the software MEGA7 (http://www.megasoftware.net/) and similarity with other published bacterial strains was verified with the nucleotide BLAST tool. Sequences were deposited in the GenBank database with the accession numbers for Str21: *nos*Z (MW286255), *cnor*B (MW286256), *nir*S (MW286257), *nar*G (MW286258), and *nas*A (MW286259), and for Bzr02: *nap*A (MW286261), *hao* (MW286262), and *nar*G (MW286263).

Taxonomic characteristics and phylogenetic analysis. The 16S rRNA bacterial molecular marker was amplified for the 10 selected strains, with 27F / 1492R primers according to Lane[60]. PCR products were processed (purification, sequencing and nucleotide BLAST analysis) similarly as specified for the functional genes in Chapter 2.3. A neighbour-joining phylogenetic tree was constructed for bacteria using the software MEGA7. Bacterial 16S rRNA sequences were deposited in the GenBank database with the accession numbers for Str21 (MW282158), Bzr02 (MW282159), Bzr07 (MW282160), Str01 (MW282161), Sok03 (MW282162), Sok05 (MW282163), Sok41 (MW282164), Sok01 (MW282165), Sok06 (MW282166), and Sok20 (MW282167).

Additional methods to corroborate the taxonomical identification of two strains (Bzr02 and Str21) were described in supplementary material. The strain Bzr02 was incubated on GEN III Biolog MicroPlates with different carbon substrates, according to the manufacturer specifications[61], and the taxonomic characteristics of bacterium were determined using the GEN III Biolog database. For Str21, the gene *rpo*B (coding for the β subunit of the RNA bacterial polymerase) was used as a molecular marker, since it has been recommended for the

optimal differentiation between *Pseudomonas* species[62]. The DNA sequence of the *rpo*B gene was published in GenBank database for Str21 (MW286260).

Ammonium transformation. Bzr02 and Str21 were cultured overnight in LB at 25 °C and 120 rpm. Cells were harvested by centrifugation (8000 rpm, 10 min, 4 °C), and washed three times with sterile water. Then, each strain was inoculated into the nitrifying medium NM (0.1 final OD_{600}) with adjusted concentrations of NH_4^+ (N: 100 mg L^{-1}) and glucose (C: 1000 mg L^{-1}), incubation was performed at 25 °C and 150 rpm. Bacterial growth (optical density OD 600 nm) was checked at 2 h intervals using an Eppendorf Biophotometer in a 24 h experiment[22]. Supernatant was also collected during each interval (13,000 rpm, 10 min, 4 °C) for the measurement of $N–NH_4$, $N–NO_3$, $N–NO_2$ and extracellular TN. The pellet was washed three times with sterile water and used to estimate intracellular TN[32].

Nitrate transformation. Bzr02 and Str21 were inoculated into denitrifying medium (DM). The denitrifying media was similar to NM with the use of KNO_3 (N: 100 mg L^{-1} final concentration) as the source of nitrogen. The check of bacterial growth and the collection of samples were performed similarly as explained for the ammonium transformation assays in a 32 h experiment[22]. The supernatant was used to measure $N–NH_4$, $N–NO_3$, $N–NO_2$ and extracellular TN, and the bacterial pellet for intracellular TN.

Simultaneous ammonium and nitrate transformation. Bzr02 and Str21 were inoculated into the simultaneous nitrifying-denitrifying medium (SNDM). The media was similar to NM with the use of KNO_3 and NH_4CL (N: 50 mg L^{-1} each; TN: 100 mg L^{-1} final concentration) as sources of nitrogen. The check of bacterial growth and the collection of samples was performed similarly as explained for the ammonium transformation assay, in a 50 h experiment[22]. The supernatant was used to measure $N–NH_4$, $N–NO_3$, $N–NO_2$ and extracellular TN, and the bacterial pellet for intracellular TN.

The impact of hydroxylamine for ammonium transformation. Bzr02 was the only strain capable of growth in the presence of hydroxylamine during the screening experiments (described in Chapter 2.2.). Therefore, in a parallel experiment, the transformation of ammonium by Bzr02 was also investigated with different concentrations of hydroxylamine (0, 10, 20 and 50 mg L^{-1} as final concentrations) added after 4 h of growth in NM. The bacterial growth and the collection of samples were performed similarly as explained for the ammonium transformation assay, at 0 and 2 h (before the addition of hydroxylamine), and 4, 8, 12, 24 and 30 h of incubation (after the addition of hydroxylamine)[22]. The supernatant was used to measure $N–NH_4$, $N–NO_3$, $N–NO_2$ and NH_2OH concentrations.

Analytical methods. Concentration of nitrogen sources were measured with the Multiskan Sky Microplate Spectrophotometer (Thermo Fisher Scientific) according to standard methods[63]: (i) $N–NH_4$ by the Nessler's colorimetric assay, (ii) $N–NO_3$ by the ultraviolet spectrophotometric method, and (iii) $N–NO_2$ by the Griess colorimetric assay. The Hydroxylamine was measured by indirect spectrophotometry[64]. The TN was calculated with the total Kjeldal reagent set[65] as follows: (i) using the supernatant for the extracellular TN, and (ii) reconstitution of the cell pellet with sterile water for intracellular TN[32]. All measurements were performed in triplicate.

Analysis of data. Nitrogen balance was monitored with the formula:

$$N_L = (TN_{Fe} + TN_{Fi}) - TN_{Ie}$$

where N_L is the loss of nitrogen at the end of the experiment, the TN_{Fe} and TN_{Fi} are the final extracellular and intracellular TN, respectively, and the TN_{Ie} is the initial extracellular TN (adapted from Fidélis Silva et al.[32]).

Bacterial removal rates for $N–NH_4^-$, $N–NO_3^-$ and NH_2OH (mg L^{-1} h^{-1}) were estimated as follows:

$$Rr\left(\text{mg } L^{-1} \text{ } h^{-1}\right) = (C_i - C_f)/t, \text{ and}$$
$$Rr(\%) = 100 \times (C_i - C_f)/C_i$$

where C_i and C_f are the initial and final concentration of the nitrogen source, respectively, and the t is the final time of the experiment[29].

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Author contributions

A.F.N.: Formal analysis, Investigation – literature review, Writing – Original Draft, visualization. L.S.: Methodology, Supervision – microbiological and biochemical analysis, Writing – Review & editing. J.M.-B.: Conceptualization, Supervision – genetic analysis, Writing – Review & editing.

Competing interests

The authors declare no competing interests.

Additional information

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Appendix 4: Additional information for the estimation of most important results

Title: Characterization and comparison of microbial communities in sequential sedimentation-biofiltration systems for removal of nutrients in urban rivers

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The next document contains a summary of graphs and tables that were necessary to calculate the results presented in the main study. In the present document we included calculations for standardization of qPCR curves, estimation of gene copy numbers (*nos*Z and *amo*A), background calculations for BIOLOG Community level physiological analysis, the respective calculations for the statistical analysis, and BLAST results for the published gene sequences in Genbank.

Preparation of Standard Curves for qPCR analysis

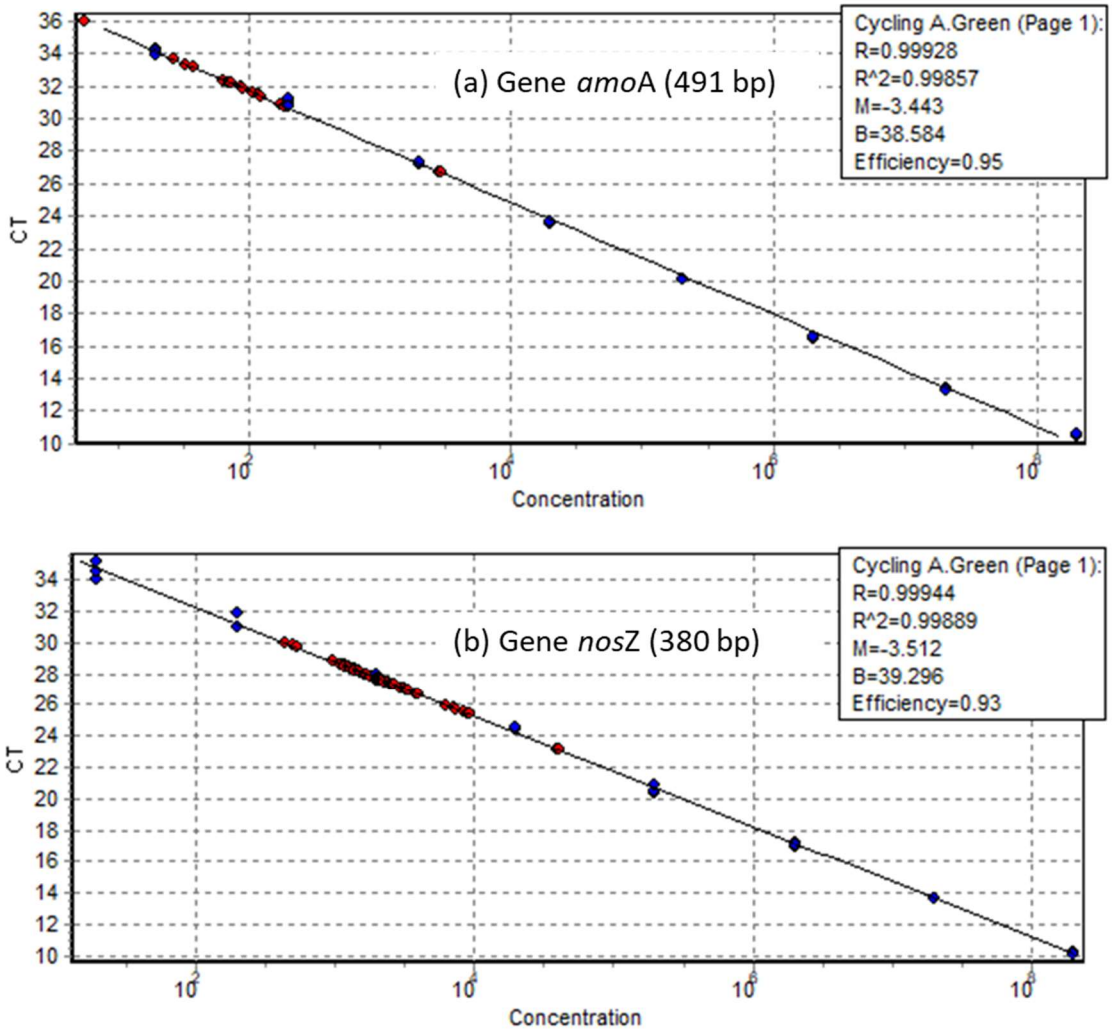

Fig. S1. Estimation of standard curve efficiency for the quantification of genes *amo*A (491 bp) and *nos*Z (380 bp) in sediment samples of both SSBSs.

Concentration refers to the amount of gene copies per µL of DNA. CT: Threshold cycle; R and R^2: Coefficients of determination for the linear regression; M: Slope; B: Intercept.

The efficiency for the gene *amo*A (491 bp) reaction was 95%, and for the gene *nos*Z (380 bp) was 93%.

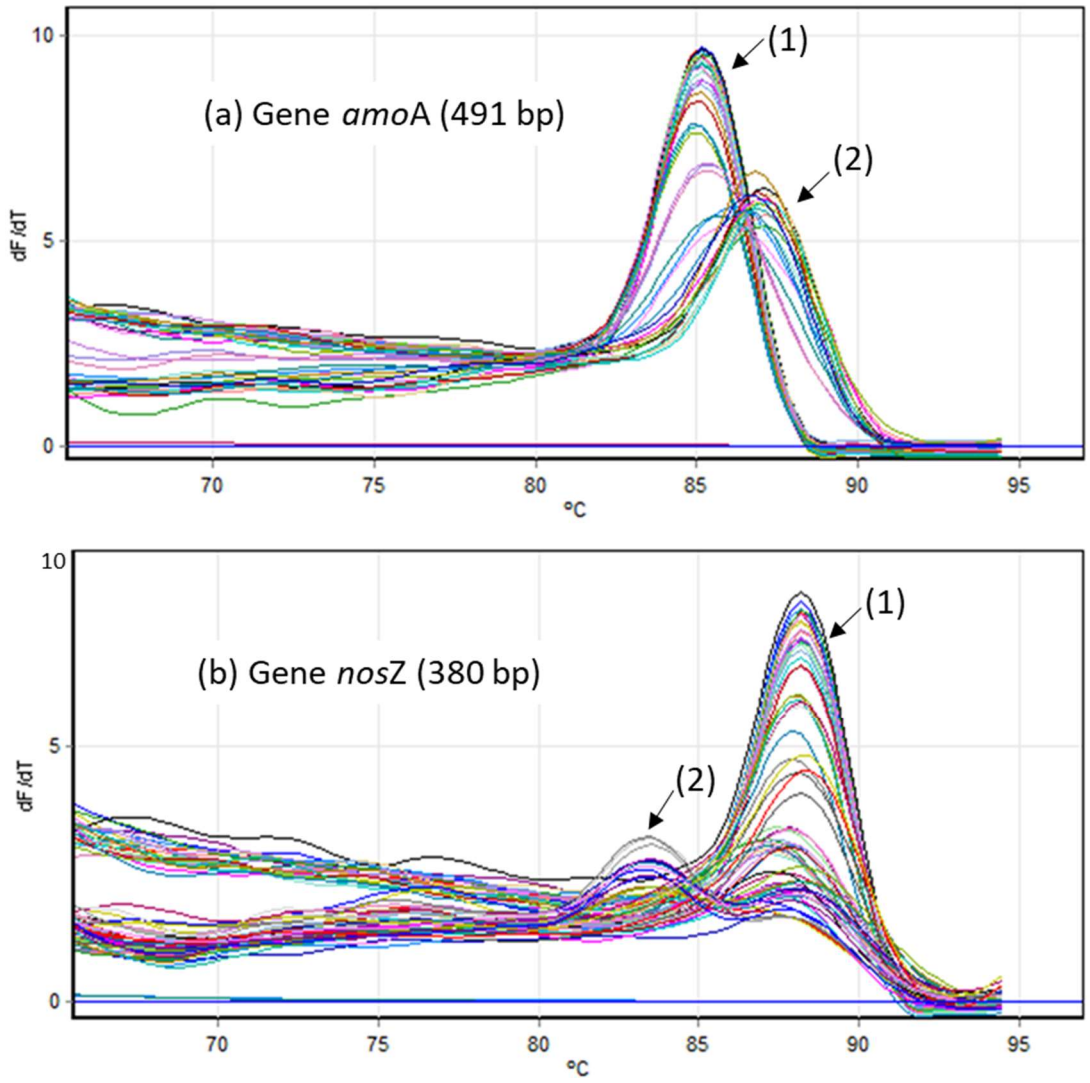

Fig. S2. Melt curve analysis for the quantification of genes *amo*A (491 bp) and *nos*Z (380 bp) in sediment samples of both SSBSs.

Defined melting peaks for both genes were marked with the number (1). In the case of gene *amo*A, plasmids were prepared from Str-SSBS sediment samples, and therefore appear as defined melting peaks with sediment samples from the same SSBS. The shoulder peaks (designated by the number 2) observed for gene *amo*A are mainly composed from sediment samples at Sok-SSBS.

The contrary case was observed for the gene *nos*Z, because plasmids were prepared from Sok-SSBS sediment samples. Defined melting peaks were composed of plasmid amplicons and sediment samples from the Sok-SSBS, while shoulder peaks appeared for samples in the Str-SSBS.

The previous results were attributed to differences in DNA polymorphism, due to different localities of the SSBSs.

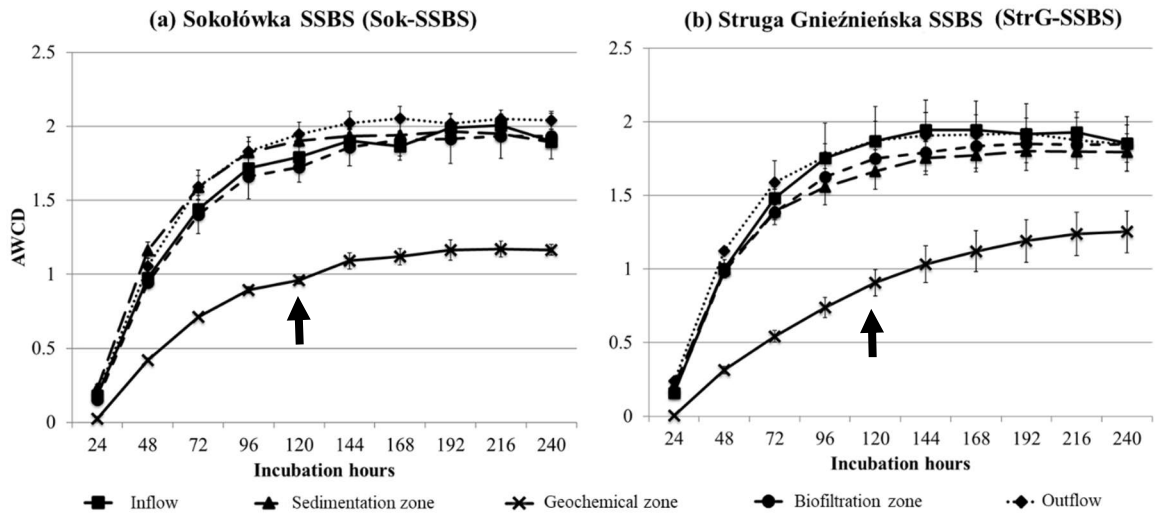

Fig. S3. Average well colour development (AWCD) in a 10-day incubation period (27°C) from sediment sample suspensions of the different areas at the: (a) Sok-SSBS and (b) Str-SSBS.

The arrow represents the time of incubation where higher differences were observed between AWCD values of samples, and therefore were selected for further diversity and richness analysis.

Table S1. Summary of indexes estimated after 120 hours from sediment sample suspensions of the different areas in both SSBSs.

Sokołówka SSBS (Sok-SSBS)

SSBS Area	AWCD		Shannon Weiver Diversity (H)		Richness (R)	
	Average	Stdev	Average	Stdev	Average	Stdev
INF (n: 3)	1.7942	0.0708	3.3100	0.0130	29.7	0.47
SEDz (n: 3)	1.9021	0.0403	3.3464	0.0134	29.7	0.47
GEOz (n: 3)	0.9598	0.0255	3.0090	0.0553	21.7	1.25
BIOz (n: 3)	1.7222	0.0997	3.3407	0.0052	30.3	0.47
OTF (n: 3)	1.9482	0.0793	3.3568	0.0324	30.0	0.82

Struga Gnieźnieńska SSBS (StrG-SSBS)

SSBS Area	AWCD		Shannon Weiver Diversity (H)		Richness (R)	
	Average	Stdev	Average	Stdev	Average	Stdev
INF (n: 3)	1.8712	0.1320	3.3205	0.0107	29.7	0.47
SEDz (n: 3)	1.6622	0.1219	3.3058	0.0088	29.0	0.82
GEOz (n: 3)	0.9062	0.0877	3.1806	0.0413	26.3	1.70
BIOz (n: 3)	1.7509	0.0587	3.2923	0.0100	29.0	0.05
OTF (n: 3)	1.8728	0.2313	3.3686	0.0287	30.3	0.47

AWCD: Average well colour development index. (n): number of measurements. INF: inflow, SEDz: sedimentation zone GEOz: geochemical zone, BIOz: biofiltration zone, OTF: outflow. Stdev: standard deviation.

Table S2. One way ANOVA test to estimate significant differences (P,0.05) between the metabolic profiles of the different SBSS areas.

Sokołówka SSBS (Sok-SSBS)

	AWCD (N: 15)				
	Sum of sqrs	df	Mean square	F	p(same)
Between groups	1.96115	4	0.49029	69.51	2.94E-07
Within groups	0.07053	10	0.00705		
Total	2.03168	14			
	Shannon Weiver diversity (H) (N: 15)				
	Sum of sqrs	df	Mean square	F	p(same)
Between groups	0.26414	4	0.06604	49.14	1.53E-06
Within groups	0.01344	10	0.00134		
Total	0.27758	14			
	Richness (R) (N: 15)				
	Sum of sqrs	df	Mean square	F	p(same)
Between groups	0.26414	4	0.06604	49.14	1.53E-06
Within groups	0.01344	10	0.00134		
Total	0.27758	14			

Struga Gnieźnieńska SSBS (StrG-SSBS)

	AWCD (N: 15)				
	Sum of sqrs	df	Mean square	F	p(same)
Between groups	1.96529	4	0.49132	16.9	0.00019
Within groups	0.29080	10	0.02908		
Total	2.25609	14			
	Shannon Weiver diversity (H) (N: 15)				
	Sum of sqrs	df	Mean square	F	p(same)
Between groups	0.05783	4	0.01446	17.06	0.00018
Within groups	0.00848	10	0.00085		
Total	0.06631	14			
	Richness (R) (N: 15)				
	Sum of sqrs	df	Mean square	F	p(same)
Between groups	27.73330	4	6.93333	5.474	0.01343
Within groups	12.66670	10	1.26667		
Total	40.40000	14			

AWCD: Average well colour development; P values marked with red colour represent significant differences.

The one way ANOVA statistical analysis indicates the existence of significant differences between the SSBSs areas (Tab. S2), however a Tukey statistical analysis was proposed to discriminate which samples are responsible for such differences (Tab S3).

Table S3. Tukey's honesty significant difference (HSD) tests to discriminate significant differences (p<0.05) between the metabolic profiles (CLPP) in the different areas of each SSBS.

Sokołówka SSBS (Sok-SSBS)				Struga Gnieźnieńska SSBS (StrG-SSBS)			
SSBS area	AWCD	H	R	SSBS area	AWCD	H	R
INF-SEDz	0.540000	0.742500	1.000000	INF-SEDz	0.584200	0.968400	0.945700
INF-GEOz	0.000176	0.000179	0.000178	INF-GEOz	0.000432	0.001244	0.029770
INF-BIOz	0.827000	0.838400	0.899000	INF-BIOz	0.903700	0.758500	0.808800
INF-OTF	0.238900	0.549100	0.991200	INF-OTF	1.000000	0.322100	0.945700
SEDz-GEOz	0.000176	0.000177	0.000178	SEDz-GEOz	0.002192	0.002717	0.091240
SEDz-BIOz	0.139000	0.999700	0.899000	SEDz-BIOz	0.965400	0.977100	0.995700
SEDz-OTF	0.958000	0.996400	0.991200	SEDz-OTF	0.577900	0.134800	0.612400
GEOz-BIOz	0.000177	0.000177	0.000176	GEOz-BIOz	0.001012	0.005969	0.157300
GEOz-OTF	0.000176	0.000176	0.000177	GEOz-OTF	0.000423	0.000242	0.009892
BIOz-OTF	0.049700	0.981100	0.991200	BIOz-OTF	0.899600	0.056760	0.417200

AWCD: Average well colour development; H: Shannon Weiver diversity index; R: Richness index. Numbers inside the table represent P values. Red colour represents the SSBSs areas that show significant differences. INF: Inflow, SEDz: Sedimentation zone, GEOz: Geochemical zone, BIOz: Biofiltration zone, OTF: Outflow.

It is possible to observe that the GEOz in both SSBSs is the area with higher significative differences when compared to the other areas in the horizontal profiles of both SSBSs (See Tab. S3)

Table S4. T-test for independent samples to estimate significant differences (P<0.05) between the metabolic profiles (CLPP) of analogous SSBSs areas.

AWCD index between Sok-SSBS (N = 3) and Str-SSBS (N = 3)

Sok-SSBS vs Str-SSBS	t-value	df	p	F-ratio	p
INF	-0.73	4	0.51	3.47	0.45
SEDz	2.64	4	0.06	9.16	0.20
GEOz	0.83	4	0.45	11.88	0.16
BIOz	-0.35	4	0.74	2.88	0.51
OTF	0.44	4	0.69	8.52	0.21

Shannon Weiver diversity (H) between Sok-SSBS (N = 3) and Str-SSBS (N = 3)

Sok-SSBS vs Str-SSBS	t-value	df	p	F-ratio	p
INF	-0.89	4	0.43	1.47	0.81
SEDz	3.59	4	0.02	2.30	0.61
GEOz	-3.51	4	0.02	1.79	0.72
BIOz	6.05	4	0.00	3.77	0.42
OTF	-0.39	4	0.72	1.27	0.88

Richness (R) index between Sok-SSBS (N = 3) and Str-SSBS (N = 3)

Sok-SSBS vs Str-SSBS	t-value	df	p	F-ratio	p
INF	0.00	4	1.00	1.00	1.00
SEDz	1.00	4	0.37	3.00	0.50
GEOz	-3.13	4	0.04	1.86	0.70
BIOz	4.08	4	0.02	100.00	0.02
OTF	-0.50	4	0.64	3.00	0.50

AWCD: Average well colour development. Red colour represents the SSBSs areas that show significant differences. INF: Inflow, SEDz: Sedimentation zone, GEOz: Geochemical zone, BIOz: Biofiltration zone, OTF: Outflow.

Table S5. Correlation of carbon substrate utilization by microorganisms with first (PC1) and second principal component (PC2) in Sok-SSBS and Str-SSBS.

Carbon substrate group	Sok-SSBS		Str-SSBS	
	PC1 (78%)	PC2 (10%)	PC1 (80.5%)	PC2 (8%)
Carbohydrates	0.95*	0.01	0.93*	0.14
Polymers	0.86*	0.28	0.89*	0.17
Carboxylic and ketonic acids	0.85*	-0.48	0.88*	-0.29
Amino acids	0.92*	-0.2	0.87*	-0.38
Amines and amides	0.80*	0.43	0.86*	0.38

*Correlation values are statistically significant.

Table S6. Summary of average well colour development (AWCD) categorized by group of carbon sources from the different sediment samples collected at both SSBSs.

Sokołówka SSBS (Sok-SSBS)

SSBS area	Carbohydrates		Polymers		Carboxylic and Ketonic acids		Amino acids		Amines and Amides	
	X	Stdev	*X*	Stdev	*X*	Stdev	*X*	Stdev	*X*	Stdev
INF	1.8783	0.1812	2.3670	0.1355	1.5057	0.0521	1.6795	0.1114	1.8707	0.2187
SEDz	1.9193	0.1157	2.1513	0.1130	1.7461	0.0412	1.8979	0.2104	2.0318	0.0163
GEOz	0.4921	0.0317	0.9712	0.2157	1.3108	0.0925	1.1859	0.0114	1.0175	0.0705
BIOz	2.0546	0.0229	2.3852	0.2110	1.7715	0.0793	1.7910	0.0809	1.4917	0.3585
OTF	2.0293	0.0146	1.9273	0.2102	1.8554	0.0847	1.9872	0.1424	1.8858	0.1958

Struga Gnieźnieńska SSBS (StrG-SSBS)

SSBS area	Carbohydrates		Polymers		Carboxylic and Ketonic acids		Amino acids		Amines and Amides	
	X	Stdev	*X*	Stdev	*X*	Stdev	*X*	Stdev	*X*	Stdev
INF	2.0114	0.2491	2.0339	0.0199	1.5749	0.1000	1.9821	0.3371	1.8450	0.4174
SEDz	1.7037	0.0987	2.0604	0.0688	1.4897	0.2136	1.6547	0.1094	1.4572	0.4381
GEOz	0.7511	0.0904	1.3403	0.0620	0.8653	0.1206	1.0216	0.1386	0.6520	0.0559
BIOz	1.6793	0.1103	2.0933	0.1027	1.5691	0.2180	1.9288	0.2998	1.7075	0.3745
OTF	1.9154	0.1688	1.8750	0.2142	1.7664	0.3082	1.9198	0.4234	1.9927	0.2397

X: media, Stdev: Standard deviation.

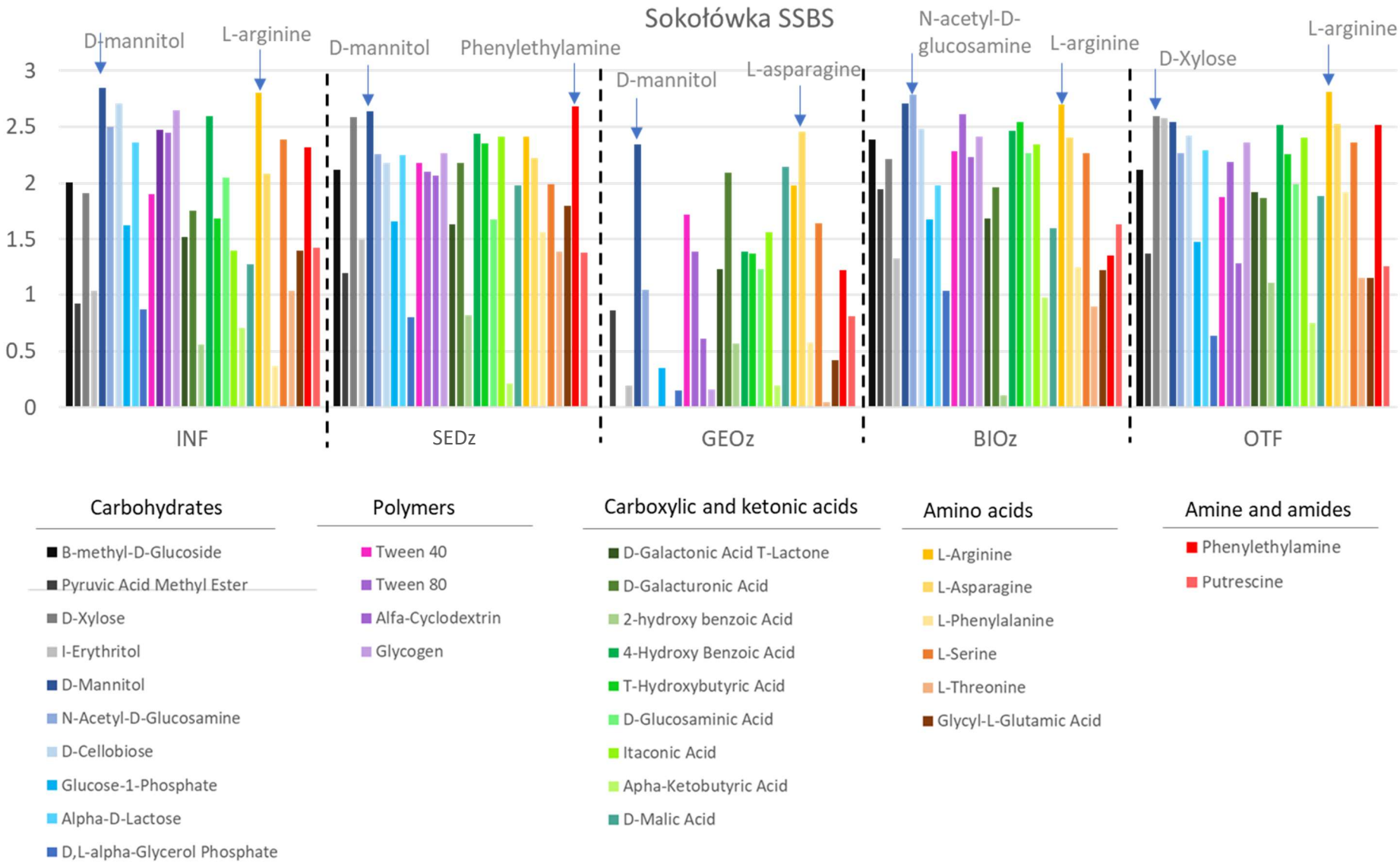

Fig. S4. Summary of carbon substrate utilization by heterotrophic microbial communities along the horizontal profile of Sokołówka SSBS

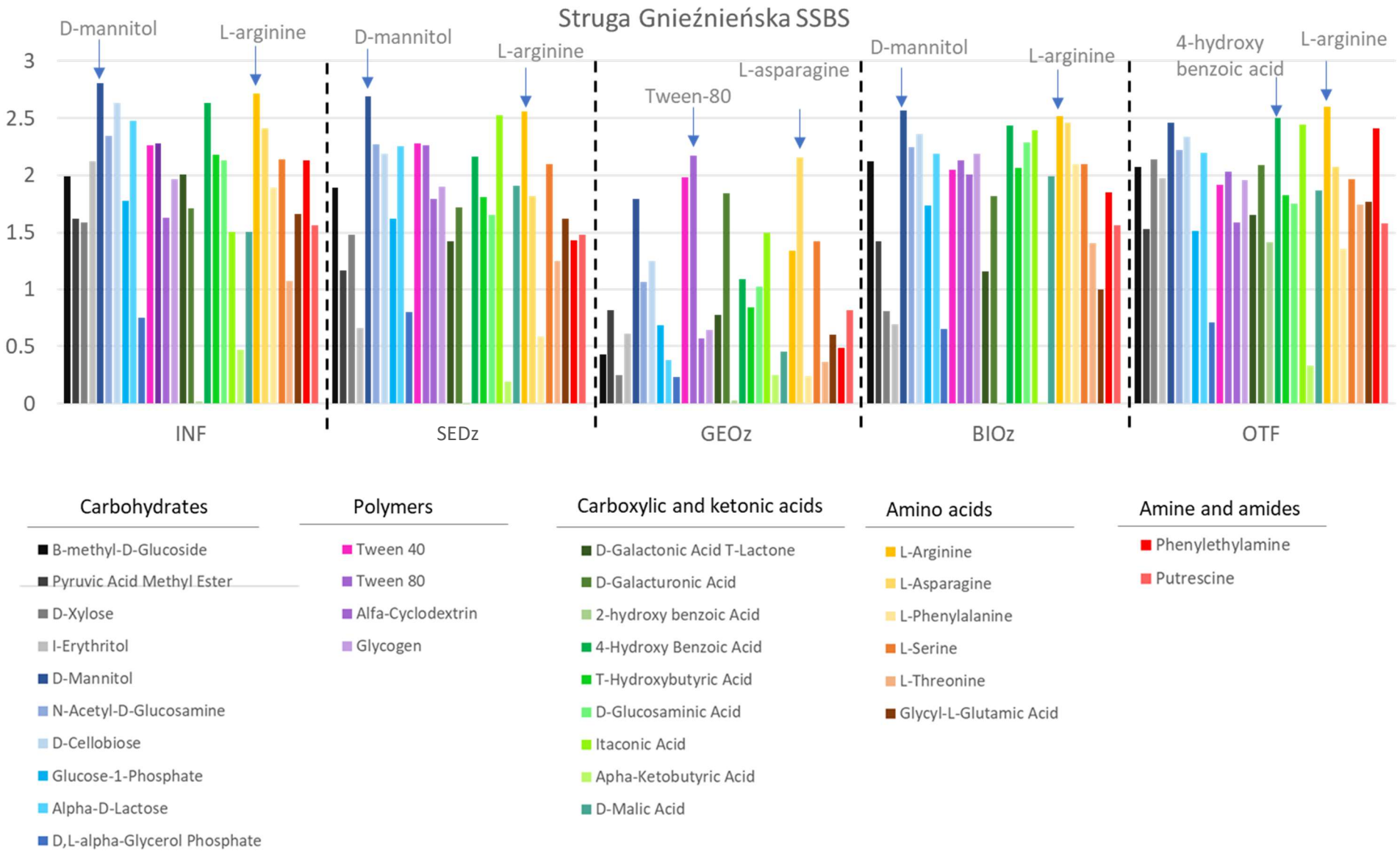

Fig. S5. Summary of carbon substrate utilization by heterotrophic microbial communities along the horizontal profile of Struga Gnieźnieńska SSBS

Table S7. Average copy numbers of key functional genes in samples (g^{-1} dry sediment) collected in August and October 2017 from both SSBSs.

SSBS	area	August 2017				October 2017			
		nosZ (380 bp)		*amoA* (380 bp)		*nosZ* (380 bp)		*amoA* (380 bp)	
		X	Stdev	*X*	Stdev	*X*	Stdev	*X*	Stdev
Sok	INF	8.28E+06	1.97E+06	2.27E+05	3.36E+04	5.84E+05	7.49E+04	2.62E+05	3.71E+03
	SEDz	**4.81E+07**	3.80E+05	**5.10E+05**	5.19E+04	1.40E+06	8.69E+04	**2.80E+05**	2.74E+04
	BIOFz	5.28E+06	2.86E+05	7.47E+04	5.92E+03	9.27E+05	4.58E+04	1.03E+05	4.56E+03
	OTFz	1.16E+07	4.27E+05	3.76E+05	2.38E+04	**1.86E+06**	4.11E+04	3.63E+05	3.99E+04
Str	INF	1.83E+07	1.15E+07	5.34E+04	1.09E+04	1.51E+06	8.37E+04	7.35E+04	6.69E+03
	SEDz	**9.97E+07**	4.86E+07	4.61E+04	1.03E+04	1.49E+06	3.05E+04	3.22E+04	8.49E+03
	BIOFz	3.98E+07	1.45E+07	1.81E+04	5.56E+03	8.17E+05	8.65E+04	1.65E+04	3.61E+03
	DENz	**1.16E+08**	8.92E+06	4.45E+04	5.78E+03	**7.41E+06**	1.50E+06	**5.68E+05**	4.43E+02
	OTFz	6.24E+07	8.45E+06	2.58E+05	8.49E+04	1.23E+06	8.63E+04	1.66E+05	1.77E+04

Values highlighted in black bold fonts are mentioned in the text. X: Media; Stdev: Standard deviation

Table S8. Tukey's honesty significant difference (HSD) tests to discriminate differences (p<0.05) between the copy numbers of key functional genes along the SSBSs horizontal profiles.

SSBS area (N:3-N:3)	Sokołówka SSBS (Sok-SSBS)			
	August		October	
	nosZ	*amoA*	*nosZ*	*amoA*
INF-SEDz	0.000176	0.999939	0.000176	0.999939
INF-BIOz	0.001678	0.791727	0.001678	0.791727
INF-OTF	0.000176	0.950962	0.000176	0.950962
SEDz-BIOz	0.000261	0.726636	0.000261	0.726636
SEDz-OTF	0.000261	0.975166	0.000261	0.975166
BIOz-OTF	0.000176	0.410786	0.000176	0.410786

SSBS area (N:3-N:3)	Struga Gnieźnieńska SSBS (Str-SSBS)			
	August		October	
	nosZ	*amoA*	*nosZ*	*amoA*
INF-SEDz	1.000000	0.001152	1.000000	0.001152
INF-BIOz	0.745026	0.000195	0.745026	0.000195
INF-DENz	0.000159	0.000159	0.000159	0.000159
INF-OTF	0.992704	0.000159	0.992704	0.000159
SEDz-BIOz	0.000159	0.000161	0.763993	0.317466
SEDz-DENz	0.000244	0.000159	0.000159	0.000159
SEDz-OTF	0.000159	0.000159	0.994640	0.000159
BIOz-DENz	0.000159	0.000159	0.000159	0.000159
BIOz-OTF	0.958595	0.000159	0.958595	0.000159
DENz-OTF	0.000159	0.065657	0.000159	0.065657

Red values indicate significant differences. N: number of measurements.

Table S9. BLAST sequence identity for genes *amo*A and *nos*Z in sediment samples from SSBSs, October 2017

Gene	SSBS	Sample	Microbial Blast				Nucleotide Blast			
			Query cover (%)	Identity (%)	Result	Accession number	Query cover (%)	Identity (%)	Result	Accession number
*amo*A (491 bp)	Sokolowka	Sediment	100	93	*Nitrosospira lacus* APG3	CP021106	100	99	Uncultured bacteria from environmental samples	KJ093945
	Struga Gnieźnieńska	Sediment	99	97	*Nitrosospira multiformis* NI1	FNKY01000001	100	98	Uncultured bacteria from environmental samples	KC773929
		Plasmid	92	81	*Nitrosomonas oligotropha* Nm75	FNOE01000063	100	98	Uncultured bacteria from environmental samples	KC735831
*nos*Z (380 bp)	Struga Gnieźnieńska	Sediment	99	87	*Thiobacillus denitrificans* RGTG21	LDUG01000021	99	96	Uncultured bacteria from environmental samples	KF543988
	Sokolowka	Sediment	99	88	*Thiobacillus thioparus* DSM505	KB891326	100	97	Uncultured bacteria from environmental samples	KF543962
		Plasmid	97	80	*Paracoccus sphaerophysae* HAMBI 3106	JRKS01000018	100	88	Uncultured bacteria from environmental samples	HQ115482

Appendix 5: SUPLEMENTARY MATERIAL

Seasonal and spatial changes of N-transforming microbial communities in sequential sedimentation-biofiltration systems - influence of system design and environmental conditions

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Table S1. General physical description and nutrient removal performances of SSBSs in previous studies.

SSBS river		Sokołówka	Bzura	Struga Gnieźnieńska
General Information	Code	Sok-SSBS	Bzr-SSBS	Str-SSBS
	City	Łódź	Łódź	Gniezno
	Dimension (width x length)	65 x16 m^2	130 x 20 m^2	17 x 7 m^2
	Area	1040 m^2	2600 m^2	119 m^2
	Maximum water capacity	440 m^3	10 000 m^3	55 m^3
	Operation time	9 years	5 years	3 years
SSBS horizontal profile (zones)	(i) Sedimentation (SEDz)	√	√	√
	(ii) Geochemical (GEOz)	√ **(limestone)**	√ **(dolomite)**	√ **(limestone)**
	(iii) Biofiltration (BIOz)	√	√	√
	(iv) Denitrification (DENz)	NC	NC	√ **(brown coal)**
Maximum nutrient removal performance (%)	NO_2^-	55.0 % [1]	NS	27.0 % [6]
	NO_3^-	46.0 % [4, 6]	91.3 % [5]	70.0 % [3]
	NH_4^+	2.8 % [4]	59.8 % [5]	48.0 % [6]
	PO_4^-	30.4 % [2]	49.0 % [5]	50.0 % [3]
	TN	56.0 % [4]	56.9 % [5]	32.0 % [6]
	TP	93.0 % [6]	57.1 % [5]	50.0 % [3]

NC: Not Constructed; NS: Not specified; [1]Zalewski et al., 2012; [2]Negussie et al., 2012; [3]Serwecińska et al., 2017; [4]Szklarek et al., 2018; [5]Jurczak et al. 2019; [6]Font-Nájera et al., 2020.

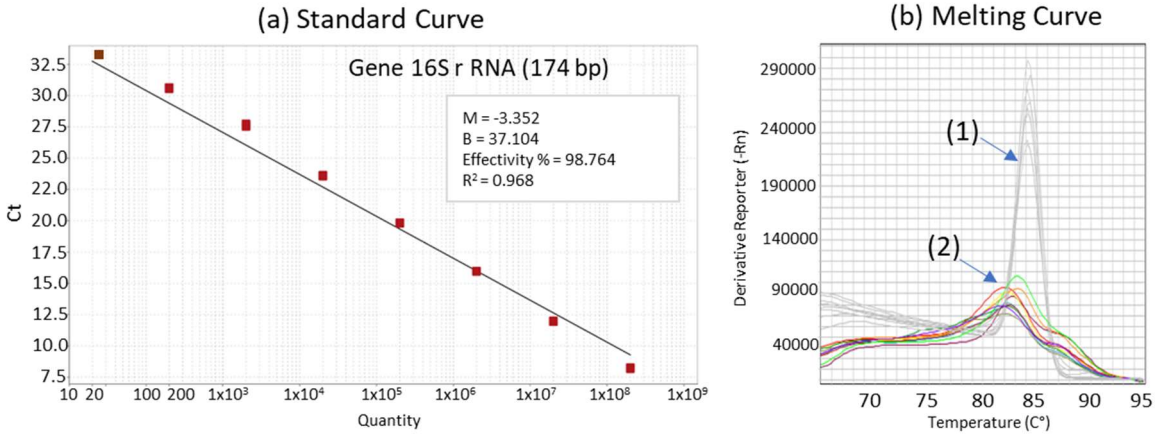

Fig S1. Estimation of standard curve efficiency (a) and the melting curve analysis (b) for the quantification of the gene 16S rRNA (174 bp) in SSBSs sediment samples.

The quantity refers to the amount of gene copies per µL of DNA. CT: Threshold cycle; R^2: Coefficient of determination for the linear regression; M: Slope; B: Intercept (Fig S1a).

The efficiency for the gene 16S rRNA was 98.7% (Fig S1a).

The plasmid used for the standard curve estimation was prepared from a sediment sample of the Bzr-SSBS, and therefore appears as defined melting peaks (several 10 fold dilutions) marked with the number (1) in grey colour. The environmental samples were diluted ten times (approximately 10-20 ng of total DNA), and are shown as colour melting peaks marked by the number (2). The slight differences in melting temperature between environmental samples was attributed to differences in DNA polymorphism, which was expected for the 16S rRNA gene representing the total amount/diversity of bacteria.

Table S2. Summary of physico-chemical parameters, nutrient concentrations and nutrient removal efficiencies in three sampling seasons for SSBSs.

Season	Area	Physico-chemical parameters			Concentration of nutrients										
					Surface Water					Interstitial Water					Dry sediments
		pH	Temp.	Oxygen	N-NO_3	N-NH_4	TN	P-PO_4	TP	N-NO_3	N-NH_4	TN	P-PO_4	TP	Organic Matter
			C°	mg L^{-1}	mg L^{-1}	mg L^{-1}	mg L^{-1}	mg L^{-1}	mg L^{-1}	mg L^{-1}	mg L^{-1}	mg L^{-1}	mg L^{-1}	mg L^{-1}	mg g^{-1}
Sok-SSBS															
Spring	Inflow	7.62	11.70	10.74	1.72	0.85	3.40	0.03	**0.10**	0.03	6.14	17.83	0.06	0.06	83.17
	Outflow	7.41	10.30	3.23	0.45	0.34	2.50	0.01	**0.11**	0.03	3.43	12.67	0.02	0.31	40.38
Summer	Inflow	8.07	20.00	6.40	0.55	**0.81**	5.90	0.40	0.45	0.17	**0.23**	**3.80**	0.23	0.30	**55.20**
	Outflow	7.43	20.60	2.74	0.11	**0.81**	2.00	0.16	0.18	0.05	**0.25**	**7.20**	0.12	0.10	**65.83**
Autumn	Inflow	7.74	9.20	13.15	**0.55**	**0.10**	3.30	0.18	0.47	0.40	**0.67**	**2.75**	0.03	0.23	**51.42**
	Outflow	7.45	9.70	5.91	**1.05**	**0.87**	2.18	0.08	0.08	0.03	**4.40**	**5.10**	0.02	0.08	**132.92**
Media	Inflow	7.81	13.63	10.10	0.94	**0.59**	4.20	0.20	0.34	0.20	**2.35**	**8.13**	0.11	0.20	**63.26**
	Outflow	7.43	13.53	3.96	0.54	**0.67**	2.23	0.08	0.12	0.04	**2.69**	**8.32**	0.05	0.16	**79.71**
Nutrient removal efficiency (%)					42.91	**NR**	46.98	59.02	64.68	81.83	**NR**	**NR**	50.00	17.42	**NR**
Bzr-SSBS															
Spring	Inflow	7.70	13.81	10.10	0.17	0.06	1.90	0.03	0.08	0.04	6.95	14.67	**0.05**	**0.06**	92.00
	Outflow	8.26	15.80	6.17	0.01	0.02	0.50	0.00	0.00	0.02	1.85	7.70	**0.05**	**0.06**	26.61
Summer	Inflow	7.83	20.30	9.65	0.37	0.07	3.40	0.22	0.32	**0.05**	**0.76**	**2.30**	0.12	0.32	51.38
	Outflow	7.30	23.80	5.45	0.03	0.05	3.30	0.08	0.09	**0.05**	**1.96**	**3.30**	0.07	0.05	22.22
Autumn	Inflow	7.61	10.60	10.40	**0.18**	0.71	1.19	**0.02**	0.28	**0.03**	**0.62**	0.98	**0.00**	0.15	107.13
	Outflow	7.61	9.50	8.80	**0.23**	0.17	0.50	**0.02**	0.15	**0.03**	**0.66**	0.78	**0.04**	0.12	30.14
Media	Inflow	7.71	14.90	10.05	0.24	0.28	2.16	0.09	0.23	0.04	2.78	5.98	0.06	0.18	83.50
	Outflow	7.72	16.37	6.81	0.09	0.08	1.43	0.03	0.08	0.03	1.49	3.93	0.05	0.08	26.32
Nutrient removal efficiency (%)					62.50	71.43	33.74	62.96	65.25	16.67	46.34	34.36	5.88	56.41	68.50
Str-SSBS															
Spring	Inflow	8.29	16.30	7.50	2.18	0.17	3.63	0.54	0.56	0.03	**6.44**	19.00	0.38	2.65	26.34
	Outflow	8.00	17.30	11.60	0.95	0.09	2.40	0.02	0.09	0.02	**8.78**	10.47	0.04	1.68	14.53
Summer	Inflow	7.62	21.60	1.20	0.08	0.55	4.70	0.46	0.47	**0.04**	6.26	13.30	1.16	1.33	**15.50**
	Outflow	7.51	23.60	1.40	0.03	0.26	3.10	0.14	0.15	**0.04**	4.67	6.80	0.22	0.45	**31.72**
Autumn	Inflow	8.06	14.50	10.40	0.49	0.69	2.10	0.26	0.30	0.05	1.67	2.30	0.73	0.89	**21.34**
	Outflow	8.00	13.90	8.40	0.03	0.02	1.50	0.03	0.12	0.04	0.20	1.30	0.10	0.10	**28.67**
Media	Inflow	7.99	17.47	6.37	0.92	0.47	3.48	0.42	0.44	0.04	4.79	11.53	0.76	1.62	**21.06**
	Outflow	7.84	18.27	7.13	0.34	0.12	2.33	0.06	0.12	0.03	4.55	6.19	0.12	0.74	**24.97**
Nutrient removal efficiency (%)					63.27	73.76	32.89	84.92	72.50	16.67	5.01	46.33	84.14	54.20	**NR**

The media for each parameter was calculated from the measurements obtained in three different sample collections (n=3). Nutrient removal efficiency (%) was estimated with the difference in nutrient concentration between inflow and outflow (media from three different sampling periods). Bold numbers indicate no nutrient reduction or increment in the nutrient concentration, from inflow (INF) to outflow (OTF) direction. NR: removal efficiency was not calculated, due to an overall nutrient increment in all three sampling seasons.

Table S3. Summary data of AWCD microbial metabolic activity, Shannon weaver diversity and richness indexes, after 120 h incubation of SSBSs sediment suspension samples.

		Metabolic activity						**Shannon Weaver Diversity (*H*)**						**Richness (*R*)**					
SSBS	zone	Spring		Summer		Autumn		Spring		Summer		Autumn		Spring		Summer		Autumn	
		Media	Stdev	Media	Stdev	Media	Stdev	Media	Stdev	Media	Stdev	Media	Stdev	Spring	Stdev	Media	Stdev	Media	Stdev
Sokolówka	INF	1.8150	±0.0450	1.7535	±0.1003	1.1949	±0.0959	3.3894	±0.0023	3.3793	±0.0017	3.2739	±0.0052	30.7	±0.2	29.7	±0.2	30.3	±0.2
	SEDz	1.8552	±0.0965	1.7233	±0.0813	1.2421	±0.0470	3.3168	±0.0063	3.2518	±0.0072	3.2442	±0.0088	29.0	±0.3	26.7	±0.4	28.3	±0.2
	GEOz	0.7465	±0.0694	1.0328	±0.0164	0.1573	±0.0868	2.6972	±0.0157	2.9636	±0.0057	1.6427	±0.0579	17.0	±0.3	21.0	±0.3	3.3	±0.3
	BIOz	1.2874	±0.0495	1.8081	±0.0283	0.8934	±0.1352	3.0148	±0.0238	3.3353	±0.0044	2.9927	±0.0349	21.7	±0.6	30.0	±0.3	20.3	±0.8
	OTF	1.6362	±0.1837	2.0656	±0.0328	1.2784	±0.1012	3.3268	±0.0073	3.3816	±0.0048	3.2859	±0.0141	28.3	±0.3	30.3	±0.2	28.0	±0.5
	SSBS zone	Media	Stdev	Media	Stdev	Media	Stdev	Spring	Stdev	Media	Stdev	Media	Stdev	Spring	Stdev	Media	Stdev	Media	Stdev
Bzura	INF	1.5395	±0.2347	1.8051	±0.1715	1.0520	±0.0645	3.2784	±0.0039	3.3552	±0.0006	3.1945	±0.0054	27.7	±0.2	29.7	±0.2	25.7	±0.2
	SEDz	1.6480	±0.0472	1.8090	±0.2335	1.2741	±0.0415	3.2011	±0.0095	3.2481	±0.0366	3.2797	±0.0042	25.7	±0.4	28.7	±0.2	28.3	±0.2
	GEOz	0.0707	±0.0661	0.1038	±0.0270	0.0298	±0.0069	1.4206	±0.0263	1.4864	±0.0709	0.4638	±0.0000	2.3	±0.6	2.7	±0.3	1.0	±0.3
	BIOz	1.0517	±0.0123	1.0257	±0.0272	1.0539	±0.2136	2.8903	±0.0193	3.2338	±0.0127	2.8765	±0.0076	19.3	±0.4	25.3	±0.7	18.3	±0.2
	OTF	0.8918	±0.0265	1.0834	±0.2018	0.2128	±0.0951	2.8834	±0.0100	2.9403	±0.0584	1.9710	±0.1057	19.3	±0.4	20.3	±1.1	6.3	±0.8
	SSBS zone	Media	Stdev	Media	Stdev	Media	Stdev	Spring	Stdev	Media	Stdev	Media	Stdev	Spring	Stdev	Media	Stdev	Media	Stdev
Struga Gnieznienska	INF	1.8172	±0.0321	1.6038	±0.1602	0.8610	±0.0943	3.2880	±0.0031	3.1073	±0.0200	2.9031	±0.0187	28.3	±0.2	23.7	±0.4	18.7	±0.4
	SEDz	1.9122	±0.0797	1.9399	±0.0441	0.5483	±0.0977	3.3137	±0.0080	3.2713	±0.0073	2.5791	±0.0431	28.7	±0.3	27.3	±0.2	13.3	±0.6
	GEOz	0.7048	±0.0387	1.0850	±0.0737	0.1862	±0.0296	2.7209	±0.0272	2.9776	±0.0146	1.9223	±0.0416	15.3	±0.4	20.7	±0.4	5.7	±0.2
	BIOz	1.7393	±0.1045	2.0481	±0.0338	1.0208	±0.1056	3.3254	±0.0027	3.3170	±0.0081	3.1102	±0.0144	29.3	±0.2	28.6	±0.1	25.6	±0.8
	OTF	1.9941	±0.0915	1.8913	±0.0306	1.1436	±0.0392	3.3237	0.0078	3.2697	0.0066	3.1903	0.0091	28.7	±0.2	27.3	±0.2	25.3	±0.3

Table S4. Statistical one way ANOVA analysis (a) and Tukey honesty test (b) for the microbial metabolic activity (AWCD value).

(a) One-way ANOVA

Sokołówka SSBS (Sok SSBS)

	INF (n=3)					SEDz (n=3)					GEOz (n=3)					BIOz (n=3)					OTF (n=3)				
	Sum of sqrs	df	Mean square	F	p(same)	Sum of sqrs	df	Mean square	F	p(same)	Sum of sqrs	df	Mean square	F	p(same)	Sum of sqrs	df	Mean square	F	p(same)	Sum of sqrs	df	Mean square	F	p(same)
Between groups	0.7002	2	0.3501	32.9	5.84E-04	0.6248	2	0.3124	34.5	5.13E-04	1.1956	2	0.5978	94.8	2.89E-05	1.2632	2	0.6316	58.7	1.15E-04	0.9321	2	0.4661	20.7	2.03E-03
Within groups	0.0639	6	0.0106			0.0544	6	0.0091			0.0378	6	0.0063			0.0646	6	0.0108			0.1352	6	0.0225		
Total	0.7641	8	0.0233			0.6792	8	0.0105			1.2334	8	0.0037			1.3277	8	0.0029			1.0673	8	0.0040		
Bzura SSBS (Bzr SSBS)																									
Between groups	0.8755	2	0.4377	9.9	0.01265	0.4519	2	0.2259	7.7	0.02188	0.0083	2	0.0041	1.6	**0.2769**	0.0015	2	0.0007	0.0	**0.9689**	1.2555	2	0.6277	24.9	1.25E-03
Within groups	0.2660	6	0.0443			0.1755	6	0.0292			0.0155	6	0.0026			0.1396	6	0.0233			0.1515	6	0.0252		
Total	1.1415	8	0.0142			0.6274	8	0.0210			0.0237	8	0.2927			0.1410	8	0.8057			1.4069	8	0.0143		
Struga Gnieźnieńska SSBS (Str SSBS)																									
Between groups	1.5114	2	0.7557	42.5	2.87E-04	3.7977	2	1.8989	212.8	2.69E-06	1.2213	2	0.6107	156.5	6.66E-06	1.6669	2	0.8335	71.8	6.45E-05	1.2929	2	0.6464	119.2	1.48E-05
Within groups	0.1068	6	0.0178			0.0535	6	0.0089			0.0234	6	0.0039			0.0696	6	0.0116			0.0326	6	0.0054		
Total	1.6182	8	0.0058			3.8513	8	0.0196			1.2447	8	0.0038			1.7365	8	0.0011			1.3254	8	0.0107		

(b) Tukey's HSD test

Sokołówka SSBS (Sok SSBS)

	INF (n=3)			SEDz (n=3)			GEOz (n=3)			BIOz (n=3)			OTF (n=3)		
	Spring	Summer	Autumn	Spring	Summer	Autumn	Spring	Summer	Autumn	Spring	Summer	Autumn	Spring	Summer	Autumn
Spring		0.7557	7.87E-04		0.2811	5.39E-04		1.07E-02	2.45E-04		2.06E-03	8.33E-03		2.96E-02	6.01E-02
Summer	1.033		1.38E-03	2.4		1.98E-03	6.243		2.51E-05	8.695		9.19E-05	4.955		1.64E-03
Autumn	10.41	9.378		11.16	8.756		12.85	19.09		6.579	15.27		4.129	9.084	
Bzura SSBS (Bzr SSBS)															
Spring		0.3371	6.68E-02		0.52	8.14E-02		0.7174	0.6101		0.9763	0.9998		0.3651	4.69E-03
Summer	2.185		1.11E-02	1.63		2.02E-02	1.13		0.2518	0.2954		0.9722	2.089		1.30E-03
Autumn	4.01	6.196		3.787	5.418		1.398	2.527		0.02516	0.3206		7.401	9.49	
Struga Gnieźnieńska SSBS (Str SSBS)															
Spring		0.2033	2.98E-04		0.9325	4.77E-06		7.34E-04	1.30E-04		2.93E-02	4.44E-04		0.2772	1.90E-05
Summer	2.77		1.19E-03	0.5067		4.22E-06	10.54		4.88E-06	4.965		5.86E-05	2.416		4.08E-05
Autumn	12.41	9.644		25.01	25.51		14.38	24.92		11.55	16.52		20	17.58	

Significant values are marked in red ($p < .05$) and blue ($p < 0.1$) colour.

Table S5. Summary of carbon substrate utilization in the horizontal profile of Sokolówka -SSBS, after 120 h of sediment sample incubation.

	Carbon substrate	Spring					Summer					Autumn				
		INF	SEDz	GEOz	BIOz	OTF	INF	SEDz	GEOz	BIOz	OTF	INF	SEDz	GEOz	BIOz	OTF
Carbohydrates	D-Mannitol	2.195	2.662	1.788	2.908	2.252	2.061	2.669	1.299	2.328	2.598	1.782	1.949	0.513	2.053	1.898
	N-Acetyl-D-Glucosamine	2.172	2.444	2.079	2.525	2.080	2.055	2.500	1.231	2.335	2.462	1.695	1.807	0.361	2.028	1.808
	D-Cellobiose	2.054	2.612	1.978	0.496	2.013	1.740	2.501	1.207	2.561	2.414	1.827	1.492	0.101	1.925	1.895
	B-methyl-D-Glucoside	2.097	2.425	1.130	1.490	2.173	2.105	2.455	1.210	2.313	2.420	1.172	1.273	0.002	1.549	1.299
	D-Xylose	2.345	2.687	1.136	0.722	2.764	2.252	1.911	1.440	2.620	2.880	1.189	1.460	**0.000**	0.268	1.897
	Pyruvic Acid Methyl Ester	1.837	1.763	0.794	1.516	1.709	2.150	1.687	0.930	1.650	1.860	0.958	0.927	**0.000**	1.494	0.851
	Alpha-D-Lactose	1.970	2.274	0.957	0.491	1.908	2.102	2.281	1.131	2.223	2.263	1.301	1.460	1.116	0.079	1.828
	Glucose-1-Phosphate	1.808	1.917	1.033	1.274	1.597	1.546	1.579	0.867	1.393	1.734	1.428	1.326	**0.000**	1.237	1.268
	I-Erythritol	1.627	1.108	0.240	1.332	1.830	0.631	0.288	0.890	1.472	1.779	0.544	0.914	0.227	0.886	1.132
	D,L-alpha-Glycerol Phosphate	0.938	0.718	0.344	1.072	0.890	1.024	1.115	0.753	0.901	1.506	0.393	0.216	0.007	0.291	0.363
Polymers	Glycogen	1.858	2.226	0.944	1.616	1.639	2.338	2.334	1.352	2.273	2.704	1.676	1.825	**0.000**	1.062	1.885
	Tween 80	1.755	1.987	1.367	1.401	1.777	1.822	2.238	1.178	1.667	2.356	1.208	1.592	0.113	1.051	1.705
	Tween 40	1.807	2.058	0.990	1.203	1.606	1.586	2.294	1.130	1.969	2.260	1.305	1.695	**0.000**	1.355	1.740
	Alfa-Cyclodextrin	1.988	1.899	0.279	0.073	1.599	1.979	2.102	1.081	1.891	2.162	1.575	1.724	0.316	0.051	1.332
Carboxylic acids	T-Hydroxybutyric Acid	1.961	2.363	0.238	0.532	2.384	1.900	2.331	0.812	2.349	1.624	1.865	1.620	**0.000**	1.690	1.706
	4-Hydroxy Benzoic Acid	2.090	2.026	1.140	2.197	1.794	2.170	2.579	1.322	1.675	2.644	1.678	1.563	**0.000**	0.893	1.873
	D-Galacturonic Acid	1.885	2.039	0.710	1.582	1.830	1.976	1.898	1.233	1.848	2.466	1.464	0.595	1.087	1.612	1.256
	D-Glucosaminic Acid	2.264	2.005	0.126	1.723	2.064	1.985	0.506	1.042	2.153	2.085	1.416	1.274	**0.000**	0.305	1.519
	D-Malic Acid	1.683	1.403	0.025	2.217	1.404	1.654	0.894	1.103	1.794	2.205	0.751	1.739	**0.000**	0.224	1.140
	D-Galactonic Acid T-Lactone	2.029	2.015	0.810	1.055	2.025	1.551	1.382	1.046	1.635	2.092	1.229	1.014	0.009	1.382	0.935
	Itaconic Acid	2.225	1.693	0.015	1.792	1.889	1.763	1.753	1.380	2.707	2.760	1.811	1.313	**0.000**	0.739	0.718
	2-hydroxy benzoic Acid	1.487	0.618	**0.000**	**0.000**	0.714	0.413	**0.000**	0.188	0.594	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.050
	Apha-Ketobutyric Acid	0.273	**0.000**	**0.000**	0.368	**0.000**	0.974	0.863	0.565	0.213	1.130	**0.000**	0.002	**0.000**	**0.000**	0.015
Amino acids	L-Asparagine	1.882	2.507	0.989	2.895	1.846	1.943	2.365	1.242	2.091	2.484	1.673	1.841	0.629	1.753	1.786
	L-Arginine	2.176	2.692	0.349	2.460	2.350	2.335	1.203	1.107	2.425	2.214	1.413	1.499	0.040	0.212	1.216
	L-Serine	1.964	2.229	1.694	1.506	1.846	1.828	2.234	1.172	2.144	2.344	1.710	1.678	**0.000**	1.123	1.694
	Glycyl-L-Glutamic Acid	1.515	1.320	0.529	0.540	1.534	1.495	1.872	1.121	1.442	2.241	1.094	1.412	**0.000**	0.361	1.482
	L-Phenylalanine	1.433	0.533	0.092	0.244	0.286	1.901	1.204	0.530	0.654	1.059	0.578	0.756	0.001	0.498	0.554
	L-Threonine	1.003	1.449	0.637	0.351	1.385	0.977	1.592	0.681	0.785	1.361	0.406	0.285	**0.000**	0.043	0.683
Amines Amides	Phenylethylamine	2.236	2.336	**0.000**	0.934	0.515	2.472	0.952	0.903	2.472	1.807	1.343	1.386	**0.000**	0.736	1.331
	Putrescine	1.704	1.541	0.747	1.401	1.172	1.628	1.887	0.873	1.473	1.746	0.931	0.915	0.591	0.846	0.771

INF: inflow, SEDz: sedimentation zone, GEOz: geochemical zone, BIOz: biofiltration zone, OTF: outflow. No metabolic activity registered (0.000) for the specific carbon source was marked with bold numbers.

Table S6. Summary of carbon substrate utilization in the horizontal profile of Bzura-SSBS, after 120 h of sediment sample incubation.

	Carbon substrate	Spring					Summer					Autumn				
		INF	SEDz	GEOz	BIOz	OTF	INF	SEDz	GEOz	BIOz	OTF	INF	SEDz	GEOz	BIOz	OTF
Carbohydrates	D-Mannitol	2.285	2.436	0.073	3.010	2.340	2.210	2.350	0.305	2.764	2.346	1.849	1.855	**0.000**	1.921	0.747
	N-Acetyl-D-Glucosamine	2.213	2.655	0.012	1.536	1.807	2.179	2.460	**0.000**	1.623	2.542	1.892	1.865	**0.000**	1.497	**0.000**
	D-Cellobiose	2.249	2.686	**0.000**	1.822	1.770	2.070	2.253	0.003	0.027	1.946	1.764	1.905	**0.000**	1.926	0.787
	B-methyl-D-Glucoside	2.219	2.419	0.004	0.705	1.265	2.223	2.274	0.011	1.114	1.644	1.312	1.389	**0.000**	1.377	0.102
	D-Xylose	2.205	2.476	**0.000**	0.232	0.965	2.684	1.667	**0.000**	0.204	**0.000**	0.642	1.412	**0.000**	1.397	**0.000**
	Pyruvic Acid Methyl Ester	1.718	2.224	**0.000**	1.428	1.556	2.081	1.566	0.173	1.577	1.515	0.818	1.066	0.166	0.865	0.472
	Alpha-D-Lactose	0.710	1.316	0.018	0.504	0.320	2.108	2.399	**0.000**	0.301	1.039	0.681	1.309	**0.000**	0.898	0.013
	Glucose-1-Phosphate	1.541	1.737	0.020	0.033	0.625	1.527	1.749	0.019	1.027	1.036	1.513	1.302	**0.000**	1.464	**0.000**
	I-Erythritol	0.949	0.674	**0.000**	0.373	0.320	0.991	0.139	**0.000**	0.176	0.418	0.576	0.475	**0.000**	0.384	**0.000**
	D,L-alpha-Glycerol Phosphate	0.638	0.523	**0.000**	0.449	0.384	1.086	0.811	0.003	0.199	0.377	0.544	0.420	**0.000**	0.321	0.172
Polymers	Glycogen	2.057	2.207	**0.000**	1.690	1.362	2.414	2.849	0.035	0.020	2.582	1.733	1.728	**0.000**	1.413	1.456
	Tween 80	1.537	1.802	0.481	1.680	1.576	2.322	2.091	0.414	1.689	1.656	1.621	1.595	**0.000**	1.116	0.230
	Tween 40	1.434	2.148	0.611	1.077	1.165	1.823	1.715	1.655	1.766	1.855	1.125	1.574	**0.000**	1.397	0.112
	Alfa-Cyclodextrin	1.521	0.962	**0.000**	0.027	0.118	1.122	1.653	0.288	0.010	0.170	0.669	1.740	**0.000**	1.449	0.022
Carboxylic acids	T-Hydroxybutyric Acid	1.351	1.531	0.055	0.221	0.742	2.079	2.455	0.205	2.076	0.947	1.448	2.128	**0.000**	1.403	**0.000**
	4-Hydroxy Benzoic Acid	1.907	1.548	0.205	0.660	0.071	2.428	2.226	**0.000**	1.278	0.677	1.364	1.297	**0.000**	1.144	**0.000**
	D-Galacturonic Acid	2.015	2.308	**0.000**	2.736	1.045	2.028	1.886	**0.000**	2.445	0.996	1.467	1.462	**0.000**	1.569	0.476
	D-Glucosaminic Acid	1.696	2.335	0.416	1.513	0.872	1.779	2.305	0.014	1.674	0.682	0.881	1.665	**0.000**	1.395	**0.000**
	D-Malic Acid	2.132	2.168	**0.000**	0.286	0.348	2.264	1.718	0.015	0.078	0.377	0.953	1.461	**0.000**	1.185	0.618
	D-Galactonic Acid T-Lactone	1.623	1.831	**0.000**	1.370	1.438	2.120	1.740	**0.000**	1.154	0.973	0.894	0.957	**0.000**	0.992	0.014
	Itaconic Acid	1.624	0.658	**0.000**	1.074	0.002	0.992	2.087	0.016	0.807	0.859	0.594	1.355	**0.000**	0.997	**0.000**
	2-hydroxy benzoic Acid	**0.000**	0.129	**0.000**	**0.000**	**0.000**	**0.000**	0.628	0.019	**0.000**	0.044	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
	Apha-Ketobutyric Acid	0.083	0.007	**0.000**	0.006	**0.000**	0.967	0.099	**0.000**	**0.000**	0.009	**0.000**	0.100	**0.000**	**0.000**	**0.000**
Amino acids	L-Asparagine	2.711	2.840	0.001	2.780	2.244	2.474	2.351	0.008	2.471	1.988	1.841	1.838	**0.000**	1.649	0.698
	L-Arginine	2.324	2.534	**0.000**	1.778	1.394	2.272	2.430	0.079	2.683	2.288	1.318	1.597	0.382	1.610	0.620
	L-Serine	2.066	2.380	**0.000**	2.568	1.598	2.245	2.236	0.008	2.117	1.779	1.539	1.725	**0.000**	1.406	0.004
	Glycyl-L-Glutamic Acid	1.030	0.993	0.105	0.573	0.568	1.860	2.071	0.007	0.536	1.206	0.757	0.810	**0.000**	0.299	**0.000**
	L-Phenylalanine	0.151	0.709	0.061	0.014	0.251	0.806	0.876	0.007	0.031	0.554	0.212	0.773	**0.000**	0.415	0.258
	L-Threonine	0.389	0.442	0.002	0.135	0.142	1.512	0.905	0.034	0.010	0.572	0.430	0.712	**0.000**	0.251	0.087
Amines Amides	Phenylethylamine	1.965	1.060	**0.000**	1.413	0.450	2.075	2.381	0.021	0.847	0.046	0.827	1.154	**0.000**	0.804	**0.000**
	Putrescine	1.408	1.350	0.279	0.937	0.987	1.433	1.709	**0.000**	1.108	0.476	1.397	0.867	0.324	0.423	0.023

INF: inflow, SEDz: sedimentation zone, GEOz: geochemical zone, BIOz: biofiltration zone, OTF: outflow. No metabolic activity registered (0.000) for the specific carbon source was marked with bold numbers.

Table S7. Summary of carbon substrate utilization in the horizontal profile of Struga Gnieźnieńska-SSBS, after 120 h of sediment sample incubation.

	Carbon substrate	Spring					Summer					Autumn				
		INF	SEDz	GEOz	BIOz	OTF	INF	SEDz	GEOz	BIOz	OTF	INF	SEDz	GEOz	BIOz	OTF
Carbohydrates	D-Mannitol	2.827	2.547	1.047	2.618	2.857	2.833	2.591	2.873	2.487	2.187	0.780	0.411	0.118	1.683	1.967
	N-Acetyl-D-Glucosamine	2.058	2.407	0.723	2.418	2.714	2.750	2.594	1.442	2.650	2.584	1.641	1.196	0.306	1.716	1.742
	D-Cellobiose	2.609	2.239	0.026	2.683	2.729	2.758	2.418	2.056	2.737	2.523	1.225	0.718	0.003	1.634	1.937
	B-methyl-D-Glucoside	2.103	2.348	0.016	2.034	2.296	2.138	2.373	1.286	2.466	2.083	0.383	1.263	0.012	1.263	1.333
	D-Xylose	2.504	2.854	**0.000**	2.808	2.778	0.286	2.133	0.200	2.218	0.624	0.307	**0.000**	**0.000**	0.294	0.484
	Pyruvic Acid Methyl Ester	1.398	1.704	0.829	1.610	2.057	2.088	2.243	0.996	2.498	2.155	0.940	1.004	0.130	0.855	1.414
	Alpha-D-Lactose	2.567	2.738	0.010	2.371	2.216	0.153	1.996	0.237	2.022	2.628	**0.000**	0.430	**0.000**	**0.000**	0.649
	Glucose-1-Phosphate	1.466	1.716	0.015	1.730	1.319	1.154	1.972	0.033	1.855	1.862	0.365	1.043	**0.000**	1.020	1.538
	I-Erythritol	2.057	1.894	0.004	1.171	1.979	0.671	0.154	0.575	0.127	0.332	0.364	**0.000**	**0.000**	0.045	0.708
	D,L-alpha-Glycerol Phosphate	0.729	1.038	0.034	0.933	0.977	0.687	0.889	0.274	0.953	0.849	0.339	0.137	0.201	0.184	0.220
Polymers	Glycogen	3.072	2.470	0.046	1.358	2.194	1.767	2.208	0.906	2.462	2.411	1.857	1.382	1.087	1.912	1.795
	Tween 80	2.192	2.163	1.776	1.673	2.238	2.132	2.334	1.581	2.250	2.313	1.475	1.114	1.286	1.495	1.471
	Tween 40	1.641	2.016	1.732	1.921	1.840	2.208	2.021	1.461	2.129	2.525	1.513	1.235	0.002	1.530	1.717
	Alfa-Cyclodextrin	1.356	1.573	0.004	1.976	1.979	1.760	2.701	**0.000**	2.574	2.226	1.926	1.194	**0.000**	1.216	1.140
Carboxylic acids	T-Hydroxybutyric Acid	1.379	2.164	1.034	0.730	1.771	2.509	2.899	0.579	2.942	2.979	1.979	1.420	0.938	1.893	1.779
	4-Hydroxy Benzoic Acid	2.285	2.200	1.178	2.528	2.560	2.332	2.610	1.705	2.279	2.480	1.636	0.469	**0.000**	1.700	1.677
	D-Galacturonic Acid	2.317	2.430	1.573	2.129	2.545	2.226	2.101	2.085	1.854	2.486	0.011	0.582	**0.000**	0.148	1.171
	D-Glucosaminic Acid	2.369	2.209	1.595	2.208	2.467	1.137	1.600	1.104	2.462	0.831	0.516	0.014	**0.000**	1.063	1.447
	D-Malic Acid	2.011	2.109	0.484	1.562	2.225	2.165	2.190	0.424	2.133	2.396	1.366	0.836	0.088	0.963	0.677
	D-Galactonic Acid T-Lactone	1.792	2.225	1.028	2.146	2.148	1.392	2.008	1.753	2.067	1.616	0.610	0.005	0.004	1.246	1.071
	Itaconic Acid	1.415	2.640	0.230	1.623	2.368	0.011	1.923	1.689	2.233	2.224	**0.000**	**0.000**	**0.000**	1.235	1.188
	2-hydroxy benzoic Acid	0.261	0.316	0.014	**0.000**	0.057	**0.000**	**0.000**	**0.000**	0.405	0.435	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
	Apha-Ketobutyric Acid	0.087	**0.000**	0.015	0.129	0.089	0.009	0.003	**0.000**	0.077	0.026	0.034	**0.000**	**0.000**	**0.000**	**0.000**
Amino acids	L-Asparagine	2.728	2.323	2.411	2.424	2.666	2.725	2.755	2.736	2.390	2.582	1.670	0.256	0.999	1.730	1.794
	L-Arginine	2.273	2.696	1.770	2.379	2.672	2.658	2.777	2.087	2.904	2.271	1.689	1.059	**0.000**	1.447	1.688
	L-Serine	2.207	2.458	1.947	2.097	2.507	2.430	2.626	2.366	2.739	2.547	0.646	**0.000**	0.509	1.797	1.403
	Glycyl-L-Glutamic Acid	1.602	1.338	0.480	1.510	1.167	1.001	1.506	0.805	1.650	1.383	0.543	0.046	0.079	0.423	0.529
	L-Phenylalanine	0.323	0.727	0.026	0.234	0.406	1.190	1.562	0.276	2.218	1.841	0.311	0.253	0.007	0.927	0.580
	L-Threonine	0.845	0.895	0.058	0.904	1.568	0.035	0.743	0.140	1.100	0.893	0.132	**0.000**	0.039	0.143	0.085
Amines Amides	Phenylethylamine	2.423	1.447	0.700	2.102	2.505	2.830	2.839	0.776	2.752	2.936	1.737	0.727	0.001	1.483	1.634
	Putrescine	1.436	1.411	1.051	1.995	1.923	1.700	1.379	1.258	1.861	1.402	0.739	0.315	0.098	0.675	0.692

INF: inflow, SEDz: sedimentation zone, GEOz: geochemical zone, BIOz: biofiltration zone, OTF: outflow. No metabolic activity registered (0.000) for the specific carbon source was marked with bold numbers.

Table S8. Summary of total bacteria and N-transforming gene abundances in SSBSs for three different seasons (Spring, Summer and Autumn), year 2018.

Season	Area	Sok-SBSS						Bzr-SBSS						Str-SBSS					
		16s rRNA		*amo*A		*nos*Z		16s rRNA		*amo*A		*nos*Z		16s rRNA		*amo*A		*nos*Z	
		Media (χ)	Stdev (±)	Media (χ)	Stdev (±)	Media (χ)	Stdev (±)	Media (χ)	Stdev (±)	Media (χ)	Stdev (±)	Media (χ)	Stdev (±)	Media (χ)	Stdev (±)	Media (χ)	Stdev (±)	Media (χ)	Stdev (±)
Spring	INF	1.60E+08	9.91E+06	7.16E+05	2.00E+05	4.44E+05	1.59E+04	5.31E+08	7.77E+07	1.21E+06	4.24E+05	5.95E+05	3.43E+04	2.72E+08	4.08E+06	6.07E+05	2.50E+05	4.94E+05	1.77E+04
	SEDz	3.63E+08	1.02E+08	7.60E+06	5.09E+05	8.79E+05	1.44E+05	3.53E+08	1.26E+07	2.09E+06	8.24E+05	6.62E+05	3.54E+04	3.65E+08	7.48E+06	1.31E+06	3.38E+05	5.42E+05	5.44E+04
	GEOz	3.10E+08	3.05E+07	8.95E+07	1.79E+07	2.84E+06	5.25E+04	7.22E+05	6.16E+04	1.50E+02	2.20E+01	5.18E+03	2.21E+02	1.84E+08	4.79E+06	5.73E+07	4.00E+06	1.34E+06	6.90E+03
	DENz	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2.94E+08	5.59E+07	2.70E+06	4.72E+04	7.21E+05	4.53E+04
	BIOz	8.10E+08	1.96E+07	2.34E+06	4.19E+05	8.99E+05	1.22E+05	5.88E+07	2.54E+07	5.78E+04	1.04E+04	1.27E+05	1.54E+04	4.87E+07	8.34E+07	1.46E+07	1.34E+06	5.27E+05	1.84E+04
	OTF	3.75E+08	1.53E+07	3.15E+06	5.77E+05	4.65E+05	3.03E+04	1.25E+08	1.08E+08	1.63E+04	3.19E+03	3.45E+05	2.57E+04	1.92E+08	4.26E+07	5.39E+06	1.43E+06	3.37E+05	1.60E+04
Summer	INF	1.21E+10	2.08E+08	1.50E+05	6.55E+03	2.42E+09	1.94E+08	1.52E+10	1.45E+09	2.21E+05	1.92E+04	1.85E+07	2.14E+06	2.88E+10	7.12E+08	2.14E+04	1.94E+03	6.07E+07	1.79E+06
	SEDz	3.89E+10	5.33E+09	1.34E+05	1.27E+04	2.92E+08	3.02E+07	4.02E+10	3.51E+09	6.54E+05	3.42E+04	7.65E+07	4.84E+06	5.60E+10	1.12E+09	1.09E+04	4.78E+03	1.97E+08	2.04E+07
	GEOz	8.26E+09	6.24E+08	7.03E+04	1.52E+04	9.11E+07	6.34E+07	3.83E+07	1.44E+07	4.51E+02	2.50E+01	2.99E+04	2.74E+03	4.42E+09	2.55E+08	2.15E+03	3.56E+02	3.09E+08	1.32E+07
	DENz	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1.94E+11	1.02E+09	1.56E+05	2.18E+04	2.02E+10	5.32E+09
	BIOz	2.56E+10	2.33E+09	2.35E+04	3.47E+03	4.25E+07	3.66E+06	1.63E+09	1.50E+08	3.23E+03	1.28E+03	9.82E+06	1.22E+06	5.48E+10	1.64E+10	6.92E+03	2.54E+02	2.45E+08	3.85E+07
	OTF	1.09E+10	9.13E+08	9.71E+04	1.07E+04	5.78E+07	3.13E+06	4.28E+09	8.36E+07	2.27E+03	1.24E+03	1.22E+07	9.45E+05	3.97E+10	1.07E+10	1.51E+04	6.03E+02	4.85E+08	1.37E+08
Autumn	INF	1.03E+09	4.03E+06	9.12E+05	1.10E+05	4.90E+07	2.23E+06	1.53E+09	8.13E+07	4.79E+05	4.54E+04	1.14E+07	1.55E+06	5.16E+09	2.08E+08	6.25E+04	2.50E+04	5.46E+07	3.93E+06
	SEDz	3.49E+09	3.83E+08	6.53E+05	7.56E+04	2.39E+08	2.95E+07	3.65E+09	2.24E+08	8.63E+05	2.72E+03	2.06E+07	2.12E+06	6.28E+09	1.09E+08	1.64E+05	1.43E+04	4.41E+07	1.45E+06
	GEOz	5.11E+09	3.32E+08	3.14E+05	4.16E+04	5.29E+08	8.27E+07	4.68E+06	8.02E+05	1.41E+02	1.00E+01	2.80E+04	1.84E+02	1.89E+09	8.00E+07	6.61E+04	7.97E+03	1.49E+08	2.94E+06
	DENz	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1.32E+10	4.12E+09	5.55E+03	9.54E+02	1.34E+07	4.67E+05
	BIOz	3.59E+09	2.10E+08	4.29E+04	8.58E+03	2.71E+07	1.24E+06	5.49E+08	1.43E+08	3.78E+05	8.04E+04	1.00E+07	8.56E+05	8.42E+08	3.20E+07	3.09E+04	1.44E+04	4.11E+07	1.69E+06
	OTF	1.07E+09	6.52E+07	3.55E+04	8.44E+03	1.32E+07	6.08E+05	5.07E+09	7.68E+07	1.81E+04	1.50E+03	3.97E+07	3.26E+05	5.19E+09	4.67E+07	1.41E+05	1.26E+04	5.66E+07	6.06E+06

NA: Not applicable. Stdev: Standard deviation.

Table S9. Description of N-transforming and 16S rRNA gene ratios expressed in percentages (%).

	Sok-SSBS					
	*amo*A			*nos*Z		
SSBS zone	Spring	Summer	Autumn	Spring	Summer	Autumn
INF	0.4	0.00124	0.089	0.3	20.0	4.8
SEDz	2.1	0.00034	0.019	0.2	0.8	6.9
GEOz	28.9	0.00085	0.006	0.9	1.1	10.3
BIOz	0.3	0.00009	0.001	0.1	0.2	0.8
OT	0.8	0.00089	0.003	0.1	0.5	1.2
	Bzr-SSBS					
	*amo*A			*nos*Z		
SSBS zone	Spring	Summer	Autumn	Spring	Summer	Autumn
INF	0.23	0.00145	0.0313	0.1	0.1	0.7
SEDz	0.59	0.00163	0.0236	0.2	0.2	0.6
GEOz	0.02	0.00118	0.0043	0.7	7.8	6.0
BIOz	0.10	0.00020	0.0689	0.2	0.6	1.8
OT	0.01	0.00005	0.0004	0.3	0.3	0.8
	Str-SSBS					
SSBS zone		*amo*A			*nos*Z	
INF	0.2	0.00007	0.0012	0.2	0.2	1.1
SEDz	0.4	0.00002	0.0026	0.1	0.4	0.7
GEOz	31.1	0.00005	0.0035	0.7	7.0	7.9
DENz	2.8	0.00008	0.0007	0.7	10.4	1.6
BIOz	5.0	0.00001	0.0002	0.2	0.4	0.3
OT	5.0	0.00001	0.0002	0.2	0.4	0.3

Ratio was calculated between the copy numbers of N-transforming genes, with the 16s rRNA representing total bacteria (*amo*A/16s RNA and *nos*Z/16S rRNA). The ratio was multiplied by a 100 to represent the fraction of the selected community in percentage.

Table S10. Statistical Kruskal Wallis (a) and Mann Whitney pairwise analysis (b) to test significant differences between the N-transforming gene copy number and the season of collection.

(a) Kruskal Wallis test

Parameter	Sok-SSBS		Bzr-SSBS		Str-SSBS	
	*amo*A (n = 15)	*nos*Z (n = 15)	*amo*A (n = 15)	*nos*Z (n = 15)	*amo*A (n = 18)	*nos*Z (n = 18)
H (chi2):	28.18	28.87	2.40	13.59	37.94	45.31
Hc (tie corrected):	28.18	28.87	2.40	13.59	37.94	45.31
p (same):	7.59E-07	5.38E-07	0.301	1.12E-03	5.78E-09	1.45E-10

Significant values are marked in red ($p < .05$) colour.

(b) Mann Whitney pairwise test

	Sok-SSBS		Bzr-SSBS		Str-SSBS	
	*amo*A		*amo*A		*amo*A	
	Summer	Autumn	Summer	Autumn	Summer	Autumn
Spring	3.39E-06	2.80E-05	0.199	0.648	3.23E-07	3.23E-07
Summer		0.097		0.199		0.01691
	*nos*Z		*nos*Z		*nos*Z	
Spring	7.45E-06	7.45E-06	1.62E-03	1.62E-03	3.23E-07	3.23E-07
Summer		0.070		0.868		2.62E-06

Significant values are marked in red ($p < .05$) colour.

Table S11. Description of PCA scores for the sediment samples analysed in SSBSs.

Season	SSBS area	Sok-SSBS				Bzr-SSBS				Str-SSBS			
		PC 1	PC 2	PC 3	PC 4	PC 1	PC 2	PC 3	PC 4	PC 1	PC 2	PC 3	PC 4
Spring	INF	1.9222	0.5561	-1.0086	-0.2829	1.9349	0.3008	1.1347	-0.6181	1.6108	0.2278	-0.7436	1.6494
	SEDz	2.2686	-0.7160	-0.1965	0.4136	2.0932	-0.0364	1.5664	-0.4264	2.1030	0.5861	0.1810	-0.4882
	BIOz	2.5181	-1.5479	-0.2666	0.5221	2.2862	-0.6313	-0.3563	-0.3746	3.1036	0.5634	0.2989	-0.5032
	OTF	2.3740	0.7716	-0.8265	-0.6059	2.7910	-2.1045	-0.7248	0.1151	2.8052	0.8885	0.1513	-0.0679
Summer	INF	-2.7896	1.1952	-0.5108	0.6486	-2.2794	-0.7579	1.2364	-0.3075	-1.3225	0.2850	1.2571	0.8077
	SEDz	-0.5453	-0.1568	0.8170	1.7931	-2.8013	-0.0243	1.9439	0.2207	-2.3950	1.1800	-0.9587	-0.6120
	BIOz	-2.6338	-1.8500	-0.2205	-0.9495	-2.3674	-1.7468	-0.8770	0.0465	-2.0887	1.0379	1.0829	0.2883
	OTF	-2.0816	-0.5861	-0.8687	-0.5684	-1.6771	-1.4396	-1.4864	-0.3977	-1.9333	1.0570	0.4416	0.2092
Autumn	INF	-1.0065	1.2543	-0.6488	-0.1019	-0.0140	1.8007	-0.6987	0.3125	-0.2723	-1.3811	1.1595	-1.2322
	SEDz	-1.1186	-0.3073	0.7286	0.4241	0.1284	2.1627	0.2378	0.4369	-0.1310	-3.4007	0.1981	0.6074
	BIOz	0.6419	0.3719	2.3977	-1.1130	-0.7571	2.4146	-1.6389	-0.8977	-0.7176	-0.9718	-1.1078	-0.6023
	OTF	0.4508	1.0150	0.6036	-0.1800	0.6627	0.0619	-0.3369	1.8902	-0.7621	-0.0720	-1.9601	-0.0562

Only the first four components were detailed.

Table S12. Description of PCA loadings for the correlation between environmental parameters and the abundance of N transforming functional genes.

Parameter	Sok-SSBS				Bzr-SSBS				Str-SSBS			
	PC 1	PC 2	PC 3	PC 4	PC 1	PC 2	PC 3	PC 4	PC 1	PC 2	PC 3	PC 4
pH	-0.6772	0.2911	-0.1214	0.2036	0.4847	-0.2500	0.3750	-0.0503	0.6428	-0.3279	-0.4838	-0.1843
Temperature C°	-0.7436	-0.4063	-0.1905	0.1466	-0.4417	-0.8041	0.1125	-0.3124	-0.7326	0.4313	0.3981	0.1698
Oxygen (mg L^{-1})	-0.0841	0.6429	-0.2327	-0.1166	0.3832	0.5024	0.0009	0.0387	0.7844	-0.3749	-0.2671	-0.2141
N-NO3 (mg L^{-1})	-0.7072	0.3575	-0.0871	0.0306	-0.7559	-0.5185	-0.0679	-0.0290	-0.7561	-0.0244	-0.1962	-0.2127
N-NH4 (mg L^{-1})	0.8769	0.1943	0.0285	0.3582	-0.2870	0.6760	0.5391	-0.3951	-0.2097	0.4512	0.6673	0.3019
P-PO4 (mg L^{-1})	-0.4090	-0.7060	-0.6283	0.5492	-0.0553	-0.8189	0.5054	0.1093	-0.3686	0.7189	-0.2961	0.2705
OMc (mg g^{-1})	-0.0802	0.4337	0.4697	0.0797	0.0610	0.3468	0.8141	-0.0149	-0.0940	-0.0989	0.2584	-0.0985
*amo*A	0.6512	-0.0119	-0.4310	0.2754	0.2015	0.7104	0.6467	-0.2323	0.9773	0.0142	-0.0775	-0.0258
*nos*Z	-0.8799	0.1668	0.2880	0.3090	-0.8139	0.3015	0.0214	0.4456	-0.9662	-0.1120	0.0732	-0.1114

Only the first four components were detailed. OMc: Organic matter content.\

Table S13. Statistical one way ANOVA analysis (a) and Tukey HSD test (b) for the most important principal component (PC1).

(a) One-way ANOVA test

	Sum of sqrs	df	Mean square	F	p (same)
Sok-SSBS					
Between groups:	37.0931	2	18.5466	28.04	1.36E-04
Within groups:	5.95368	9	0.66152		
Total:	43.0468	11	0.00047		
Bzr-SSBS					
Between groups:	41.544	2	20.772	89.58	1.15E-06
Within groups:	2.087	9	0.231888		
Total:	43.631	11	0.00012		
Str-SSBS					
Between groups:	39.0101	2	19.505	76.96	2.19E-06
Within groups:	2.28114	9	0.25346		
Total:	41.2912	11	0.00018		

Significant values ($p < .05$) are marked in red colour.

(b) Tukey's HSD test

	Sok-SSBS			Bzr-SSBS			Str-SSBS		
	Spring	Summer	Autumn	Spring	Summer	Autumn	Spring	Summer	Autumn
Spring		1.03E-04	4.42E-03		8.19E-07	2.42E-04		1.81E-06	5.43E-05
Summer	10.5		0.03341	18.9		2.30E-04	17.2		6.66E-03
Autumn	6.2	4.3		9.4	9.5		11.4	5.8	

Significant values ($p < .05$) are marked in red colour.

Table S14. Abundance (%) of most representative bacterial taxa in biofilm formations from geochemical (GEOz) and denitrifying (DENz) zones in Sok-SSBS and Str-SSBSs.

CLASS

Abundance range (%)	Sok-SSBS GEOz				Str-SSBS GEOz				Str-SSBS DENz			
	Spring		Summer		Spring		Summer		Spring		Summer	
	Genera	(%)	Genera	(%)	Genera	(%)	Genera	(%)	Genera	(%)	Genera	(%)
Major >10%	*β-Proteobacteria*	17.0	*β-Proteobacteria*	20.6	*α-Proteobacteria*	20.2	*β-Proteobacteria*	17.8	*β-Proteobacteria*	27.6	*β-Proteobacteria*	20.4
	α-Proteobacteria	15.1	*α-Proteobacteria*	15.6	*ϒ-Proteobacteria*	10.9	*ϒ-Proteobacteria*	15.3	*α-Proteobacteria*	17.3	*ϒ-Proteobacteria*	12.1
			δ-Proteobacteria	11.4			*α-Proteobacteria*	10.2	*ϒ-Proteobacteria*	15.0		
Medium 5-10%	*δ-Proteobacteria*	8.7	*Actinobacteria*	7.8	*Actinobacteria*	9.8	*Sphingobacterii*	7.2	*Actinobacteria*	5.8	*Flavobacteria*	9.5
	Unclassified	8.3	Unclassified	7.7	*β-Proteobacteria*	9.3	*Nostocophycideae*	6.9	*Oscillatoriophycideae*	5.1	*α-Proteobacteria*	9.2
	Actinobacteria	8.1	*ϒ-Proteobacteria*	6.4	*Oscillatoriophycideae*	9.2	*Oscillatoriophycideae*	6.6			*Sphingobacterii*	7.1
	Nostocophycideae	6.0	*Anaerolineae*	4.1	*Sphingobacterii*	7.2	*δ-Proteobacteria*	5.6			*δ-Proteobacteria*	7.0
	Sphingobacterii	5.7			*Nostocophycideae*	5.2	Unclassified	5.6			Unclassified	6.0
Minor 1-5%	*δ-Proteobacteria*	4.4	*Clostridia*	3.7	Unclassified	4.9			Unclassified	3.6		
									Sphingobacterii	3.4		
									Verrucomicrobiae	3.2		
Lower <1%	Others	26.7	Others	22.7	Others	23.3	Others	24.8	Others	17.0	Others	28.7
Total sequences	7.91E+04	100.0	1.42E+05	100.0	1.12E+05	100.0	1.20E+05	100.0	1.92E+05	100.0	2.03E+05	100.0

FAMILY

Abundance range (%)	Sok-SSBS GEOz				Str-SSBS GEOz				Str-SSBS DENz			
	Spring		Summer		Spring		Summer		Spring		Summer	
	Family	(%)	Family	(%)	Family	(%)	Family	(%)	Family	(%)	Family	(%)
Major >10%	*Comamonadaceae*	10.5	*Rhodocyclaceae*	9.3	*Sphingomonadaceae*	7.7	*Comamonadaceae*	8.9	*Comamonadaceae*	18.5	*Comamonadaceae*	12.9
											Flavobacteriaceae	10.2
Medium 5-10%	*Rhodocyclaceae*	6.6			*Comamonadaceae*	5.4	*Rhodocyclaceae*	6.8	*Crenotrichaceae*	6.9	*Rhodocyclaceae*	6.1
	Nostocaceae	5.6			*Xanthomonadaceae*	5.3	*Nostocaceae*	6.3				
Minor 1-5%	*Flavobacteriaceae*	4.9	*Comamonadaceae*	4.6	*Rhodobacteriaceae*	4.3	*Verrucomicrobiaceae*	4.6	*Hyphomicrobiaceae*	4.4	*Sphingobacteriaceae*	3.3
	Rhodobacteraceae	3.8	*Anaerolinaceae*	4.4	*Micrococcaceae*	3.9	*Rhodobacteriaceae*	4.0	*Rhodobacteriaceae*	4.3	*Nitrospiraceae*	3.3
	Sphingomonadaceae	3.3	*Gallionellaceae*	3.9	*Verrucomicrobiaceae*	3.7	*Crenotrichaceae*	3.5	*Rhodocyclaceae*	4.1	*Verrucomicrobiaceae*	2.3
	Sphingobacteriaceae	3.2	*Hyphomicrobiaceae*	3.8	*Crenotrichaceae*	3.3	*Flavobacteriaceae*	3.0	*Verrucomicrobiaceae*	3.6	*Rhodobacteriaceae*	2.2
	Nitrospiraceae	2.9	*Sphingobacteriaceae*	2.0	*Flavobacteriaceae*	3.3	*Pedosphaeraceae*	2.8	*Gallionellaceae*	3.2	*Crenotrichaceae*	1.9
	Nocardioidaceae	2.6	*Sphingomonadaceae*	1.9	*Hyphomicrobiaceae*	3.2	*Sinobacteraceae*	2.6	*Nitrospiraceae*	3.1	*Nostocaceae*	1.6
	Hyphomicrobiaceae	2.3	*Nitrospiraceae*	1.7	*Cyanobacteriaceae*	2.9	*Thiotrichaceae*	2.1	*Methylophilaceae*	2.4	*Hyphomicrobiaceae*	1.5
	Xanthomonadaceae	2.3	*Crenotrichaceae*	1.4	*Scytonemataceae*	2.6	*Sphingomonadaceae*	2.0	*Nostocaceae*	2.0	*Xanthomonadaceae*	1.4
	Verrucomicrobiaceae	1.9	*Pedosphaeraceae*	1.3	*Gemmatimonadaceae*	2.6	*Xanthomonadaceae*	1.9	*Sphingomonadaceae*	2.0	*Pedosphaeraceae*	1.2
	Crenotrichaceae	1.4			*Nostocaceae*	2.4	*Sphingobacteriaceae*	1.7	*Flavobacteriaceae*	2.0	*Sinobacteraceae*	1.1
	Scytonemataceae	1.1			*Nocardioidaceae*	2.3	*Hyphomicrobiaceae*	1.4	*Xanthomonadaceae*	1.8		
	Pedosphaeraceae	1.0			*Rhodocyclaceae*	1.6	*Methylophilaceae*	1.1	*Micrococcaceae*	1.8		
Lower <1%	Others	46.4	Others	65.7	Others	45.5	Others	47.5	Others	39.7	Others	51.0
Total sequences	7.91E+04	100	1.42E+05	100	1.12E+05	100	1.20E+05	100	1.92E+05	100	2.03E+05	100

Table S14. Abundance (%) of most representative bacterial taxa in biofilm formations from geochemical (GEOz) and denitrifying (DENz) zones in Sok-SSBS and Str-SSBSs, continuation.

GENERA												
Abundance range (%)	Sok-SSBS GEOz				Str-SSBS GEOz				Str-SSBS DENz			
	Spring		Summer		Spring		Summer		Spring		Summer	
	Genera	(%)	Genera	(%)	Genera	(%)	Genera	(%)	Genera	(%)	Genera	(%)
Major >10%									*Limnohabitans*	10.8		
Medium 5-10%					*Novosphingobium*	5.1			*Crenothrix*	7.5	*Flavobacterium*	9.4
Minor 1-5%	*Flavobacterium*	4.5	*Gallionella*	2.1	*Rhodobacter*	3.8	*Rhodobacter*	4.1	*Rhodobacter*	4.2	*Nitrospira*	3.5
	Nitrospira	3.3	*Thiobacillus*	2.0	*Crenothrix*	3.6	*Dechloromonas*	4.0	*Gallionella*	3.4	*Limnohabitans*	3.4
	Pedobacter	3.3	*Sulfuritalea*	2.1	*Arthrobacter*	3.2	*Crenothrix*	3.9	*Nitrospira*	3.3	*Dechloromonas*	2.7
	Rhodobacter	3.0	*Desulfobulbus*	1.3	*Cyanobacterium*	3.0	*Luteolibacter*	3.5	*Rhodoferax*	2.8	*Rhodoferax*	2.2
	Dechloromonas	2.4	*Geobacter*	2.0	*Flavobacterium*	3.0	*Pedosphaera*	3.1	*Methylotenera*	2.5	*Crenothrix*	2.0
	Rubrivivax	2.1	*Nitrospira*	1.9	*Scytonema*	2.8	*Steroidobacter*	2.9	*Luteolibacter*	2.0	*Rhodobacter*	1.9
	Runella	2.0	*Dechloromonas*	1.6	*Gemmatimonas*	2.7	*Thiothrix*	2.3	*Arthrobacter*	1.9	*Geobacter*	1.8
	Nocardioides	2.0	*Crenothrix*	1.5	*Runella*	2.6	*Calothrix*	2.1	*Flavobacterium*	1.7	*Pedobacter*	1.7
	Novosphingobium	1.9	*Pedobacter*	1.4	*Luteolibacter*	2.5	*Methylomonas*	2.1	*Novosphingobium*	1.3	*Luteolibacter*	1.7
	Crenothrix	1.6	*Pedosphaera*	1.4	*Nocardioides*	2.2	*Rubrivivax*	1.9			*Rubrivivax*	1.4
	Geobacter	1.6			*Methylotenera*	1.3	*Flavobacterium*	1.8			*Pedosphaera*	1.3
	Scytonema	1.3			*Calothrix*	1.1	*Runella*	1.8			*Steroidobacter*	1.1
	Pedosphaera	1.2					*Novosphingobium*	1.5				
	Rhodoferax	1.2					*Geobacter*	1.2				
	Luteolibacter	1.1					*Methylotenera*	1.1				
							Pedobacter	1.1				
Lower <1%	Others	67.4	Others	82.7	Others	63.1	Others	61.6	Others	58.4	Others	66.0
Total sequences	7.91E+04	100.0	1.42E+05	100.0	1.12E+05	100.0	1.20E+05	100.0	1.92E+05	100.0	2.03E+05	100.0

Table S15. Description of PCA scores for the geochemical and denitrification zones.

SSBS	zone	season	PC 1	PC 2	PC 3	PC 4	PC 5
Sok	GEOz	spring	5.055	-1.590	-0.455	-7.925	1.476
		summer	-10.920	-5.757	-5.411	0.393	-0.759
Str	GEOz	spring	6.829	-7.710	4.418	3.027	-2.153
		summer	-4.739	2.101	4.359	2.329	5.052
	DENz	spring	8.416	5.201	-6.113	3.433	0.469
		summer	-4.642	7.755	3.201	-1.257	-4.084

Table S16. Description of PCA loadings (correlations) for the environmental parameters and bacterial families

Parameter	PC 1	PC 2	PC 3	PC 4	PC 5
pH	0.421	-0.313	-0.017	0.046	-0.024
Temperature	-0.698	0.248	0.238	0.589	-0.112
Oxygen	0.488	-0.260	-0.296	0.105	-0.160
N-NH4	0.282	0.638	0.215	0.189	-0.013
N-NO3	-0.003	0.729	-0.003	0.000	-0.001
TN	0.581	0.033	0.124	-0.140	0.065
P-PO4	-0.616	0.028	0.034	0.020	0.005
TP	-0.676	0.010	0.016	0.149	-0.053

Family	PC 1	PC 2	PC 3	PC 4	PC 5
Comamonadaceae	0.305	0.697	-0.336	0.033	0.027
Rhodocyclaceae	-0.672	0.035	-0.192	-0.237	0.213
Flavobacteriaceae	0.008	0.835	0.397	-0.306	-0.511
Nostocaceae	0.054	0.025	0.213	-0.190	0.578
Rhodobacteraceae	0.182	0.046	0.134	0.046	0.192
Crenotrichaceae	0.553	0.119	-0.125	0.318	0.142
Sphingomonadaceae	0.522	-0.326	0.162	0.104	-0.042
Hyphomicrobiaceae	0.050	-0.068	-0.203	0.104	-0.068
Verrucomicrobiaceae	0.107	0.079	0.181	0.180	0.208
Xanthomonadaceae	0.355	-0.309	0.209	0.073	-0.066
Nitrospiraceae	0.022	0.546	-0.164	-0.174	-0.181
Sphingobacteriaceae	-0.095	0.067	0.049	-0.272	-0.034
Gallionellaceae	-0.051	-0.031	-0.352	0.138	-0.029
Pedosphaeraceae	-0.091	0.033	0.074	-0.026	0.195
Nocardioidaceae	0.094	-0.117	0.078	-0.150	-0.022
Methylophilaceae	0.078	0.026	-0.036	0.162	0.078

Anaerolinaceae	-0.157	-0.136	-0.206	0.019	-0.067
Sinobacteraceae	-0.057	0.075	0.128	0.051	0.174
Cyanobacteriaceae	0.065	-0.120	0.111	0.097	-0.125
Scytonemataceae	0.058	-0.107	0.099	0.087	-0.112
Gemmatimonadaceae	0.058	-0.107	0.099	0.087	-0.112
Thiotrichaceae	-0.032	0.024	0.079	0.054	0.213
Scytonemataceae	0.018	-0.009	-0.004	-0.096	0.033

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Appendix 6: SUPPLEMENTARY MATERIAL

Culturable nitrogen-transforming bacteria from sequential sedimentation biofiltration systems and their potential for nutrient removal in urban polluted rivers

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Table S1. Nucleotide BLAST sequence identity analysis for the gene 16S rRNA of nitrogen transforming bacterial strains.

SSBS	#	Strain	Query cover (%)	Identity (%)	Result	Accession
Sok	1	Sok01	100	99.32	*Hydrogenophaga taeniospiralis* NBRC 102512	AB681846
	2	Sok03	98	99.45	*Pseudomonas guineae* LMGT 24016	AM491810
	3	Sok06	100	99.63	*Bacillus aerius* 24K	AJ831843
	4	Sok20	99	99.34	*Janthinobacterium lividum* DSM 1522	NR_164625
	5	Sok41	99	99.29	*Acidovorax radicis* N35	NR_117776
	6	Sok05	100	99.08	*Kocuria rosea* DSM 20447	NR_044871
Bzr	7	**Bzr02**	99	99.39	***Citrobacter freundii* NBRC 12681**	NR_113596
	8	Bzr07	100	99.83	*Pseudomonas migulae* NBRC 103157	NR_114223
Str	9	Str01	100	98.36	*Bacillus simplex* LMG 11160	NR_114919
	10	**Str21**	99	99.55	***Pseudomonas mandelii* NBRC 103147**	NR_114216

Sok: Sokolówka SSBS, Bzr: Bzura-SSBS, Str: Struga Gnieznienska SSBS.

(c)

Query cover (%)	Identity (%)	Result	Accession
100	99.65	*Pseudomonas mandelii* strain LMG 21607 genome assembly, chromosome: I	LT629796.1
100	99.65	*Pseudomonas mandelii* partial *rpoB* gene for DNA-directed RNA polymerase beta chain, strain CIP 105273T	AJ717435.1
100	99.30	*Pseudomonas* sp. B14-6 chromosome, complete genome	CP053929
100	99.30	*Pseudomonas mandelii* JR-1, complete genome	CP005960
98	99.65	*Pseudomonas mandelii* partial *rpoB* gene for RNA polymerase beta-subunit, strain CFML 96-122	AJ786271
100	99.77	*Pseudomonas mandelii* partial *rpoB* gene for RNA polymerase beta-subunit, strain CFML 96-122	AJ786271

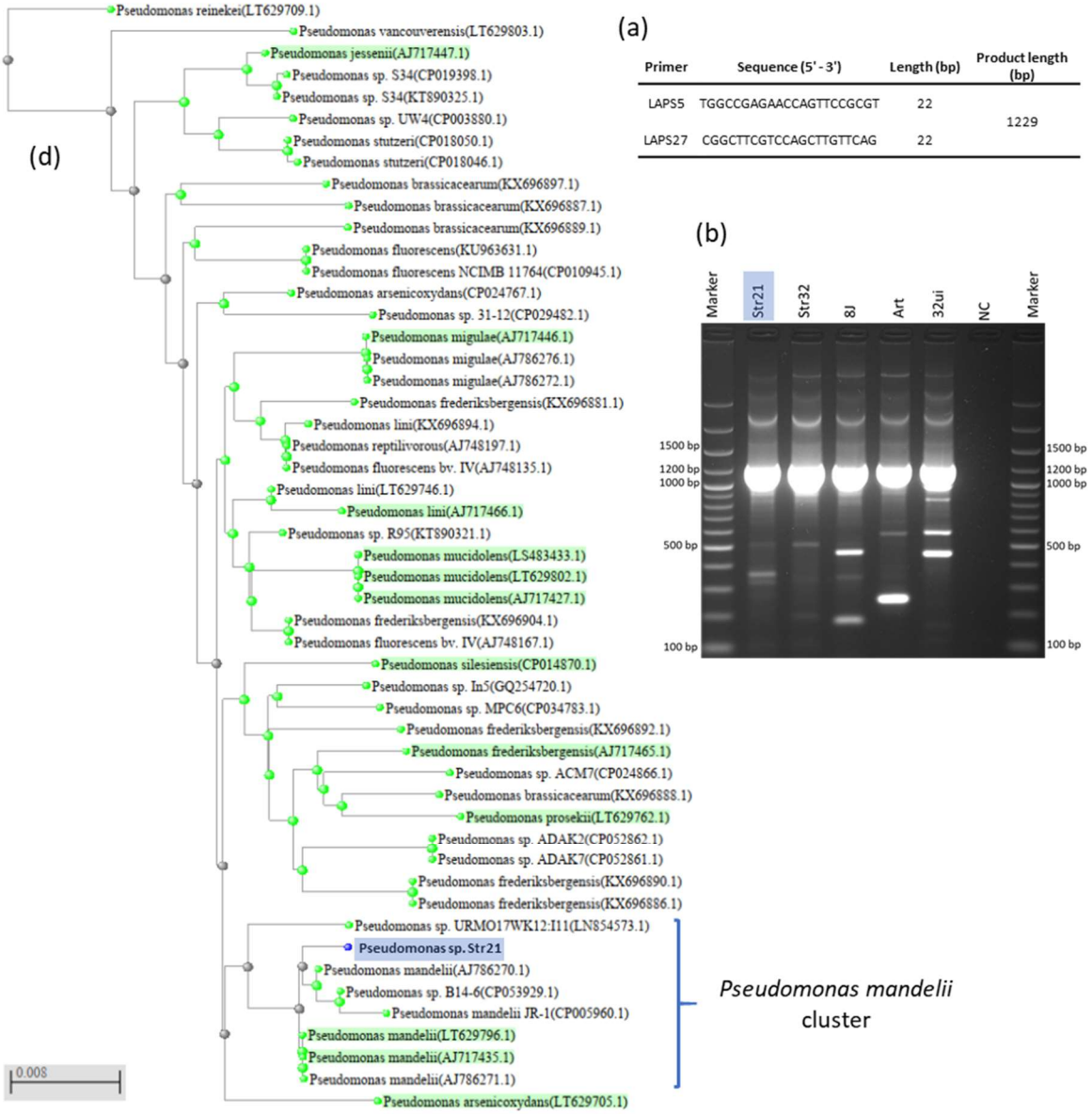

Fig S1. Taxonomical identification of strain Str21 with the detection and sequence analysis of gene encoding for the β subunit of bacterial RNA polymerase (*rpo*B); (a) description of primer sets, (b) detection in agarose gel electrophoresis using conventional PCR, (c) nucleotide BLAST similarity results, and (d) Phylogenetic analysis with other related *Pseudomonas* spp. published strains. PCR conditions for primer sets were described in Tayeb et al., (2005). Molecular marker Blirt-Gdansk (100-3000 bp); positive *Pseudomonas* spp. strains: Str32, 8J, Art, 32UI. NC: negative control. Strains highlighted in green were retrieved from full genomic published sequences. The strain Str21, highlighted in blue, is closely associated to other strains that have been identified as *Pseudomonas mandelii*, and therefore, the bacterium Str21 was believed to be a different strain of the same species.

Table S2. Taxonomical identification of strain Bzr02 according to the metabolic profile obtained in BIOLOG GEN III MicroPlates.

Protocol A					Protocol B				
No.	PROB	SIM	DIST	Best results	No.	PROB	SIM	DIST	Best results
24h of incubation					**24h of incubation**				
1	0.803	0.803	2.778	*Citrobacter freundii*	1	0.646	0.646	5.114	*Citrobacter freundii*
2	0.325	0.325	2.979	*Citrobacter braakii*	2	0.126	0.126	5.655	*Citrobacter sedlakii*
3	0.007	0.007	5.318	*Citrobacter youngae*	3	0.096	0.096	5.821	*Citrobacter braakii*
4	0.007	0.007	5.388	*Citrobacter sedlakii*	4	0.091	0.091	5.846	*Citrobacter youngae*
48h of incubation					**48h of incubation**				
1	0.865	0.865	1.921	*Citrobacter freundii*	1	0.717	0.526	3.762	*Citrobacter freundii*
2	0.37	0.37	2.072	*Citrobacter braakii*	2	0.135	0.09	4.833	*Citrobacter braakii*
3	0.005	0.005	4.633	*Citrobacter werkmanii*	3	0.127	0.084	4.873	*Citrobacter sedlakii*
4	0.004	0.004	4.894	*Citrobacter youngae*	4	0.021	0.012	6.029	*Citrobacter youngae*

The isolated strain Bzr02 was incubated into plates using two different liquid media: protocol A) for general metabolic description of heterotrophic aerobes, and Protocol B) to identify a false positive from the results obtained in protocol A, according to the manufacturer instructions (BIOLOG Hayward, California). The PROB (probability), SIM (similitude) and DIST (distance) indexes were measured at 24h and 48h, and were used to identify the most probable metabolic profile of the strain Bzr02 with the GEN III database provided by BIOLOG. The best results were always observed for the metabolic profile presented by *Citrobacter freundii*, and therefore, the bacterium Bzr02 is believed to be a different strain of the same species.

Table S3. Nucleotide BLAST sequence identity analysis for key functional genes detected in strains Bzr02 and Str21.

N-transforming process	Gene	Strain	Query cover (%)	Identity (%)	Result	Accession	Observations
Denitrification	*nos*Z	Str21	100	99.93	*Pseudomonas mandelii* JR-1, complete genome, nitrous oxide reductase gene	CP005960	extracted from full genome
	*nor*B	Str21	100	94.41	*Pseudomonas lini* PD 28 *cnor*B gene	DQ420250	
	*nir*S	Str21	99	96.15	*Pseudomonas lini* PD 28 *nir*S gene	DQ518188	
	*nar*G	Bzr02	100	98.94	*Citrobacter freundii* complex sp. CFNIH3 chromosome, complete genome, nitrate reductase subunit alpha	CP026235	extracted from full genome
		Str21	99	98.39	*Pseudomonas mandelii* strain LMG 21607 genome assembly, chromosome: I, nitrate reductase subunit alpha	LT629796	extracted from full genome
	*nap*A	Bzr02	100	95.96	*Citrobacter freundii* complex sp. CFNIH3 chromosome, complete genome, nitrate reductase catalytic subunit n*ap*A	CP026235	extracted from full genome
Nitrification	*hao*	Bzr02	100	99.49	*Citrobacter freundii* complex sp. CFNIH3 chromosome, complete genome, hydroxylamine reductase	CP026235	extracted from full genome
Assimilation	*nas*A	Str21	99	93.4	*Pseudomonas* sp. WP5m-11 assimilatory nitrate reductase (*nas*A) gene, partial cds	JX533707	

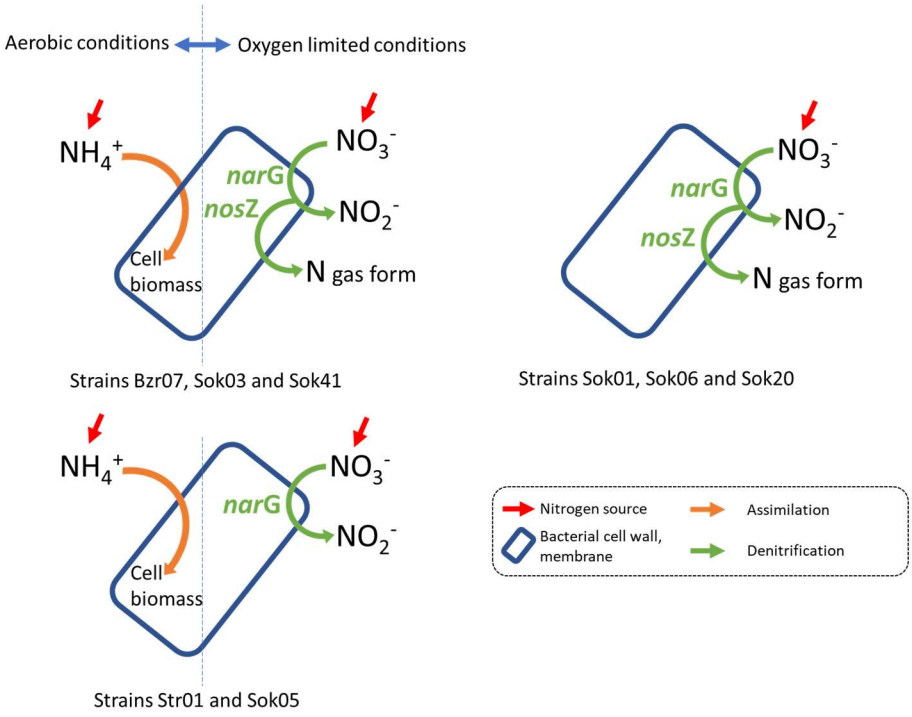

Fig S2. Metabolic pathways for the transformation of nitrogen compounds in selected bacteria.

Supplementary description of media

a) Giltay denitrifying medium (GidM), according to Alexander 1965

Prepare solution 1 and solution 2 separately, in distilled water,

Solution 1	500 mL	Solution 2	500 mL
$C_4H_8N_2O_3$ Asparagine	0.05 g	$Na_3C_6H_5O_7$ Trisodium citrate	2.50 g
$C_6H_{12}O_6$ Glucose	10.0 g	KH_2PO_4 Potassium dihydrogen phosphate	2.0 g
KNO_3 Potassium nitrate	2.0g	$MgSO_4$ Magnesium sulphate	0.97 g
		$CaCl_2$	0.1014 g

Mix both solutions and filter sterilize (0.2 µm).

Before inoculation of bacteria, add 45 µL of a stock solution of FeCl (200 mg mL^{-1}) for every 50 mL of GidM.

b) Glucose nitrifying medium (GNM), adapted from Padhi et al., 2017

Prepare basal and trace elements solution separately,

Basal solution	1000 mL	Trace elements solution	1000 mL
$MgSO_4 * 7H_2O$ Magnesium sulphate heptahydrate	0.10 g	EDTA	1.25 g
K_2HPO_4 Dipotassium phosphate	3.84 g	$ZnSO_4$ Zink sulphate	75 mg
KH_2PO_4 Potassium dihydrogen phosphate	1.50 g	$MnCl_2 * 4H_2O$ Manganese (II) chloride dihydrate	125 mg
NH_4Cl Ammonium chloride	0.802 g	$FeSO_4 * 7H_2O$ Iron (II) sulphate heptahydrate	125 mg
		$CuSO_4 * 5H_2O$ Copper (II) sulphate pentahydrate	50 mg
$C_6H_{12}O_6$ Glucose	5.3 g	$CoCl_2 * 6H_2O$ Cobalt (II) chloride hexahydrate	75 mg

Adjust the pH to 7.2 and filter sterilize (0.2 µm), for each solution separately.

To prepare working solution (GNM), add 2 mL of trace element solution for every litre of basal medium.

c) Succinate nitrifying medium (SNM)

Replace Glucose from the basal medium of GNM with:

11.9 g of $C_4H_4Na_2O_4 * 6H_2O$ (sodium succinate dibasic hexahydrate)

d) Acetate nitrifying medium (ANM)

Replace Glucose from the basal medium of GNM with:

10.0 g of $C_2H_3NaO_2$ (sodium acetate)

e) Citrate nitrifying medium (CNM)

Replace Glucose from the basal medium of GNM with:

8.65 g of $C_6H_5Na_3O_7 * 2H_2O$ (trisodium citrate dihydrate)

f) Nitrifying medium (NM)

Modify the content of Glucose and NH_4Cl from the basal medium of GNM with:

0.3818 g of NH_4Cl (100 mg L^{-1} of nitrogen content)
2.5000 g of Glucose (1000 mg L^{-1} of carbon content)

a) Denitrifying medium (DM)

Modify the content of Glucose and replace NH_4Cl for $NaNO_3$ from the basal medium of GNM with:

0.6068 g of $NaNO_3$ (100 mg L^{-1} of nitrogen content)
2.5000 g of Glucose (1000 mg L^{-1} of carbon content)

a) Simultaneous Nitrifying Denitrifying medium (SNDM)

Modify the content of Glucose and NH_4Cl and add $NaNO_3$ from the basal medium of GNM with:

0.1909 g of NH_4Cl
0.3034 g of $NaNO_3$
2.5000 g of Glucose (1000 mg L^{-1} of carbon content)

Table S4. PCR primers for detection of bacterial key functional genes.

Process	Gene	Name / Function	Primer pair sequence (5'→3')	Size (bp)	Thermal cycling conditions
Nitrification	*hao*	Hydroxylamine reductase $NH_2OH \rightarrow NO_2$ $NH_2OH \rightarrow N_2O$	Hyrxl_f2 ATGTTTTGTGTNCAATGTGA Hyrxl_r2 GCYTTCAGYTCRAACCA	1485[a]	Initial denaturation at 94°C-5min, followed by 36 cycles of denaturation at 94°C-1min, annealing at 51.8°C-1min, extension at 72°C-1min, and final extension at 72°C-9min.
Denitrification	*nap*A	Periplasmic nitrate reductase $NO_3 \rightarrow NO_2$	NAP1 TCTGGACCATGGGCTTCAACCA NAP2 ACGACGACCGGCCAGCGCAG	786[b]	Initial denaturation at 95°C-7min, followed by 37 cycles of denaturation at 95°C-30sec, annealing at 61°C-30sec, extension at 72°C-1min, and final extension at 72°C-4min.
	*nar*G	Respiratory nitrate reductase $NO_3 \rightarrow NO_2$	narG1960f TAYGTSGGSCARGARAA narG2650r TTYTCRTACCABGTBGC	650[c]	Initial denaturation at 95°C-5min, followed by 8 cycles of denaturation at 94°C-30sec, annealing at X°C-30sec (Touch down PCR -0.5°C per cycle, starting in 54°C and finalizing in 50°C), extension at 72°C-45sec, followed by 30 cycles with fixed annealing at 50°C-30sec, and final extension at 72°C-6min.
	*nir*S	Nitrite reductase $NO_2 \rightarrow NO$	nirS1F CCTAYTGGCCGCCRCART nirS6R CGTTGAACTTRCCGGT	890[d]	Initial denaturation at 95°C-5min, followed by 10 cycles of denaturation at 95°C-30sec, annealing at X°C-40sec (Touch down PCR -0.5°C per cycle, starting in 57°C and finalizing in 52.5°C), extension at 72°C-1min, followed by 30 cycles with fixed annealing at 55°C-40sec, and final extension at 72°C-5min.
	*nor*B	Nitric oxide reductase $NO \rightarrow N_2O$	cnorB2F GACAAGNNNTACTGGTGGT cnorB6R GAANCCCCANACNCCNGC	389[e]	
	*nos*Z	Nitrous oxide reductase $N_2O \rightarrow N_2$	nosZ-F-1181 CGCTGTTCXTCGACAGYC nosZ-R-1880 ATGTGCAKXGCRTGGCAG	700[f]	Initial denaturation at 94°C-3min, followed by 25 cycles of denaturation at 94°C-45sec, annealing at 58°C-60sec, extension at 72°C-2min, and final extension at 72°C-7min.
Nitrogen Assimilation	*nas*A	Assimilatory nitrate reductase $NO_3 \rightarrow NO_2$	nasA964 CARCCNAAYGCNATGGG nasA1735 ATNGTRTGCCAYTGRTC	775[g]	Initial denaturation at 95°C-5min, followed by 20 cycles of denaturation at 94°C-30sec, annealing at X°C-30sec (Touch down PCR -0.3°C per cycle, starting in 56°C and finalizing in 50°C), extension at 72°C-1min, followed by 10 cycles with fixed annealing at 50°C-30sec, and final extension at 72°C-7min.

Descriptions for PCR assays can be found in: [a]Padhi et al. 2017; [b]Qing et al. 2018; [c]Philippot et al. 2002; [d]Braker et al. 1998; [e]Braker and Tiedje, 2003; [f]Rich et al. 2003; [g]Allen et al. 2001

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IX. Abstract

Microorganisms have been identified as a key biotic element in nutrient transformations in both natural freshwater and constructed ecosystems. Considering microbial metabolic activity, the nitrogen cycle is accomplished by a series of biochemical reactions where nitrification and denitrification are known to be one of the most important in the environment, and both are performed by different bacterial communities. In turn, phosphorus removal has been attributed to a different communities known as the polyphosphate accumulating bacteria, which belong to a more variable group known as the polyphosphate accumulating organisms (PAOs). In the present study, three sequential sedimentation biofiltrations systems (SSBSs) were periodically monitored to describe the dynamics of occurrence, diversity, and activity of microbial communities associated to nutrient transformations. SSBSs are directly constructed in urban freshwater ecosystems (rivers connected with recreational lake/ponds) for the treatment of pollutants carried from storm water runoff and snowmelts, among others. These systems are constructed with different zones for the sequential treatment of contaminated water from inflow to outflow direction: i) the sedimentation zone (SEDz) for the removal of coarse and particulate organic matter, ii) the geochemical zone (GEOz) for the removal of phosphorus, iii) the denitrification zone (DENz) to improve microbial activity in the reduction of nitrogen compounds, and iv) the biofiltration zone (BIOz) dedicated to phytoremediation. The rivers are connected with downstream lakes and ponds, and therefore they play an essential role for the removal of nutrients in order to control cyanobacterial harmful algal blooms (CyanoHABs) in recreational areas of cities. Moreover, they are considered as ecohydrological and nature-based solutions (NBS), since they help to increase the resilience of urban ecosystems by mimicking ecological processes for the treatment of water.

Microbial communities involved in nutrient transformations have not been previously studied in SSBSs. These communities have been investigated in natural ecosystems, however, the major focus is dedicated to constructed wetlands (CWs) and waste water treatment plants (WWTPs). Moreover, bacteria capable of polyphosphate accumulation have been scarcely studied in natural environments, and in the case of SSBSs, this work represents the first attempt of investigation. Therefore, the present study proposed the following two hypotheses: 1. *Dynamics of microbial metabolic activity and the abundance of nutrient-transforming bacteria in SSBSs are subject to change according to specific environmental conditions observed through the seasons and the different designs in the SSBSs multi-complex structure,* and 2.

Culturable heterotrophic bacteria isolated from SSBSs have biotechnological potential to improve the efficiency in nutrient removal.

Sampling was performed in five periods (representing spring, summer, and autumn) of the years 2017 (August and October) and 2018 (April, August, and November), for three different SSBSs: i) the Sokołówka SSBS (Sok-SSBS, constructed in 2011) and the i) Bzura SSBS (Bzr-SSBS, constructed in 2013) located in the city of Lodz, and iii) the Struga Gnieźnieńska SSBS (Str-SSBS, constructed in 2016) in the city of Gniezno. Each SSBS contains the SEDz, GEOz (containing limestone barrier in Sok-SSBS and Str-SSBS, and dolomite barrier at Bzr-SSBS), and BIOz. Only the Str-SSBS contains the DENz, between the GEOz and BIOz, with the addition of brown coal. Water, sediment, rock (limestone or dolomite), and brown coal samples were collected through the horizontal profile from each SSBS. Physico-chemical parameters (temperature, oxygen concentration, and pH) were measured for surface water, and chemical analysis was performed to estimate concentrations of nutrients ($N\text{-}NH_4$, $N\text{-}NO_2$, $N\text{-}NO_3$, TN, $P\text{-}PO_4$, and TP) in surface water and interstitial water extracted from the sediments. Spatial and temporal differences by microbial diversity, community and metabolic activity was analysed using the Community Level Physiological Profile approach (CLPP) through the Biolog Ecoplate ™. Quantitative PCR (qPCR) was performed to estimate the abundance of nitrifying and denitrifying bacterial communities in SSBSs with the use of functional genes involved in such nitrogen cycling processes (*amo*A and *nos*Z, respectively). The gene 16S rRNA was used to estimate their relative abundance from the total community of bacteria. Microbial community analysis – with high-through put sequencing of the gene 16S rRNA – was performed in biofilm formations in limestone barriers (GEOz) and brown coal (DENz) from Sok-SSBS and Str-SSBS, since higher quantities of nutrient transforming bacteria were observed during qPCR analysis. Moreover, culturable heterotrophic bacteria were isolated from SEDz and were tested for their ability to transform nitrogen compounds in controlled experiments with selective media, and their genetic characteristics were investigated to corroborate the metabolic pathways involved in nitrogen transformation.

Results indicated that the temperature was one of the most important factors modifying microbial metabolic activity, diversity and abundance in SSBS's sediments. According to the CLPP analysis, microbial metabolic activity was higher in warmer periods during spring and summer. In the case of bacterial communities, the nitrifying bacteria were more abundant in temperate spring season (14.2 ± 2.5 °C), and positive significant correlation between the $N\text{-}NH_4$ and the gene copy numbers of *amo*A suggested that they were more active during this

season of collection. In contrast, denitrifying bacteria were more abundant in warm summer season (21.9 ± 1.9 °C), and a positive significant correlation between the $N\text{-}NO_3$ and the gene copy numbers of the *nos*Z suggested that they were more active during this season of collection. The design of the system was another important factor modifying microbial communities in SSBSs, and the biofilm formations in GEOz and DENz were the most outstanding in comparison to the other zones. Microbial communities in GEO zones were less metabolically active when compared to the other zones. In turn, considering the rock substrate, the metabolic activity in dolomite (in Bzr-SSBS) was significantly lower than barriers constructed with limestone (in Sok-SSBS and Str-SSBS). Moreover, nitrifying communities were significantly higher in GEOz containing limestone, when compared to dolomite barriers or other zones containing sediments. Bacterial community analysis – using 16S rRNA – suggested that nitrogen transforming bacteria in GEOz with limestone were represented by: *Commamonadaceae*, *Rhodobacter*, and *Crenothrix*. In the case of *Rhodocyclaceae* (*Dechloromona*s), the results indicated that they could be involved in the removal of phosphorus. In turn, the denitrifying bacteria were observed with the highest abundances in biofilm formations over brown coal in DENz, when compared to any other SSBS zone. Moreover, bacterial community analysis – using 16S rRNA – suggested that nitrogen transforming bacteria in DENz were represented by: *Commamonadaceae*, *Flavobacteriaceae (Flavobacterium)*, *Crenotrichaceae* (*Crenothrix*) and *Rhodobacter*. In conclusion, the above results not only allowed to confirm the first hypothesis, but also helped to identify preferable conditions that increased abundance and metabolic activity of microbial communities in SSBSs. The results were used to suggest better planning in the construction of SSBSs, that could aid to increase their removal efficiency of nutrients.

From a total of 150 bacterial strains isolated from SSBSs, two (*Citrobacter freundii* Bzr02, and *Pseudomonas mandelii* Str21) were observed to perform effective removal of nitrogen compounds in controlled experiments with selective media. The strain Bzr02 contained the genes *nap*A/*nar*G which are involved in the reduction of nitrate to nitrite, and therefore it was identified as a nitrate reducer. Moreover, the Bzr02 was also able to remove hydroxylamine from the medium - confirmed by the presence of the *hao* gene. In the case of Str21, the strain was found to contain all genes involved in the dissimilatory nitrate reduction (*nar*G, *nir*S, norB, and *nos*Z), and therefore indicating that it was a denitrifying bacterium. Both strains demonstrated that assimilation of nitrogen compounds ($N\text{-}NH_4$ and $N\text{-}NO_3$) is also an important process occurring simultaneously with dissimilatory nitrate reduction. In conclusion,

the above results not only allowed to confirm the second hypothesis, but also suggested that both strains are potential candidates with biotechnological applications to increase the removal efficiency of nutrients in SSBSs.

X. Streszczenia

Mikroorganizmy jako kluczowy element biotyczny są odpowiedzialne za przemiany biogenów – związków azotu i fosforu, zarówno w naturalnych ekosystemach słodkowodnych, jak i tych zmodyfikowanych przez człowieka. Cykl azotowy powiązany jest z aktywnością metaboliczną bakterii, a reakcje nitryfikacji i denitryfikacji są jednymi z najważniejszych procesów, za które odpowiedzialne są różne społeczności bakteryjne. Z kolei usuwanie fosforu jest związane z aktywnością bakterii akumulujących polifosforany, które należą do szeroko pojętej grupy, znanej jako organizmy akumulujące polifosforany (*ang*. Polyphosphate accumulating organisms, PAOs). W niniejszych badaniach, trzy sekwencyjne systemy sedymentacyjno biofiltracjne (*ang*. sequential sedimentation biofiltration systems, SSBSs) były okresowo monitorowane w celu opisania dynamiki występowania, różnorodności i aktywności społeczności mikroorganizmów związanych z przemianami biogenów. SSBSs są budowane w miejskich ekosystemach słodkowodnych (rzeki połączone ze zbiornikami/stawami rekreacyjnymi) w celu oczyszczania wód powierzchniowych z zanieczyszczeń niesionych m.in. ze spływów burzowych i roztopów. W systemach tych funkcjonują różne strefy dla intensyfikacji procesu oczyszczania wody, wśród których można wymienić następujące: i) strefa sedymentacji (*ang*. sedimntation zone, SEDz) do usuwania gruboziarnistej i cząsteczkowej materii organicznej, ii) strefa geochemiczna (*ang*. geochemical zone, GEOz) do usuwania fosforu, iii) strefa denitryfikacji (*ang*. denitrification zone, DENz) w celu intensyfikacji aktywności mikroorganizmów w redukcji związków azotu, oraz iv) strefa biofiltracji (*ang*. biofiltration zone, BIOz) dedykowana fitoremediacji.

Rzeki miejskie są połączone ze zbiornikami i stawami, dlatego istotne jest usuwanie nadmiaru substancji biogennych, poprzez konstrukcje SSBSs powyżej w/w zbiorników, w celu m.in. kontroli szkodliwych sinicowych zakwitów wód (*ang*. cyanobacterial harmful algal blooms, CyanoHABs). Ponadto, SSBSs reprezentują rozwiązania bliskie naturze (*ang*. nature-based solution, NBS), z wykorzystaniem koncepcji ekohydrologii, pomagając zwiększyć odporność ekosystemów miejskich poprzez naśladowanie procesów ekologicznych w oczyszczaniu wody.

Zespoły mikroorganizmów biorące udział w przemianach biogenów – związków azotu i fosforu nie były wcześniej badane w SSBSs. Takie społeczności są analizowane w naturalnych ekosystemach, jednakże większość badań koncentruje się na sztucznych mokradłach (*ang*. constructed wetlands, CW) i oczyszczalniach ścieków (*ang*. wastewater treatment plants, WWTP). Ponadto, bakterie zdolne do akumulacji polifosforanów są rzadko badane w środowisku naturalnym, a w przypadku SSBSs, niniejsza praca stanowi pierwszą próbę ich identyfikacji.

W związku z tym, postawiono dwie hipotezy: 1. *Dynamika metabolicznej aktywności mikroorganizmów i liczebność bakterii uczestniczących w transformacji biogenów w SSBSs jest zależna od warunków środowiskowych obserwowanych w różnych porach roku oraz od różnych typów konstrukcji SSBSs;* i 2. *Heterotroficzne bakterie wyizolowane z SSBSs mają potencjał biotechnologiczny dla poprawy efektywności usuwania biogenów*.

Materiał do badań został pobrany w pięciu różnych okresach, w sezonie wiosennym, letnim i jesiennym, w roku 2017 (sierpień i październik) oraz 2018 (kwiecień, sierpień i listopad), z trzech różnych SSBSs: i) SSBS Sokołówka (Sok-SSBS, wybudowany w 2011 r.), ii) i SSBS Bzura (Bzr-SSBS, wybudowany w 2013 r.) zlokalizowanych na terenie miasta Łodzi oraz iii) SSBS Struga Gnieźnieńska (Str-SSBS, wybudowany w 2016 r.) na terenie miasta Gniezno. W skład każdego z SSBSs wchodzą kolejno strefy: SEDz, GEOz (zawierająca barierę wapienną w Sok-SSBS i Str-SSBS oraz barierę dolomitową w Bzr-SSBS) i BIOz. Jedynie Str-SSBS zawiera DENz, pomiędzy GEOz i BIOz, z dodatkiem węgla brunatnego. Próbki wody, osadów, wapienia lub dolomitu oraz węgla brunatnego pobierano w profilu poziomym z każdego SSBS. W wodzie powierzchniowej mierzono parametry fizyko-chemiczne (temperaturę, stężenie tlenu i pH), a stężenie substancji biogennych ($N\text{-}NH_4$, $N\text{-}NO_2$, $N\text{-}NO_3$, TN, $P\text{-}PO_4$ i TP) analizowano w wodzie powierzchniowej i wodzie pobranej z osadów. Przestrzenne i czasowe różnice w różnorodności mikroorganizmów, ich społeczności i aktywności metabolicznej były analizowane przy użyciu metody CLPP (*ang*. Community Level Physiological Profile approach) za pomocą Biolog Ecoplate ™. Ilościowe analizy genetyczne (qPCR) zostały przeprowadzone w celu oszacowania liczebności bakterii nitryfikacyjnych i denitryfikacyjnych, z wykorzystaniem kluczowych genów funkcyjnych zaangażowanych w procesy obiegu azotu, odpowiednio *amo*A i *nos*Z. Względna liczebność całej populacji bakterii została oszacowana na podstawie ilości kopii genu 16S rRNA. Analizę zbiorowisk bakteryjnych - z wykorzystaniem wysokoprzepustowego sekwencjonowania genu 16S rRNA – wykonano dla próbek pobranych z biofilmów utworzonych na wapieniu w GEOz

i węglu brunatnym w DENz, z Sok-SSBS i Str-SSBS. Ponadto, z osadów w SEDz wyizolowano hodowalne bakterie heterotroficzne, które scharakteryzowano pod kątem zdolności do transformacji związków azotu w warunkach laboratoryjnych na zdefiniowanych podłożach mikrobiologicznych. Ponadto, dla w/w bakterii wykonano analizę genetyczną pod kątem obecności genów kluczowych dla transformacji związków azotu.

Wyniki badań wykazały, że temperatura jest jednym z najważniejszych czynników modyfikujących aktywność metaboliczną, różnorodność i liczebność mikroorganizmów w osadach, w SSBSs. Jak wynika z analizy CLPP, aktywność metaboliczna mikroorganizmów była wyższa w cieplejszych okresach - sezon wiosenny i letni. W przypadku analizy zróżnicowania czasowego zbiorowisk bakteryjnych, bakterie nitryfikacyjne były liczniejsze w okresie wiosennym, przy umiarkowanej temperaturze (14,2 ± 2,5 °C). Dodatnia korelacja między $N\text{-}NH_4$, a liczbą kopii genu *amo*A sugeruje, że były one również bardziej aktywne w w/w sezonie. Z kolei bakterie denitryfikacyjne występowały liczniej w okresie cieplejszym – sezon letni (21,9 ± 1,9 °C), a dodatnia korelacja między $N\text{-}NO_3$, a liczbą kopii genu *nos*Z sugeruje, że były one również bardziej aktywne w w/w sezonie. Kolejnym ważnym czynnikiem modyfikującym zbiorowiska mikroorganizmów w SSBSs była ich konstrukcja, przy czym biofilm w GEOz i DENz charakteryzował się największą liczebnością bakterii uczestniczących w transformacji azotu. Zbiorowiska mikroorganizmów w GEOz były mniej aktywne metabolicznie w porównaniu z pozostałymi strefami. Z kolei, biorąc pod uwagę materiał skalny użyty w GEOz, aktywność metaboliczna w dolomicie (Bzr-SSBS) była istotnie niższa niż barier zbudowanych z wapienia (Sok-SSBS i Str-SSBS). Ponadto, statystycznie istotna była większa liczebność bakterii nitryfikacyjnych w GEOz zbudowanej z użyciem wapienia, w porównaniu do barier dolomitowych i pozostałych stref SSBSs. Analiza zbiorowisk bakteryjnych - z wykorzystaniem 16S rRNA - wykazała, że bakterie transformujące azot w GEOz z wapieniem reprezentowane były przez: *Commamonadaceae*, *Rhodobacter* i *Crenothrix*. W przypadku *Rhodocyclaceae* (*Dechloromonas*) wyniki wskazały, że mogą one brać udział w usuwaniu fosforu. Z kolei bakterie denitryfikacyjne zaobserwowano w największej liczebności w biofilmie utworzonym na węglu brunatnym w DENz. Ponadto analiza zbiorowisk bakteryjnych - z wykorzystaniem 16S rRNA - wykazała, że bakterie transformujące azot w DENz reprezentowane były przez: *Commamonadaceae*, *Flavobacteriaceae* (*Flavobacterium*), *Crenotrichaceae* (*Crenothrix*) i *Rhodobacter*.

Podsumowując, powyższe wyniki pozwoliły nie tylko na potwierdzenie pierwszej hipotezy, ale również na określenie preferowanych warunków zwiększających liczebność i aktywność

metaboliczną zbiorowisk mikroorganizmów w badanych SSBSs. Uzyskane wyniki przyczynią się do optymalizacji konstrukcji SSBSs, w celu zwiększenia efektywności usuwania biogenów.

W prezentowanej pracy, badania skupiły się nad charakterystyką dwóch szczepów wyizolowanych z SSBSs, które to izolaty sposród 150 ogółem wyizolowanych i badanych, wykazywały największą, aktywność metaboliczną względem związków azotu, w warunkach laboratoryjnych. Były to szczepy *Citrobacter freundii* Bzr02 i *Pseudomonas mandelii* Str21. Szczep Bzr02 posiadał w swoim genomie geny *nap*A/*nar*G, które biorą udział w redukcji azotanów do azotynów, dlatego został zidentyfikowany jako reduktor azotanów. Ponadto, Bzr02 był również zdolny do usuwania z podłoża hydroksyloaminy, co zostało potwierdzone obecnością genu *hao*. W przypadku szczepu Str21 stwierdzono obecność wszystkich genów zaangażowanych w dysymilacyjną redukcję azotanów (*nar*G, *nirS*, *nor*B i *nos*Z), co wskazuje na to, iż jest to bakteria zdolna do denitryfikacji. Oba szczepy wykazały, że asymilacja związków azotu ($N\text{-}NH_4$ i $N\text{-}NO_3$) jest ważnym procesem zachodzącym równocześnie z dysymilacyjną redukcją azotanów. Podsumowując, powyższe wyniki pozwoliły nie tylko na potwierdzenie drugiej hipotezy, ale również zasugerowały, że oba szczepy są potencjalnymi kandydatami do zastosowań biotechnologicznych w celu zwiększenia efektywności usuwania biogenów w SSBSs.

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XII. Other activities

Other publications

Font Nájera A., Serwecińska LE., Gągała-Borowska I., Jurczak TE., Mankiewicz-Boczek JD. 2017. *The characterization of a novel bacterial strain capable of microcystin degradation from the Jeziorsko reservoir, Poland: a preliminary study*. Biologia, Cellular and Molecular Biology. 72(12). DOI: 10.1515/biology-2017-0172

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Conferences

Font Nájera A., Gągała-Borowska I., Serwecińska L., Jurczak T., Mankiewicz-Boczek J. *Characterization of isolated bacterial strain **capable** of microcystin molecule degradation*. 2nd African International Symposium Ecohydrology for Water, Biodiversity, Ecosystem Services and Resilience in Africa. Addis Ababa, Ethiopia. 7th - 9th November 2016. ISBN: 978-83-928245-3-4. Poster.

Mankiewicz-Boczek J., Gągała-Borowska I., Jaskulska A., Serwecińska L., **Font Nájera A**., Jurczak T. *Impact of microorganisms on the regulation of microcystin-producing cyanobacteria.* International Symposium Ecohydrology for the Circular Economy and Nature-Based Solutions towards mitigation/adaptation to climate change. Lodz, Poland. 26th - 28th September 2017. ISBN: 978-83-928245-5-8. Presentation.

Font Nájera A., Mankiewicz-Boczek J. *Bacterial communities involved in nutrient transformations in urban biofilters*. International Symposium Ecohydrology for the Circular Economy and Nature-Based Solutions towards mitigation/adaptation to climate change. Lodz, Poland. 26th - 28th , September 2017. ISBN: 978-83-928245-5-8. Poster.

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Mankiewicz-Boczek J., Szklarek S., Kiedrzyńska E., Bednarek A., Serwecińska L., **Font Nájera A**., Kiedrzyński M., Zalewski M. *Biomonitoring of threats and biotechnological solutions to improve the quality of surface waters*. IX National Conference of Bioindicators, Practical use of bioindication systems to assess the quality and toxicity of the environment and chemicals. Poznan, Poland. 08th - 10th April 2019. ISBN 978-83-947733-1-1. Presentation.

Mankiewicz-Boczek J., Gągała-Borowska I., Izydorczyk K., Jurczak T., Serwecińska L., Frątczak W., Jaskulska A., **Font Nájera A**., Zalewski M. *Control and mitigation of microcystin-producing cyanobacteria occurrence in lowland dam reservoirs*. 11th International Conference on Toxic Cyanobacteria. Learning from the Past to Predict the Future. Krakow, Poland, 05th – 10th May 2019. Presentation.

Font Nájera A. *Dynamics of occurrence and metabolic activity of microorganisms involved in the removal of nutrients in urban sequential sedimentation-biofiltration systems*. European Innovation Fest, University of Alcala, Spain. 14th – 16th September 2020. Presentation.

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Font Nájera A., Serwecińska L., Mankiewicz-Boczek J. *Isolation and characterization of nitrogen-transforming bacteria from nature-based solutions: sequential sedimentation biofiltration systems*. Hydromicro, Sopot, Poland. June 09th – 11th 2021. Poster.

Joanna Mankiewicz-Boczek, Liliana Serwecińska, Ilona Gągała-Borowska, Tomasz Jurczak, **Arnoldo Font Nájera**, Aleksandra Jaskulska, Jesús Morón-López. *Bakterie i wirusy w regulacji występowania sinic produkujących mikrocystyny* (*Bacteria and viruses in the regulation of microcystin-producing cyanobacteria*). Hydromicro 2011. Sopot, Poland, June 9th to 11th, 2021.

Participation in organization of conferences

International Symposium Ecohydrology for the Circular Economy and Nature-Based Solutions towards mitigation/adaptation to climate change. University of Lodz. Lodz, Poland. 26th – 28th, September 2017.

Workshops

Font Nájera A., Mankiewicz-Boczek J. *Soluciones Ecohidrológicas, la importancia de las bacterias en tecnologías aplicadas al ambiente* (Ecohydrological solutions, the importance of bacteria in technologies applied to the environment). Workshop, Ecohydrología y su aplicación en la subcuenca del Río Tomebamba (Ecohydrology and its application in the tributary of the River Tomebamba). Quito, Ecuador. 15th – 17th March, 2021. Presentation.

Participation in other activities

Festival for Science, Technology and Art
Manufaktura, April 2017 and April 2018.
Co-organization and participation in the stand for Ecohydrology

Summer School, Understanding Poland.
Centre for Asian Affairs, University of Lodz, Poland.
Preparation of Lecture, June 2017