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Denitrification Process Enhancement and Diversity of the Denitrifying Community in the Full Scale Activated Sludge System after Adaptation to Fusel Oil

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Abstract: Implementation of anaerobic digestion of primary sludge in modern wastewater treatment plants (WWTPs) limits the availability of organic carbon for denitrification in conventional nitrification-denitrification (N/DN) systems. In order to ensure efficient denitrification, dosage of the external carbon source is commonly undertaken. However, application of commercial products, such us ethanol or acetate, greatly increases operational costs. As such, inexpensive and efficient alternative carbon sources are strongly desirable. In this study, the use of the fusel oil, a by-product from the distillery industry, was validated in terms of the denitrification process enhancement and impact on the activated sludge bacterial community structure. The experiment was conducted at a full scale biological nutrient removal facility (210,000 PE), in the set of the two technological lines: the experimental line (where fusel oil was introduced at 45 cm^3/m^3 dose) and the reference line (without an external carbon source addition). During the experimental period of 98 days, conventional nitrate utilization rate (NUR) measurements were carried out on a regular basis in order to assess the biomass adaptation to the fusel oil addition and denitrification process enhancement. While the NURs remained at a stable level in the reference line (1.4 ± 0.1 mg NO_3-N/g VSS·h) throughout the entire duration of the experiment, the addition of fusel oil gradually enhanced the denitrification process rate up to 2.7 mg NO_3-N/g VSS·h. Moreover, fusel oil contributed to the mitigation of the variability of NO_3-N concentrations in the effluent from the anoxic zone. The bacterial community structure, characterized by 16S rRNA PCR—DGGE and the clone libraries of the genes involved in the denitrification process (*nirS* and *nirK*), was comparable between the reference and the experimental line during the entire experimental period. In both analyzed lines, the most frequent occurrence of denitrifiers belonging to the genera *Acidovorax*, *Alcaligenes*, *Azoarcus*, *Paracoccus* and *Thauera* was noticed. Our results proved that fusel oil would a valuable substrate for denitrification. The addition of fusel oil enhances the process rate and does not reflect a severe selection pressure on the bacterial community at applicable doses. Practical application of fusel oil generates opportunities for the WWTPs to meet effluent standards and reduce operational costs, as well as optimizing waste management for the distillery industry.

Keywords: 16SrRNA PCR-DGGE; biological nitrogen removal; denitrification; fusel oil; nirS/nirK gene library

Citation: Kowal, P.; Ciesielski, S.; Otieno, J.; Majtacz, J.B.; Czerwionka, K.; Mąkinia, J. Denitrification Process Enhancement and Diversity of the Denitrifying Community in the Full Scale Activated Sludge System after Adaptation to Fusel Oil. *Energies* **2021**, *14*, 5225. https://doi.org/10.3390/en14175225

Academic Editor: Antonio Zuorro

Received: 13 July 2021
Accepted: 17 August 2021
Published: 24 August 2021

1. Introduction

The priority task of protecting surface waters from pollution—which is caused by municipal wastewater from large and medium urban agglomerations—is to ensure its full biological treatment and enhanced removal of nutrients. Increasingly, the requirements

around indicators of the effluent quality are forcing modernization of the currently existing installations, as well as implementation of new solutions to enhance nutrient removal processes rates. For instance, in accordance with Polish regulations [1], for Wastewater Treatment Plants (WWTPs) with the size of 2000 population equivalent (PE), the maximum permissible value of total nitrogen (TN) is 30 mg N/L, while for the largest installations (>100,000 PE) this limit was set on 10 mg N/L.

One of the key factors that determines the effectiveness of nitrogen removal with the conventional activated sludge process in WWTPs is the presence of organic carbon compounds, which are easily available for the microorganisms carrying out denitrification. The concentration of biodegradable carbon compounds in raw municipal wastewater is usually limited, especially in the context of modern WWTP design, where a significant portion of the primary sludge is directed into biogas production in a separated sidestream treatment process. The primary sludge is considered to be the most efficient substrate available in WWTPs for biogas production via anaerobic digestion [2]. The benefits of energy recovery from the anaerobic digestion of sewage sludge have resulted in a steadily growing number of dedicated installations around the world. For example, in Poland there are currently 113 full-scale biogas plants operating in municipal WTTPs [3]. However, the redirection of organic compounds for biogas production limits the pool of carbon that is available for the mainstream nitrogen removal process, which is mostly dependent on denitrification processes.

Currently, scientific and commercial efforts have focused on the implementation of novel technologies based on shortcut nitrification and anaerobic ammonium oxidation (anammox) processes, such as deammonification, which are less dependent, or even fully independent, from the carbon availability [4]. Nevertheless, in medium and small WWTPs, conventional nitrification-denitrification (N/DN) remains the most sustainable technology.

A common practice of supporting the nitrogen removal process in the N/DN based systems is the dosing of external carbon sources. For such purposes, commercial products (i.e., methanol, ethanol or acetic acid) are commonly used. The application of commercial substrates ensures relatively high rates of denitrification, however, it also raises the operating costs of wastewater treatment.

Fusel oil—a by-product of the rectification (i.e., process of multistage distillation) of spirit—is characterized by a high concentration of organic compounds readily available to microorganisms, while maintaining a favorable ratio of carbon to nitrogen concentration [5]. In 2016, the global spirits market was estimated at $6.13 billion, with a further 33.4% increase prognosed up to 2025 [6]. In consequence, the amount of fusel oils—which are considered to be a post-production waste that is difficult to manage—is growing rapidly. Due to the COVID-19 pandemic, an increase in the production of pure ethanol, which is used as a basic component of the disinfectants, has been observed globally.

In countries such as Poland, where the spirit industry constitutes an important branch of the national economy and bioethanol production is growing annually [7], the implementation of solutions based on waste management from this market will be in line with the concept of the circular economy promoted by the Waste Framework Directive [8]. As a result, their energy balance could be improved through increasing the production of electricity from an elevated amount of biogas and reducing the energy demand for the aeration of activated sludge reactors, loaded with a lower load of organic compounds.

The time required to commonly implement new denitrification-supporting substrates can be significantly shortened by providing information about their impact on the bacterial communities responsible for the process. Changes in the structure of activated sludge consortia may have a direct impact on their metabolic potential and, consequently, their denitrification efficiency. In addition, knowledge of the ecology of bacterial consortia allows for a more complete interpretation of the data obtained during technological experiments.

There are numerous studies on the use of different substrates to support the biological nitrogen removal. A change in the efficiency of the denitrification process was observed, coupled with selection of the specific groups of bacteria (i.e., [9,10]).

Most studies have focused on the effects caused by sole substrates, such as methanol, ethanol or acetic acid, which are the commercial products most commonly applied as an external carbon source in practice.

When using methanol, a long period of acclimation of biomass is usually required, until an increased efficiency of nitrogen compounds removal is obtained [11]. Further investigations by Ginige et al. [12] showed that the acclimation process was associated with a change in the structure of the denitrifying bacteria population and, in particular, with the intensification of the growth of *Methylophiles* family members, as well *Paracoccus* and *Hyphomicrobium* genera. On the other hand, acetic acid may favor the growth of certain groups of denitrifying bacteria that prefer growth beyond the floc structure, which may adversely affect the sedimentation properties of the sediment and reduce the efficiency of pollutants removal [12].

Research carried out by Hwang et al. [13] has shown that when ethanol or acetic acid was applied as an external carbon source, an increased growth of denitrifying bacteria, related to representatives of the genera *Acidovorax*, *Azoarcus*, *Dechloromonas* and *Thauera*, is observed. These are also considered to be common components of activated sludge bacterial community.

Currently, to characterize complex microbial consortia, a set of molecular microbiology tools is commonly applied, including polymerase chain reaction (PCR) based techniques (e.g., PCR-denaturing gradient gel electrophoresis—DGGE) or wide range of sequencing techniques (mostly high-throughput techniques termed next-generation sequencing—NGS) [14]. Although the study of microbial communities based on the analysis of 16S rRNA gene sequences polymorphism is currently the most widely used approach for assessing overall microbial diversity, this approach is limited in terms of denitrifying bacteria population characteristics. Denitrifiers are highly diverse in terms of taxonomy and are not defined by a close phylogenetic relationship. Thus, approaches based on 16S rRNA are not sufficient [15]. More sensible approach is to analyze the functional genes that are directly involved in denitrification, especially the genes (i.e., *nar*, *nir*, *nor* and *nos*) encoding reductases that are responsible for the conversion of a particular nitrogen form [16].

An example of research that uses molecular techniques based on functional denitrifying genes analysis in order to characterize activated sludge adaptation to external carbon sources is work by Hallin et al. [17]. The authors conducted studies on a pilot scale, in two parallel systems, where methanol and ethanol were dosed as external carbon sources. The control consisted of samples from the full-scale system, in which no additional factors stimulating the denitrification process were used. Gene libraries of *nirK* and *nirS*, both encoding the alternative forms of nitrite reductase, were applied for the denitrifying population characterization. The results showed that, in the case of both substrates, the structure of bacterial subpopulation with the *nirS* genotype changed significantly throughout the experiment. The genetic diversity of microorganisms with the *nirS* genotype increased in both reactors, with methanol being a favorable factor in biodiversity being. The groups of microorganisms with the *nirK* genotype formed much more stable consortia.

In terms of the waste materials, which were applied as an external carbon source, general scientific attention in the literature has focused on food waste and fermentation products. For instance, Tang et al. [18] obtained increased microbial metabolic activity and bacterial community diversity in the activated sludge that was cultured with fermentation liquid from food waste. Investigation of the substrate showed denitrification occurring at a relatively high rate of 5.5 mg NO_3-N/g VSS·h. Microbial analyses revealed the predominance of representatives of the *Hydrogenophilaceae* family from *Betaproteobacteria*, which accounted for 36.6% of the total bacterial community in the analyzed reactor. In the systems supplemented with other carbon sources, like starch and acetate, the share of those bacteria was less than 1%.

In previous studies related to the use of fusel oil to increase the denitrification process rate, most experiments were conducted in lab-scale and their impact on the microbial

communities was assessed during relatively short time perspectives [19,20]. For full scale investigations, no analysis of microbial data was provided [21,22].

This study presents the first comprehensive research on the overall stability of the structure of denitrifying bacteria communities during the implementation of fusel oil to enhance the denitrification process rate at a full scale WWTP. The results of the technological measurements, which were carried out for 98 days in two lines (reference and experimental, without an external carbon dosage and with fusel oil addition, respectively), were supported by microbial analyses based on modern molecular biology tools (clone libraries of functional denitrifying genes and 16S rRNA PCR-DGGE). One of the key scientific aspects of the presented study was a comparison of the genetic diversity of the *nirK* and *nirS* gene variants encoding alternative forms of nitrite reductase in the biomass samples of activated sludge from the two lines. The potential selective interaction of compounds that constitute the external carbon source for microorganisms, may contribute to changes in the structure of the denitrifying bacteria population, which, in turn, affects the functional and metabolic properties of these communities.

2. Materials and Methods

2.1. Characterization of Fusel Oil Used as an External Carbon Source

Fusel oil was selected as a potential external carbon source to support the denitrification process based on analysis of the basic chemical composition that was carried out by Makinia et al. [5]. The analyzed fusel oil samples contained a high fraction of soluble, readily biodegradable organic compounds (chemical oxygen demand (COD) concentrations approx. 1,700,000 g O_2/m^3) and a favorable COD/TN ratio (approximately 1800). It was found that 2-methyl-1-butanol was a dominant component of fusel oil (almost 40% by weight), with three other important components: 2-methyl-1-propanol, 3-methyl-1-butanol and ethanol [22].

2.2. The Wastewater Treatment Plant and Experiment Design

The experiment was carried out at Poznan WWTP, situated in central Poland (N 52°43′33″, E 16°95′77″). The size of the WWTP in Poznan is estimated to be 210,000 PE with an average daily flow rate of around 35,000 m^3, while the age of the sludge is maintained in the range of 20–36 days. The average concentrations of the basic pollution indicators (including total phosphorus (TP), TN and COD) in the influent and effluent during the experimental period are presented in Table 1.

Table 1. Average values of the basic pollution indicators in the influent and effluent from Poznan WWTP during the experimental period.

Parameter	Unit	Concentration
	Influent	
COD	g COD/m^3	851 ± 120
N_{TN}	g N/m^3	72 ± 6.5
P_{TP}	g P/m^3	10.1 ± 2.1
	Effluent	
COD	g COD/m^3	26.2 ± 10.1
NO_3-N	g NO_3-N/m^3	5.4 ± 0.6
NH_4-N	g NH_4-N/m^3	0.3 ± 0.2
N_{TN}	g N/m^3	7.8 ± 1.2
P_{TP}	g P/m^3	0.5 ± 0.1

Poznan WWTP designed for biological nutrient removal (BNR) is Johannesburg (JHB) type process configuration (with an additional denitrification zone for recalculated activated sludge) consisting of four biological treatment lines and two secondary clarifiers. The schematic layout of the bioreactors and the fusel oil dosing point are shown in Figure 1.

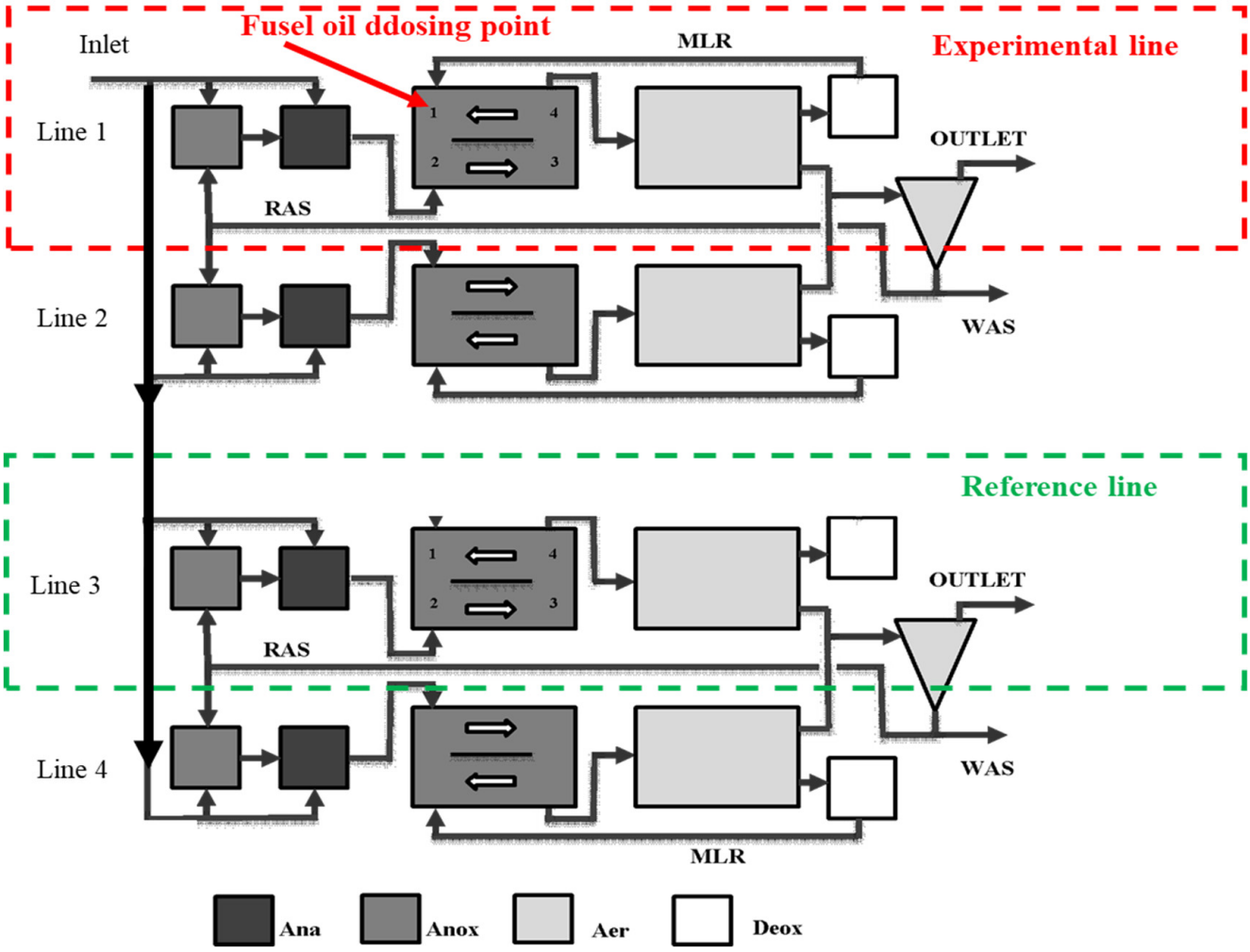

Figure 1. Layout of the full-scale JHB systems for investigating the acclimation of biomass to fusel oil at Poznan WWTP. Abbreviations: RAS—recalculated activated sludge, MLR—mixed liquor recirculation, WAS—waste activated sludge. Reactors: Ana—anaerobic, Anox—anoxic, Aer—aerobic, Deox—deoxidation of the MLR.

The average dose of fusel oil to the experimental line was approximately 0.4 m^3/d (i.e., 45 cm^3/per m^3 of the average influent flowrate). Line three was chosen as the reference line (without an external carbon source dosage). Research was carried out for 98 days (from March to July). During this period, detailed analyses of the efficiency of pollutant removal for both bioreactors were carried out five times (at 1, 21, 42, 76 and 98 days). Measurements of the variability of concentrations of the selected pollutants at four points in the circulation denitrification chamber of the experimental and reference line were performed. In addition to the in-situ measurements in the full-scale bioreactors, the conventional nitrate utilization rate (NUR) measurements were carried out under laboratory conditions, including process biomass from the experimental and reference lines. The NURs were validated during one-phase tests under anoxic conditions, with nitrate as the nitrogen source (KNO_3) and fusel oil (experimental line) or raw wastewater from Poznan WWTP (reference line) added. The substrates were added at the beginning of the test, at concentrations related to the full scale system. Each test was run for 6–7 h. At the regular time intervals (1 h), measurements of the N compounds (NO_3-N, NO_2-N) and COD were conducted. In order to estimate the NUR, the following equation (Equation (1)) was applied:

$$NUR = \frac{slope(S_{NO_3-N})}{\Delta t * X} \left[mg\ NO_3^- - N\ /g\ VSS{\cdot}h\right] \quad (1)$$

where:

S_{NO3-N}—concentration of nitrate nitrogen over time (mg N/L),
Δt—difference between the end and beginning of the measurements (h),
X—concentration of the organic fraction of activated sludge (g VSS/L).

2.3. Analytical Methods

The concentrations of inorganic nitrogen compounds (NH_4-N, NO_3-N, NO_2-N), total phosphorus (P_{TP}) and COD were determined using dedicated cuvette tests and XION 500 spectrophotometer (Hach Lange GmbH, Berlin, Germany). The total nitrogen (TN) was determined using a total organic carbon (TOC) analyzer (TOC-VCSH), coupled with a TN module (TNM-1) (SHIMADZU Corporation, Columbia, USA). The procedure for measuring the TN concentration was described in the previous paper [21]. The concentrations of nitrogen and the remaining parameters in samples of mixed liquor were determined after filtering the samples under vacuum pressure through a 1.2 pore size Millipore (Billerica, MA, USA) nitrocellulose filter. The total and volatile suspended solids (TSS and VSS) were measured by the gravimetric method, according to Polish Standards (PN-72/C-04559).

2.4. Microbial Analyses

2.4.1. Sampling and Genomic DNA Isolation

A 2-L sampler was used to collect biomass samples from a depth of approximately one meter from the surface of the effluent in the anoxic reactor. Initially, samples of the activated sludge were mixed and, after thickening, were poured into a test tube with a volume of about 15 mL. A repetition was prepared for each sample. The obtained sludge samples were frozen and kept at −20 °C prior to DNA extraction. Samples were collected during the 1st, 7th, 28th, 49th and 74th day of the experiment. The isolation of genomic DNA from a single sample was carried out as follows: 0.075 g of the biomass was washed with a phosphate buffer (0.1 M, pH 8.0) and centrifuged for 1 min at 13,000 rpm. After removing the supernatant, 1 mL of the extraction buffer (100 mM Tris-HCl, 100 mM EDTA; 1.5 M NaCl, pH = 8) and 0.3 g of a mixture of glass beads (Ø 0.25–0.5, Carl Roth, Karlsruhe, Germany) were added. The samples were subsequently shaken for 20 min. at 5000 rpm in a shaker (Uniequip, Planegg, Germany). To increase the efficiency of the cell lysis, 0.2 mL of a 10% sodium dodecyl sulfate (SDS) solution was added and incubated at 65 °C for one hour. In the next step, the cell extract was centrifuged at 13,000 rpm. for 10 min. The DNA suspension from an aqueous solution was purified using silica columns (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's recommendations. Purified DNA was suspended in 50 µL of nuclease free deionized water and stored at −20 °C. Quality and quantity analyses of DNA were checked spectrophotometrically at 280 nm and by electrophoresis on a 1% agarose gel stained with ethidium bromide.

2.4.2. PCR-DGGE of 16S rRNA Gene

The fragment of the 16S rRNA gene was amplified using the pair of F-968-GC and R-1401 primers proposed by Nübel et al. [23]. The reaction mixture consisted of the following components: 100 ng genomic DNA; 0.5 µM of each primer; 100 µM nucleotide mixture (Promega, Madison, WI, USA); 0.6 U of Hypernova DNA polymerase (DNA-Gdansk, Gdańsk, Poland); 3 µL of 10× concentrated reaction buffer (100 mM Tris-HCl, 500 mM KCl, 1.5% Triton X-100); 1.5 mM $MgCl_2$; the mixture was diluted with deionized water to a volume of 30 µL. The PCR reactions were performed in a Gene-Amp® PCR System 9700 thermocycler (Applied Biosystem, Waltham, MA, USA) with a temperature program consisting of: initial denaturation at 94 °C for 5 min; 35 cycles with denaturation at 94 °C for 45 s, primer assembly at 62 °C (which was lowered by 0.1 °C in subsequent cycles) for 45 s, elongation at 72 °C for 45 s, and final elongation at 72 °C for 10 min. The PCR reaction for each trial was carried out in triplicate. The qualitative analysis of the products were performed in a 1% agarose gel, in the presence of 5 µg molecular marker (100 bp Ladder, Promega). After verification, the products from each replication were mixed together.

Two 6% acrylamide-bisacrylamide solutions (Acrylamide: N, N′-Metylene bisacrylamide 37.5:1, Fluka, Buchs, Switzerland) that differed in terms of the concentration of the denaturant agent were mixed to prepare the gel for the DGGE analysis. The less concentrated solution contained 30%, while the second solution contained 60% of the denaturant agent (urea). Both solutions contained formamide in order to reduce the renaturation of DNA strands during electrophoresis. To ensure a uniform denaturation gradient in the range of 30% to 60%, the gels were prepared using attachments from the DCode™ Universal Mutation Detection System (Bio-Rad Laboratories Inc., Hercules, CA, USA) according to the manufacturer's instructions. TEMED (N′-Tetramethyl-ethylene-diamine) and a 1% solution of ammonium persulfate (Carl Roth GmbH, Karlsruhe, Germany) were used as initiating agents for the gel polymerization. Before the electrophoresis, up to 50 µL of the sample from the three PCR replications were mixed together and added with 10 µL of the loading buffer to the gel wells. The electrophoresis of the PCR products was carried out in a Dcode System electrophoresis apparatus (Bio-Rad Laboratories Inc., USA) in 1× concentrated TAE buffer (2 M Tris, 40 mM acetic acid, 1 mM EDTA). The electrophoresis process lasted 12 h, during which a constant temperature of 60 °C and 60 V voltage were maintained. The fingerprints contained in the gels were visualized using the SybrGold fluorescent dye (1:10,000, Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions. After rinsing off the excess dye, the gel was transilluminated with UV radiation. Images were archived and analyzed, using the KODAK 1 D 3.6 Image Analysis Software (Eastman Kodak Company, Rochester, NY, USA). In order to verify the obtained patterns of the bands, the whole process was repeated twice.

The presence/absence patterns of individual bands on the stained gels were used to prepare binary matrices for genetic distance estimation between the samples. Genetic distance was assessed by a Dice coefficient with formula (Equation (2)):

$$Sd = 1 - \frac{2j}{a+b} \quad (2)$$

where j is the number of bands common to both samples, while a and b correspond to the total number of bands in a given track [24]. In order to map the genetic distance values in a graphic form, the DGGEstat program module was applied [25] with the method of calculating the average distances between objects in clusters (UnMaged Pair Group Method with Arithmetic Mean—UPMGA).

It was assumed that a single band on the particular track that was visible on PCR-DGGE gel corresponded to an individual bacteria taxon. With this assumption and by measuring the band intensity (as a densitometric curves), it was possible to estimate the relative abundance of the particular bacterial group. The sum of the band intensities from a single track was considered as the total number of all bacteria presented in the sample. Based on the band intensity on the gel tracks, measured by the peak heights of the densitometric curves, a Shannon index of general diversity H was estimated [26], using the following formula (Equation (3)):

$$H = -\sum_i \frac{n_i}{N} * log_{\frac{ni}{N}} \quad (3)$$

where: n corresponds to the height of the densitometric curve of a single band, and N the sum of the heights of all densitometric curves on a given track.

2.4.3. *nirS* and *nirK* Genes Clone Libraries

For the purpose of constructing gene libraries, fragments of the *nirS* and *nirK* gene were amplified using PCR. Gene libraries were constructed for the samples collected during the 74th day of the experiment, from both reactors (i.e., the experimental line with the fusel oil addition and the reference line). In the case of the *nirS* gene, a pair of cd3aF/R3cd primers were used, whereas for *nirK* a pair of F1aCu/R3Cu, developed by Throbäck

et al. [27] and Hallin et al. [28], respectively, were used. The R3cd and R3Cu primers had an additional GC clamp of 33 nucleotides. The primer sequences are shown in Table 2.

Table 2. PCR primers used to amplify fragments of *nirS* and *nirK* genes (based on [27] and [28], respectively).

Marker Gene	Primer Name	Primer Sequence
nirS	GC-R3cd	5′-GGC GGC GCG CCG CCC GCC CCG CCC CCG TCG CCC GA(C/G) TTC GG(A/G) TG(C/G) GTC TTG A-3′
	cd3aF	5′-GT(C/G) AAC GT(C/G) AAG GA(A/G) AC(C/G) GG-3′
nirK	GC-R3Cu	5′-GGC GGC GCG CCG CCC GCC CCG CCC CCG TCG CCC GCC TCG ATC AG(A/G) TTG TGG TT-3′
	F1aCu	5′-ATC ATG GT(C/G) CTG CCG CG-3′

Amplification of the fragments of both genes was performed with the same composition of the PCR reaction mixture and the temperature profile. The reaction mixture contained: 50 ng of genomic DNA; 0.5 µM of each primer; 100 µM nucleotide mixture (Promega, Madison, WI, USA.); 0.15 U GoTaq® Polymerase DNA (Promega, Madison, WI, USA); 6 µL of 5× concentrated reaction buffer (50 mM Tris-HCl, pH 9.0; 50 mM NaCl;); and 1.5 mM $MgCl_2$. The obtained mixture was then diluted with deionized water to a volume of 30 µL.

The PCR reactions were performed in a Gene-Amp® PCR System 9700 thermocycler (Applied Biosystem, USA) with the following temperature profile: initial denaturation at 94 °C for 10 min; 32 cycles with denaturation at 94 °C for 60 s; primer assembly at 63 °C (which has been lowered by 0.1 °C in subsequent cycles) for 60 s; elongation at 72 °C for 60 s; and final elongation at 72 °C for 10 min. The qualitative analysis of the product was performed in a 1% agarose gel, in the presence of a 5 µg molecular marker (100 bp Ladder, Promega, Madison, WI, USA). The verified PCR products from the three replicates were mixed. The resulting mixture was subsequently cleaned using the Clean-up kit (A & A Biotechnology, Gdynia, Poland), according to the protocol provided by the manufacturer. The purified DNA was suspended in deionized water.

The recovered PCR products were cloned using the InsTAclone™ PCR Cloning Kit (Fermentas, Waltham, MA, USA), according to the manufacturer's instructions. Clones were verified by the means of a microorganism culture system on the Luria-Bertani (LB) agar nutrient medium (components in proportion to the volume of total aqueous solution: 0.5% yeast extract, 1% NaCl, 1% tryptone-peptone, 1.5% agarose), which additionally contained IPTG, Xgal and ampicillin.

The cultures were carried out in a liquid LB medium, supplemented with ampicillin, overnight at 37 °C. The cultures were subsequently centrifuged for 30 min at 4000 rpm. Plasmid DNA was isolated from the harvested cells by using the Plasmid Mini kit (A & A Biotechnology, Gdynia, Poland). The presence of the insert of correct length was verified using PCR. Four libraries in total were constructed for both genes (with comprehension of the particular gene variants diversity between the reference and experimental lines), of which each consisted of 40 vectors with an insert.

2.4.4. DNA Sequencing and Phylogenetic Analysis

Sequencing of the plasmids containing the inserts with *nirS* and *nirK* gene fragments was performed by Macrogen Europe (Amsterdam, The Netherlands) using the ABI3730XL automatic sequencer (PE Applied Biosystems, Foster City, CA, USA). The vector primer M13F (GTAAAACGACGGCCAGT) was used to initiate the sequencing reaction. Sequence readings for further analysis were provided in the form of fluorograms and text files.

Fluorograms in which the degree of contamination of the matrix prevented the unambiguous recording of the DNA sequence were rejected. The remaining reads with the *nirS* and *nirK* gene DNA sequences were aligned in the MEGA 6.06 program [29] using the ClustalW module [30]. At this stage of the analysis, the sequences of plasmids and reactions primers were removed from the reads. The sequences of the *nirS* and *nirK* gene fragments were checked by translating them with the EMBOSS Transeq tool which is included in

the EMBL-EBI service resources (http://www.ebi.ac.uk (accessed on 2 August 2021). In cases where stop codons were present and where the amino acid composition differed significantly from the DNA sequences derived from the cultivable denitrifying bacteria, the given read was not taken into account in further analysis. The obtained DNA sequences have been deposited in the Gene Bank (NCBI) with the accession numbers ranging from KP662417 to KP662440 in terms of the *nirK* gene and from KP662360 to KP662386 for the *nirS* gene.

The positively verified sequences of both genes were subsequently compared to the sequences that were deposited in the Gene Bank (NCBI, http://www.ncbi.nlm.nih.gov (accessed on 2 August 2021). The BLAST algorithm was used to search the database [31], including cultivable microorganisms, as well environmental isolates from bacteria not susceptible to growth on synthetic media. In order to establish the taxonomic position of the microorganisms from which the *nirS* and *nirK* gene DNA sequences were obtained, sets of marker DNA sequences from cultivable denitrifying bacteria were selected during a previously performed search of the Gene Bank resources. The analyzed DNA sequences and marker DNA sequences were used for the construction of phylogenetic trees, after alignment with the ClustalW program (constituting module of the MEGA 6.06 software). Phylogenetic trees were created in the MEGA 6.06 program through the use of the neighbor joining algorithm [32]. To verify the topology of the obtained dendrograms, self-sampling (bootstrap) for 500 repetitions was applied. In addition, the values of total genetic distance and mean inter-group distance were estimated using the maximum composite likelihood model included in the MEGA 6.06 program.

3. Results and Discussion

3.1. Kinetics of Denitrification during the Acclimation Experiments Effect

Conventional denitrification rate measurements showed that the NURs obtained for the experimental line with fusel oil dosage were higher than the values obtained for the reference line.

The NURs for biomass collected from the experimental line gradually increased from 1.7 mg NO_3-N/g VSS·h on the first experimental day, to 2.7 mg NO_3-N/g VSS·h in the final period of the study (representing an increase of approximately 60%). In the case of biomass from the reference line, the NUR values were significantly lower, remaining in a relatively narrow range from 1.3 to 1.5 mg NO_3-N/g VSS·h throughout the study period. Changes in the NUR values during the conducted experiment are shown graphically in Figure 2.

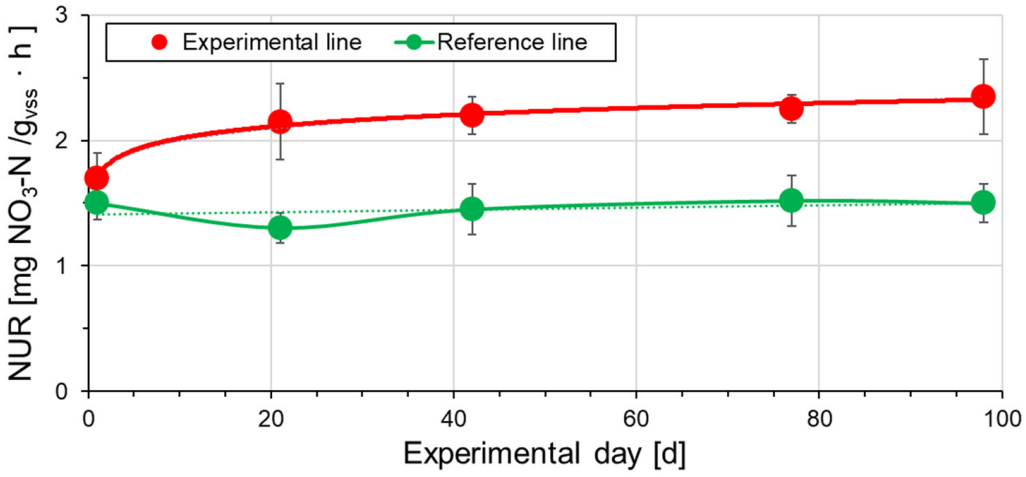

Figure 2. Denitrification rates measured in laboratory conditions for biomass from the experimental line (with fusel oil dosing) and reference line (without dosing of fusel oil) at Poznan WWTP.

The initial NURs for the non-adapted activated sludge from the Poznan WWTP were comparable to the NUR values measured in our previous lab scale studies, also for not

acclimated biomass to an external carbon source [20]. After adaptation to fusel oil, the NUR obtained in this study (2.7 mg NO_3-N/g VSS·h) was significantly lower in relation to the values obtained in lab-scale experiments, where the NUR was as high as 16.0 mg NO_3-N/g VSS·h [20,22].

There are two main explanations of the differentiation of the NURs between laboratory and full scale experiments:

1. As shown in Figure 1, lines one and two of the bioreactors had a common secondary clarifier from which the sludge was recirculated to the activated sludge reactors. With this technical solution applied at Poznan WWTP, the sludge from line one (with dosing fusel oils) was mixed with the sludge from line two (without dosing). For this reason, the actual dose of fusel oils per unit of biomass was about two times lower than in the laboratory tests.

2. Insufficient mixing of the dosed fusel oils in the reactors was observed, which resulted in their partial lifting to the surface and evaporation into the atmosphere. As a result of this loss, the actual external carbon dose was potentially lower than assumed. The study by Kim et al. [33] also indicated the necessity of verifying the results of the external carbon application obtained in laboratory scale, in relation to the full-scale tests under real operating conditions. At the same time, they indicated that there was only a limited number of relevant studies. Higher NURs were obtained during tests under laboratory conditions for various sources of organic carbon.

In the literature [34], there is a consensus that the chemical structure and molecular weight of the substrate used as a carbon source is correlated with the denitrification efficiency. In general, sole carbon sources with low molecular weights favors denitrifying activity in contrary to complex substrates, especially those with an aromatic structure. For instance, Peng et al. [35] conducted a process of adaptation to ethanol and obtained the denitrification rate of 9.6 g mg NO_3-N/g VSS·h. In contrast, Queiroz et al. [36] studied denitrification with phenol addition as an external carbon source., The observed NURs were significantly lower and ranged from 1.3 to 1.5 mg NO_3-N/g VSS·h. The highest values of NUR were reported for acetic acid (16.86 mg NO_3-N/g VSS·h) [37] and food waste materials (12.89 mg NO_3-N /g VSS·h) [38]. However, previously identified issues about the scale of the experiments have to be taken into account (e.g., in studies by Wang et al. [39], the application of acetate, despite C/N optimization, was not allowed to cross the threshold of NUR = 4.0 mg NO_3-N/g VSS·h).

Another issue that has to be taken into account is the potential toxicity of the applied substrate. In our previous studies [22], detailed analyses of the composition of ten fusel oil samples were performed. The scope of the analyses included 29 selected organic compounds, as the study was also aimed at their potential toxicity. It was shown that 2-methyl-1-butanol, 2-methyl-1-propanol, 3-methyl-1-butanol and ethanol accounted for over 68% by weight. When additionally taking into account n-propanol, this proportion increases by more than 70%. The content of the remaining compounds ranged only from 0.002 to 0.3%.

During preliminary studies, the toxicity of fusel oils (at doses equivalent to what was applied during full scale experiments) was assessed by checking their influence on the nitrification process. The results of respirometric measurements of oxygen uptake did not show any significant differences from the control sample (i.e., the differences did not exceed 3%). On this basis, it was confirmed that fusel oil will not adversely affect the biocenosis of activated sludge at applicable doses.

The results showed that the required period of biomass adaptation to the addition of fusel oil is approximately three weeks. A 5-fold analysis of nitrate nitrogen concentrations at four points located in the anoxic chamber of the experimental and reference line confirmed the increase in the denitrification efficiency as a result of fusel oil dosing (Figure 3). The average concentration of nitrate nitrogen in the area of mixed liquor recirculation from the aerobic chamber (point 1) was approximately by 3 g NO_3-N/m^3 lower in the test line compared to the reference one. The inflow of wastewater from the anoxic chamber (point 2) enabled the effective denitrification in the reference line, resulting in low concentrations of

nitrate in point 3 (mid-way of internal recirculated wastewater flow) and point 4 (outflow into the nitrification chamber).

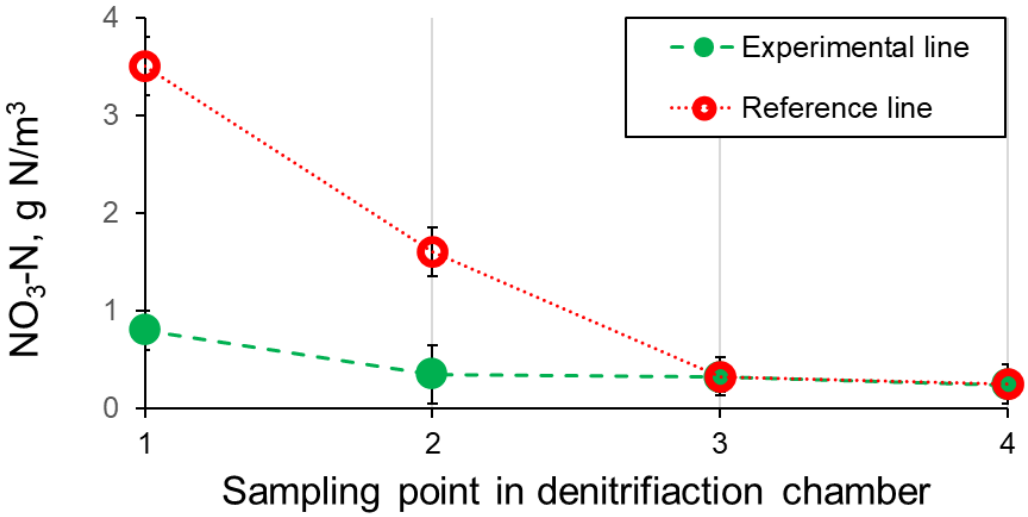

Figure 3. Variability of NO_3-N concentrations in the anoxic chamber in experimental and reference line at Poznan WWTP (average values from the entire research period).

3.2. Analysis of Activated Sludge Bacterial Community Composition during Adaptation to Fusel Oil

3.2.1. General Bacteria Population Structure

The structure of the general bacterial community analyzed with the 16S rRNA PCR-DGGE, showed that the number of bands and their distribution in the samples collected from the experimental line fed with fusel oil (FO) and the reference line (REF) were similar to each other during the entire experiment (Figure 4A). In all the samples, the same set of bands with the highest light intensity was present, and were marked with symbols from t_1 to t_9. In the inoculum sample, 29 bands were detected. In the case of a reactor FO, after the first week, the number of bands gradually increased from 29 to 33 and stabilized at this level until the end of the experiment. In the reference line, the number of observed bands initially decreased to 28 and then increased to 34 in the third sampling period (28th day of the experiment). Similarly to the FO reactor, the structure of the analyzed community was not subjected to stringent rearrangements in the further part of the research. Changes in the intensity of the single bands were observed. The course of changes was similar in both reactors. The analysis of the genetic distance, visualized as the form of the dendrogram, allowed for distinguishing two branches (Figure 4B). On the first branch, the samples collected from both reactors in the initial phase of the experiment were grouped together with the inoculum sample. The second branch of the genetic distance tree was formed by samples derived from both reactors after the 28th day of the experiment. The distance between the samples increased with the time interval between their collection.

The Shannon biodiversity index for the FO experimental line varied within a narrow range from 3.3 to 3.4 (Figure 5). These values were comparable with the value of the indicator for the inoculum sample (H = 3.3). In the reference reactor (REF) at the beginning of the experiment, there was a slight decrease in the H value to 3.2. H values then increased and stabilized at 3.3 in the final two months of the experiment.

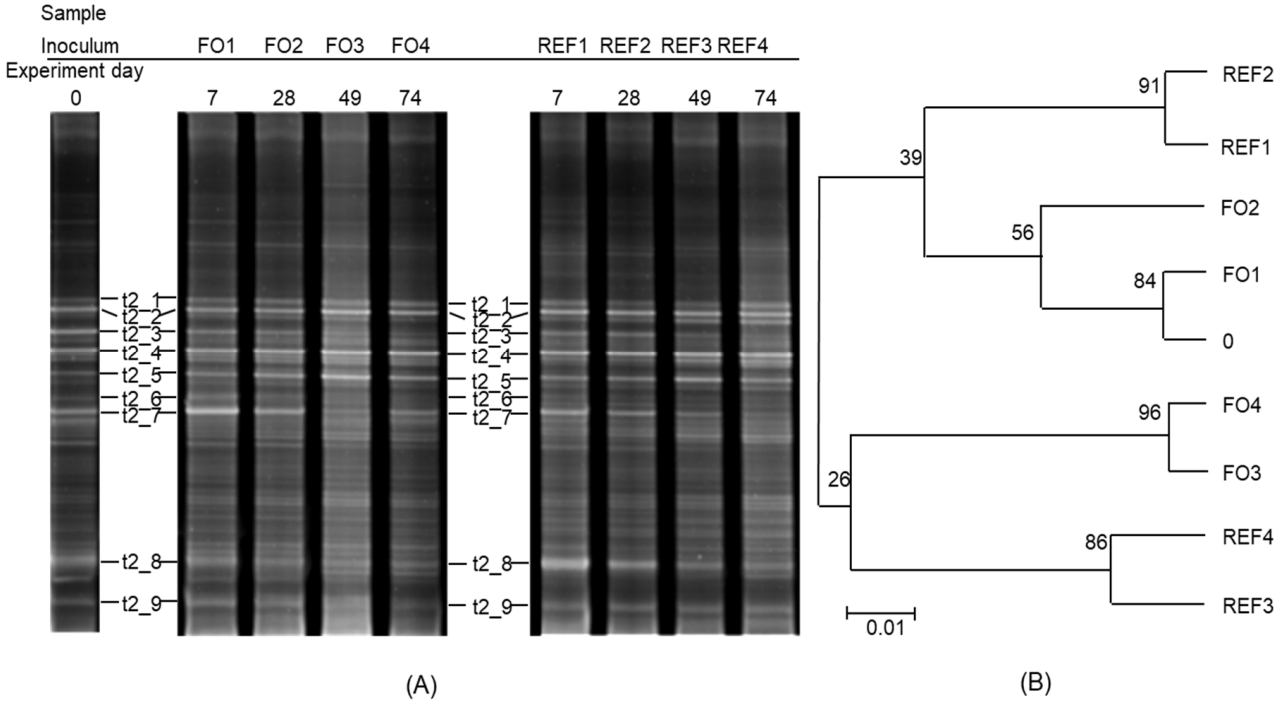

Figure 4. Band pattern obtained by 16S rDNA PCR-DGGE technique of the samples collected from the reference reactor without dosage of external carbon source (REF) and from the reactor with fusel oil addition (FO). The days of sample collection during the experiment are shown below the reactor symbols (**A**). Dendrogram created using the UPGMA algorithm (self-sampling—1000 repetitions) illustrating the genetic distance between individual samples (**B**).

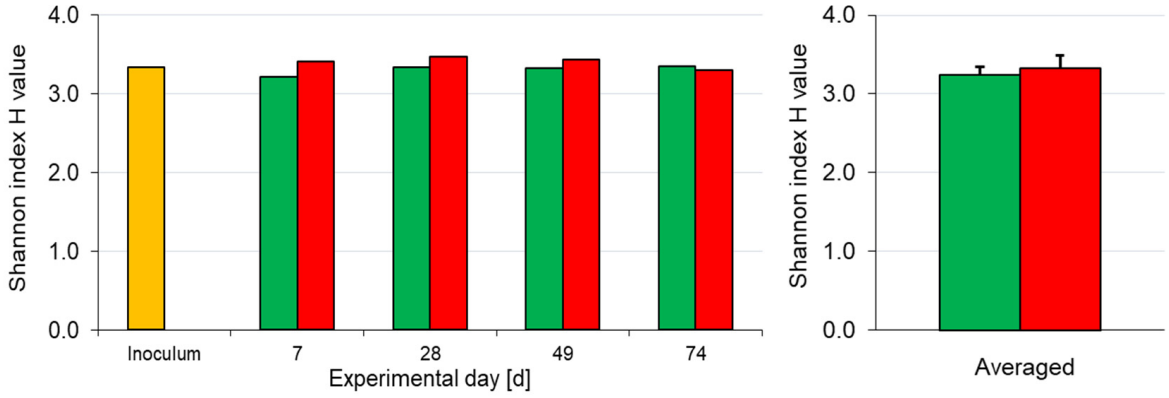

Figure 5. Shannon index H values representing biodiversity of the activated sludge samples during the experimental period: Inoculum; reference reactor (REF) reactor with fusel oil addition (FO).

The structure of the microbial communities in the activated sludge samples were characterized by a high stability in both analyzed lines (i.e., the experimental line with dosing fusel oil, as well the reference system without the addition of external carbon source). This is evidenced by the balanced values of the Shannon diversity index in both lines throughout the duration of the experiment. A direct comparison of fingerprints indicated a significant similarity of the structure of communities between the experimental and reference lines. The number of detected bands and the trends of intensity changes were similar. Arrangement of the individual samples on the genetic distance tree indicated the gradual adaptation of the analyzed communities to the operational conditions applied at the local WWTP. Adaptation proceeded in a similar manner in both lines, thus fusel oil addition at the applied concentration was considered to not be a strictly selective factor in relation to the activated sludge bacteria.

3.2.2. Diversity of *nirS* and *nirK* Gene Variants

Due to the significant phylogenetic diversity of microorganisms with the ability to carry out the denitrification process, their identification based on the sequence of the gene coding for 16S rRNA is limited [40]. Commonly, the *nirS* and *nirK* genes encoding alternative forms of nitrite reductase are applied for the analysis of denitrification bacteria groups, due to the fact that this enzymes catalyze the first step of the denitrification process where gaseous product occurs [41]. These genes show a relatively high genetic heterogeneity, which, combined with the availability of PCR primers with verified efficacy [27], enabled their widespread use for the biodiversity analysis of denitrifying communities in various natural ecosystems (e.g., soil [42] or marine sediments [43] as well as artificial ecosystems [44]). For this reason, the analysis of *nirS* and *nirK* genes libraries were applied in order to characterize and compare denitrifying bacteria population between reference and experimental line with the fusel oil addition (after adaptation period).

Of the 80 *nirS* clones analyzed, 69 DNA sequences were obtained which correctly encoded the cytochrome cd1 nitrite reductase amino acid chain. The acquired DNA sequences differed in length, ranging from 366 to 381 nucleotides. For the reference and reactor with fusel oil dosage, 34 and 35 positively verified clones were obtained, respectively. The general genetic distance between the obtained DNA sequences of the *nirS* gene fragment was 0.38. The intra-group distance values in the experimental and reference reactors were similar and reached 0.34 and 0.33 values respectively (Table 3).

Table 3. Genetic distance values, number of positively verified clones and number of unique sequences within libraries for the *nirS* gene.

Sample Origination	Positively Verified Clones Number	Unique Gene Variant	Intra-Group Distance	General Genetic Distance
Experimental reactor	35	32	0.33	0.34
Reference reactor	34	31	0.34	

The dendrogram illustrating the phylogenetic relationships between the analyzed *nirS* DNA sequences obtained from the samples collected from reactor fed with fusel oil (DNA sequences marked as OF) and the reference reactor (DNA sequences marked as KON) is presented in Figure 6. In the course of phylogenetic analysis, nine groups were distinguished. Three of them (i.e., group II, which included representatives of the genus *Halomonas*; group IV with sequences from *Cupriavidus*; and group VI that included representatives of *Herbaspirillum*) did not contained any of the experimental sequences. Group I, present on the phylogenetic tree in the form of two smaller branches, contained sequences similar to that of the representatives of the *Azoarcus tolulyticus* species (AY078272). In addition to the *Acidovorax* representatives, Group III contained three sequences from the experimental line and a single sequence isolated from the reference line. The three experimental DNA sequences were included together with the *Bradyrhizobium* representatives in the V group. Group VIII was formed from two sequences from the reference line and representatives of the *Paracoccus* genus. The sequence of OF31 (KP410866), from the reactor where the fusel oil were dosed, was found on a common branch together with representatives of *Alicycliphilus*. Group X was divided into two subgroups: XA composed of a single experimental sequence and marker sequences derived from the *Dechloromonas* genus and a subgroup of XB formed by a set of seven experimental DNA sequences along with variants of the *nirS* gene specific for the *Thauera* genus. The last group, XI, contained a set of DNA sequences showing the greatest uniqueness in relation to groups containing marker sequences. In addition, this group was not characterized by homogeneity, as evidenced by its division into two subgroups shown in the phylogenetic tree. Due to the different nucleotide composition with respect to the other analyzed sequences, the OF24 sequence (KP410874) was not included in any of the identified groups.

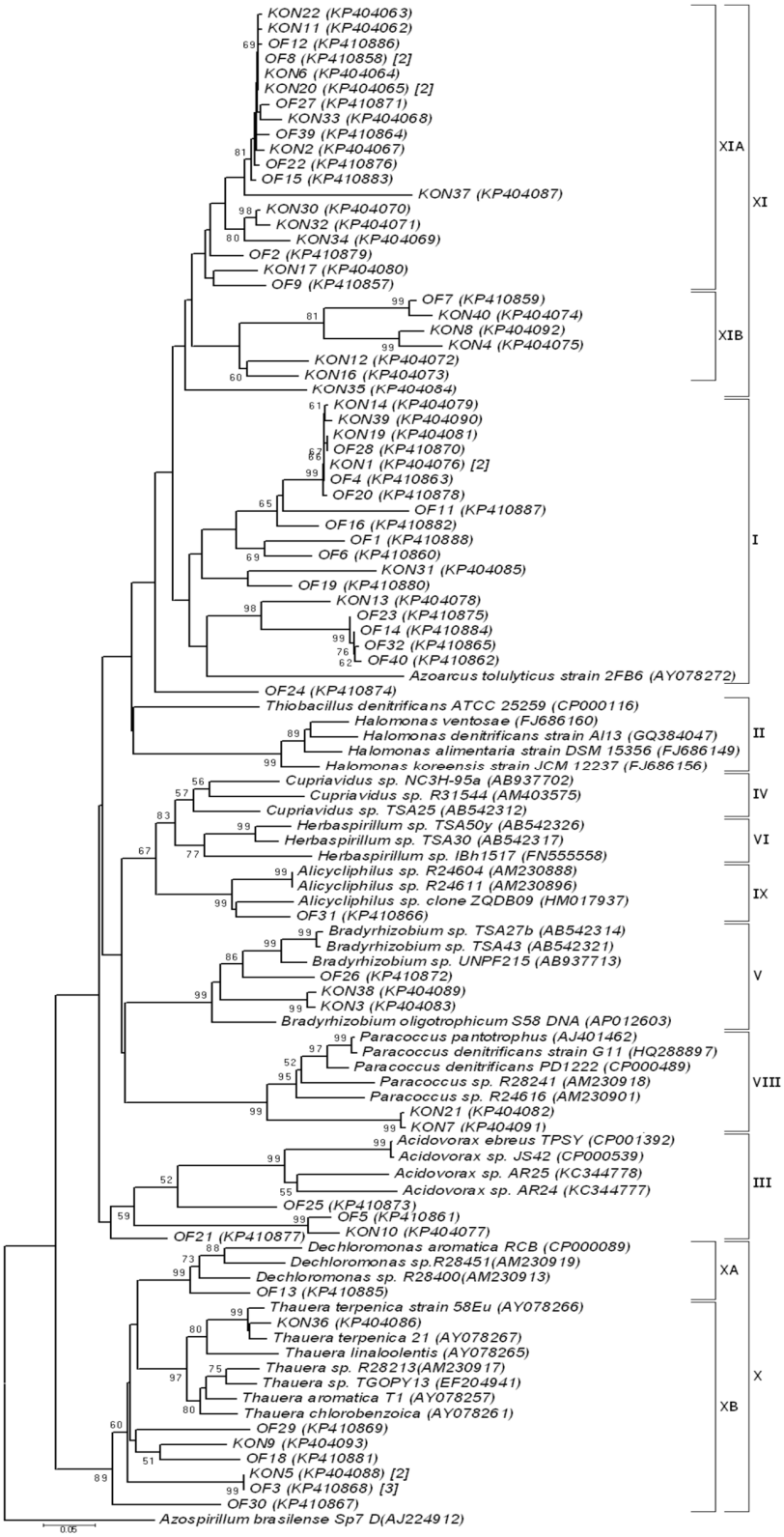

Figure 6. Phylogenetic analysis of nucleotide sequences of the *nirS* gene. The rooted phylogenetic tree was built on the basis of 102 sequences (63 test clones and 39 sequences obtained from the Gene Bank), using the algorithm of combining neighboring taxa. The figure shows self-sampling values above 50% for 500 repetitions. In parentheses () the accession numbers from the Gene Bank are shown, in square brackets [] the number of repetitions of the same sequence within the analyzed maple library.

After discarding DNA sequences that contained unreadable fluorograms and those that did not correctly encode the amino acid chain of *nirK* reductase, 34 sequences were obtained. They all had the same length, which was 439 nucleotides. In the case of samples taken from the experimental and reference lines, 15 and 19 positively verified clones were obtained, respectively, of which 15 and 16 contained unique sequences. The general genetic distance between the obtained sequences of the *nirK* gene fragment was 0.33. The intra-group distance values in the experimental and reference lines were the same and reached 0.33 (Table 4).

Table 4. Genetic distance values, number of positively verified clones and number of unique sequences within libraries for the *nirK* gene.

Sample Origin	Positively Verified Clones Number	Unique Gene Variant	Intra-Group Distance	General Genetic Distance
Experimental line	15	15	0.33	0.33
Reference line	19	16	0.33	

Figure 7 shows the results of phylogenetic analysis for *nirK* gene DNA sequences obtained from both analyzed lines. A set of 29 marker sequences was used to construct the dendrogram.

The obtained dendrogram has six clearly separated branches. Group I was divided into two subgroups, of which IA comprised a set of DNA sequences, from both the experimental and reference lines characterized by high homogeneity; and a subgroup IB, which included variants of the *nirK* gene derived from representatives of the genus *Nitrosomonas*. In addition, the unique sequence KON38 (KJ584691) was located at the branching point of the dendrogram for the IA and IB subgroups. Group II also had two branches. The first IIA focused the *nirK* gene sequences belonging to *Bradyrhizobium* representatives, while IIB included four closely related test sequences, from both reactors, and a single KON17 (KJ584684) showing a smaller degree of similarity to them. Group III was built only from a heterogeneous genetic set of DNA sequences. None of the test sequences were included in group IV, which contained the DNA sequences of the *nirK* gene derived from representatives of the genera *Rhizobium* and *Shinorhizobium*. There were no sequences related to representatives of the genus *Paracoccus*. Group VI was composed of the *nirK* sequences derived from the *Mesorhizobium*-related bacteria; it contained three unique test sequences. The marker sequences obtained from the microorganisms of the genera *Pseudomonas* and *Alcaligenes*, forming Group VII, were clearly distinguished from the other groups. Between Group VII and the sequence derived from *Starkey novella*, a single OF29 sequence was located (JK584671).

The DNA sequences of the *nirS* and *nirK* genes obtained in this study represented a wide range of phylogenetic groups of microorganisms. This fact is consistent with data presented in the literature, which emphasize the large diversity of denitrifying bacteria communities, which may include representatives of more than 50 bacterial genera belonging to the types *Proteobacteria*, *Firmicutes* and *Bacteroidetes* [16,45]. Analyses carried out in subsequent years and based on the use of molecular biology techniques, showed that the dominant share of β-proteobacteria belonged mainly to the genera *Acidovorax*, *Azoarcus*, *Curvibacter*, *Dechloromonas*, *Thauera* and *Zoogloea* [15,46,47]. The obtained results correspond to the trends presented above, especially in the context of the abundant occurrence of *nirS* gene variants derived from β-protebacteria, showing a high degree of similarity to the sequences derived from the genera *Acidovorax*, *Azoarcus* and *Thauera*. In research carried out with the use of the FISH technique [48], samples of activated sludge from 17 different wastewater treatment plants with separate denitrification zones were analyzed. The results showed that representatives of *Azoarcus* and *Thauera* may constitute between 20% and 49% of the general microbial population. It was concluded that the dominant share of the representatives of the discussed types in the activated sludge samples result from their metabolic versatility, which enables them to use substrates, such as volatile fatty acids,

ethanol and amino acids, as a carbon source. Literature data indicate that representatives of α-proteobacteria belonging to the genera *Paracoccus* and *Rhizobium* are also mentioned as a numerical component of denitrifying bacteria complexes in the activated sludge [15,49]. The presence of DNA sequences derived from microorganisms that are related to the above-mentioned species was confirmed mainly in the analysis of *nirK* gene libraries.

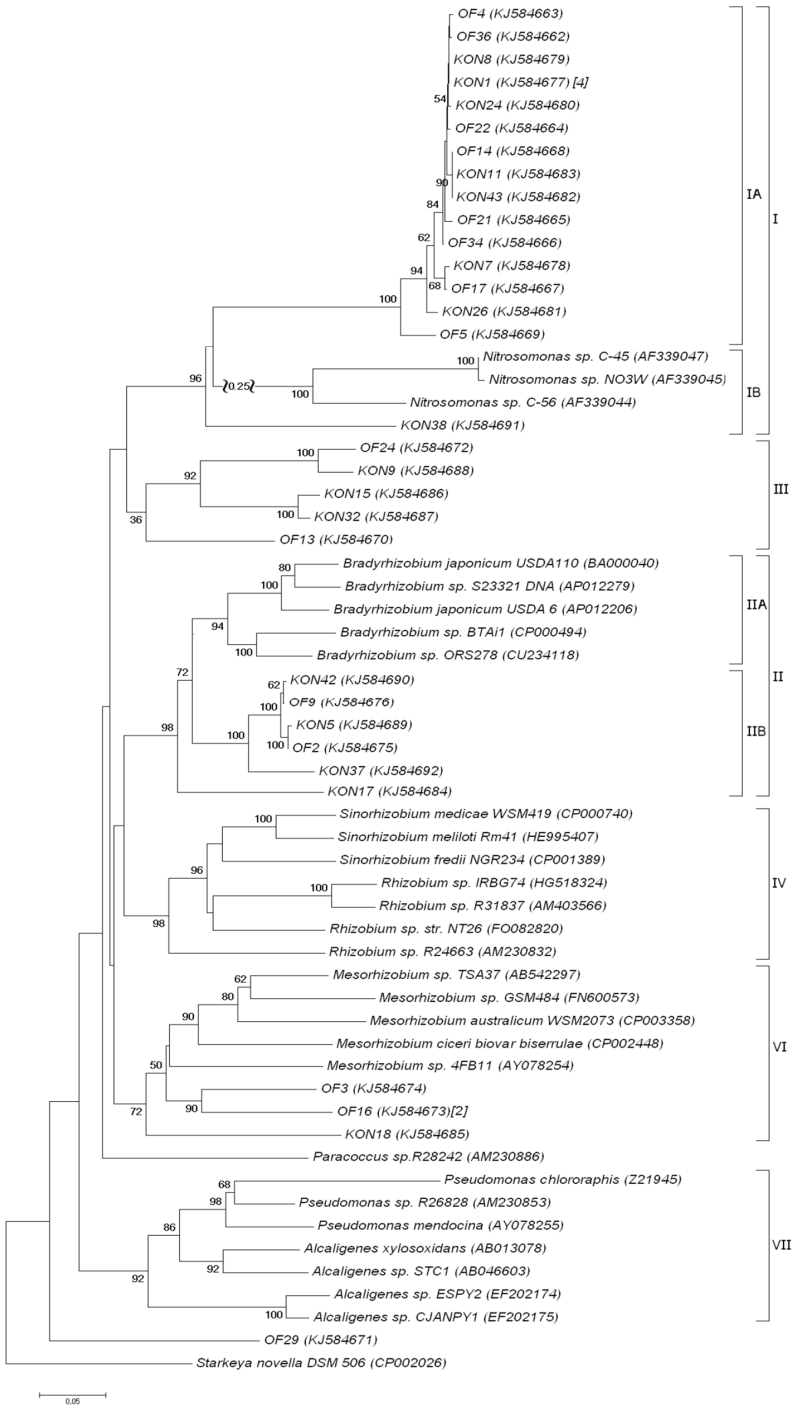

Figure 7. Phylogenetic analysis of the *nirK* gene sequence obtained from activated sludge samples from the technical scale. The rooted phylogenetic tree was built on the basis of 60 sequences (31 test clones and 29 sequences obtained from the Gene Bank), using the algorithm of combining neighboring taxa. The figure shows the bootstrap values above 50% for 500 repetitions. In parentheses () the accession numbers from the Gene Bank are shown, in square brackets [] the number of repetitions of the same sequence within the analyzed maple library.

Important information regarding the biodiversity of bacterial assemblages was provided by Heylen et al., [15] who analyzed the presence of *nirS* and *nirK* genes among 227 cultivated strains of denitrifying bacteria. Both genes were distributed mainly among the representatives of α- and β-proteobacteria and, to a lesser extent, in γ-proteobacteria. Additionally, the *nirK* gene was identified for representatives of the *Firmicutes* and *Bacteroidetes* and *Firmicutes* types. Phylogenetic analyses of the *nirS* gene sequences showed their significant convergence with the phylogenesis of the 16S rRNA gene at the family and species level, although some deviations were observed. The reverse situation was found for the sequence of the *nirK* gene, which showed no such convergence. The authors observed that most of the *nirK* sequences obtained from a specific taxonomic unit showed a greater degree of similarity with regard to the *nirK* gene variants derived from the same sample, rather than representatives of the same species isolated from different biocenoses. The obtained results suggest that DNA sequences encoding enzymes that are involved in denitrification processes are subject to horizontal gene transfer mechanisms, while the *nirK* gene is characterized by an increased frequency of exchange. This may be due to the fact that *nirK* genes, in contrast to *nirS* genes, occur mainly on plasmids, which can easily be exchanged between even unrelated microorganisms. Therefore, the *nirK* gene is of limited use in determining the taxonomic position of microorganisms. However, the authors emphasize that representatives of the genera *Rhizobium* and *Nitrosomonas* are an exception in this aspect.

In addition, in this study, the *nirK* gene libraries consisted of DNA sequences showing common phylogenesis with variants of the discussed gene derived from nitrifying bacteria of the genus *Nitrosomonas*. However, results of the experiments conducted by Beaumont et al. [50] indicated that *nirK* of nitrifying bacteria does not really participate in the denitrification process in the classical sense. The gene rather constitutes an element of protective system of the discussed group against the toxic effects of nitrite accumulation, which may occur as a result of partial denitrification or nitrification. Libraries for both analyzed genes also contained numerous variants derived from microorganisms that have not been characterized so far, and show a further degree of relatedness with regard to the known groups of denitrifiers. A significant proportion of sequences from unidentified microorganisms is commonly found in the analysis of microbial communities using molecular techniques [51]. This problem concerns both the previous and the newest generation of techniques, including metagenomics [52], and is mainly related to the lack of sufficiently rich databases. The solution to the above difficulties may be the dissemination of advanced cultivation techniques, such as co-cultivation of dependent bacterial groups or on the recreation of the conditions prevailing in a given ecosystem [53] and the use of complex bioinformatic tools that allow for obtaining entire bacterial genomes from data created as part of metagenomic analyses [54].

4. Conclusions

The presented study provided the first comprehensive insight into the overall stability of denitrifying bacterial communities during the implementation of fusel oil to enhance the denitrification process at a full-scale WWTP. After 98 days of adaptaion, with continuous introduction of the fusel oil to the experimental line at a dose of 45 cm^3/ per m^3, gradual enhancement of the denitrification process rate was achieved. In comparison with the reference line, where NURs remained at a stable level (1.4 ± 0.1 mg NO_3-N/g VSS·h) throughout the entire experimental period, the use of fusel oil approximately doubled NUR up to 2.7 mg NO_3-N/g VSS·h. Moreover, fusel oil contributed to the mitigation of the variability of NO_3-N concentrations in the effluent from the anoxic zone. The results of the 16S rRNA PCR-DGGE assay revealed a very similar composition of the bacterial community between the reference and experimental lines at the particular experimental stages. The applied doses of fusel oil did not reflect a stringent selective pressure on the bacterial community structure, but rather stimulated denitrifying bacteria activity.

Despite fusel oil addition, denitrifiers belonging to the genera *Acidovorax*, *Alcaligenes*, *Azoarcus*, *Paracoccus* and *Thauera* were predominant and similarly distributed between the clone libraries for biomasses from the experimental and reference lines. Analysis of the clone libraries reflected a greater diversity of the *nirS* gene variants in comparison with *nirK*. This suggests greater metabolic potential of the *nirS* denitrifiers in terms of variable external carbon source usage.

The practical application of fusel oil generates opportunities for WWTPs to meet effluent standards and reduce operational costs (e.g., increased biogas production can be achieved by redirecting the primary sludge to anaerobic digesters). Additional benefits of the study is the opportunity for the distillery industry to optimize waste management.

Author Contributions: Conceptualization, J.M., K.C. and S.C.; methodology, P.K. and S.C.; software, P.K.; validation, K.C., S.C. and J.M.; formal analysis, J.B.M. and P.K.; investigation, J.B.M. and P.K.; resources, J.B.M.; data curation, P.K.; writing—original draft preparation, P.K., K.C. and J.O.; writing—review and editing, P.K., K.C., S.C. and J.M.; visualization, P.K. and J.O.; supervision, K.C., J.M. and S.C.; project administration, K.C.; funding acquisition, J.M. All authors have read and agreed to the published version of the manuscript.

Funding: This study has been financially supported by the European Regional Development Fund within the framework of the Innovative Economy Operational Programme 2007–2013 under the project no. UDA-POIG.01.03.01-22-140/09-04.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The DNA sequences obtained in this study have been deposited in the Gene Bank NCBI (https://www.ncbi.nlm.nih.gov/genbank/) with the accession numbers ranging from KP662417 to KP662440 in terms of the *nirK* gene and from KP662360 to KP662386 for the *nirS* gene.

Acknowledgments: The participation of the Poznan WWTP staffs gratefully acknowledged. The authors would like to thank the reviewers for valuable suggestions and critical comments that allowed to improve the quality of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Regulation of the Minister of Maritime Economy and Inland Navigation of 12 July 2019 on Substances Particularly Harmful to the Aquatic Environment and the Conditions to Be Met When Discharging Sewage into Waters or Soil, as Well as When Discharging Rainwater or Meltwater into Waters or into Devices Aquatic. Available online: https://Isap.Sejm.Gov.Pl/Isap.Nsf/Download.Xsp/WDU20190001311/O/D20191311.Pdf (accessed on 6 August 2021).
2. Wilinska-Lisowska, A.; Ossowska, M.; Czerwionka, K. The Influence of Co-Fermentation of Agri-Food Waste with Primary Sludge on Biogas Production and Composition of the Liquid Fraction of Digestate. *Energies* **2021**, *14*, 1907. [CrossRef]
3. Urząd RegulacjiEnergetyki, Mapaodnawialnych Źródeł Energii [In Polish]. Available online: https://Www.Ure.Gov.Pl/Pl/Oze/Potencjal-Krajowy-Oze/8108,Instalacje-Odnawialnych-Zrodel-Energii-Stan-Na-31-Grudnia-2020-r.html (accessed on 6 August 2021).
4. Sharif Shourjeh, M.; Kowal, P.; Lu, X.; Xie, L.; Drewnowski, J. Development of Strategies for AOB and NOB Competition Supported by Mathematical Modeling in Terms of Successful Deammonification Implementation for Energy-Efficient WWTPs. *Processes* **2021**, *9*, 562. [CrossRef]
5. Makinia, J.; Czerwionka, K.; Oleszkiewicz, J.; Kulbat, E.; Fudala-Ksiazek, S. A Distillery By-Product as an External Carbon Source for Enhancing Denitrification in Mainstream and Sidestream Treatment Processes. *Water Sci. Technol.* **2011**, *64*, 2072–2079. [CrossRef]
6. Craft Spirits Market Size, Share & Trends Analysis Report by Distiller Size (Large, Medium, Small), by Product (Whiskey, Vodka, Gin, Rum, Brandy, Liqueur), by Region, and Segment Forecasts, 2018–2025 h. Available online: Https://Www.Grandviewresearch.Com/Industry-Analysis/Craft-Spirits-Market (accessed on 6 August 2021).
7. Kupczyk, A.; Mączyńska, J.; Redlarski, G.; Tucki, K.; Bączyk, A.; Rutkowski, D. Selected Aspects of Biofuels Market and the Electromobility Development in Poland: Current Trends and Forecasting Changes. *Appl. Sci.* **2019**, *9*, 254. [CrossRef]
8. Directive 2008/98/EC of The European Parliament and of the Council 2008. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex%3A32008L0098 (accessed on 6 August 2021).
9. Cherchi, C.; Onnis-Hayden, A.; El-Shawabkeh, I.; Gu, A.Z. Implication of Using Different Carbon Sources for Denitrification in Wastewater Treatments. *Water Environ. Res.* **2009**, *81*, 788–799. [CrossRef] [PubMed]

10. Wang, X.; Xia, K.; Yang, X.; Tang, C. Growth Strategy of Microbes on Mixed Carbon Sources. *Nat. Commun.* **2019**, *10*, 1279. [CrossRef]
11. Isaacs, S.H.; Henze, M.; Søeberg, H.; Kümmel, M. External Carbon Source Addition as a Means to Control an Activated Sludge Nutrient Removal Process. *Water Res.* **1994**, *28*, 511–520. [CrossRef]
12. Ginige, M.P.; Hugenholtz, P.; Daims, H.; Wagner, M.; Keller, J.; Blackall, L.L. Use of Stable-Isotope Probing, Full-Cycle RRNA Analysis, and Fluorescence in Situ Hybridization-Microautoradiography To Study a Methanol-Fed Denitrifying Microbial Community. *Appl. Environ. Microbiol.* **2004**, *70*, 588–596. [CrossRef]
13. Hwang, C.; Wu, W.-M.; Gentry, T.J.; Carley, J.; Carroll, S.L.; Schadt, C.; Watson, D.; Jardine, P.M.; Zhou, J.; Hickey, R.F.; et al. Changes in Bacterial Community Structure Correlate with Initial Operating Conditions of a Field-Scale Denitrifying Fluidized Bed Reactor. *Appl. Microbiol. Biotechnol.* **2006**, *71*, 748–760. [CrossRef]
14. Kumar, P.; Singh, A.; Sachan, A.; Sachan, S.G. Molecular Tools for Microbial Diversity Analysis. In *Wastewater Treatment*; Elsevier: Amsterdam, The Netherlands, 2021.
15. Heylen, K.; Gevers, D.; Vanparys, B.; Wittebolle, L.; Geets, J.; Boon, N.; de Vos, P. The Incidence of NirS and NirK and Their Genetic Heterogeneity in Cultivated Denitrifiers. *Environ. Microbiol.* **2006**, *8*, 2012–2021. [CrossRef]
16. Zumft, W.G. Cell Biology and Molecular Basis of Denitrification. *Microbiol. Mol. Biol. Rev. MMBR* **1997**, *61*, 533–616. [CrossRef]
17. Hallin, S.; Throbäck, I.N.; Dicksved, J.; Pell, M. Metabolic Profiles and Genetic Diversity of Denitrifying Communities in Activated Sludge after Addition of Methanol or Ethanol. *Appl. Environ. Microbiol.* **2006**, *72*, 5445–5452. [CrossRef] [PubMed]
18. Tang, J.; Wang, X.C.; Hu, Y.; Pu, Y.; Huang, J.; Hao Ngo, H.; Zeng, Y.; Li, Y. Nitrogen Removal Enhancement Using Lactic Acid Fermentation Products from Food Waste as External Carbon Sources: Performance and Microbial Communities. *Bioresour. Technol.* **2018**, *256*, 259–268. [CrossRef] [PubMed]
19. Tuszynska, A.; Kaszubowska, M.; Kowal, P.; Ciesielski, S.; Makinia, J. The Metabolic Activity of Denitrifying Microorganisms Accumulating Polyphosphate in Response to Addition of Fusel Oil. *Bioprocess Biosyst. Eng.* **2019**, *42*, 143–155. [CrossRef] [PubMed]
20. Czerwionka, K.; Luczkiewicz, A.; Majtacz, J.; Kowal, P.; Jankowska, K.; Ciesielski, S.; Pagilla, K.; Makinia, J. Acclimation of Denitrifying Activated Sludge to a Single vs. Complex External Carbon Source during a Start-up of Sequencing Batch Reactors Treating Ammonium-Rich Anaerobic Sludge Digester Liquors. *Biodegradation* **2014**, *25*, 881–892. [CrossRef]
21. Czerwionka, K.; Makinia, J.; Kaszubowska, M.; Majtacz, J.; Angowski, M. Distillery Wastes as External Carbon Sources for Denitrification in Municipal Wastewater Treatment Plants. *Water Sci. Technol.* **2012**, *65*, 1583–1590. [CrossRef]
22. Makinia, J.; Czerwionka, K.; Kaszubowska, M.; Majtacz, J. Distillery Fusel Oil as an Alternative Carbon Source for Denitrification—from Laboratory Experiments to Full-Scale Applications. *Water Sci. Technol.* **2014**, *69*, 1626–1633. [CrossRef]
23. Nübel, U.; Garcia-Pichel, F.; Muyzer, G. PCR Primers to Amplify 16S RRNA Genes from Cyanobacteria. *Appl. Environ. Microbiol.* **1997**, *63*, 3327–3332. [CrossRef]
24. Nei, M.; Li, W.H. Mathematical Model for Studying Genetic Variation in Terms of Restriction Endonucleases. *Proc. Natl. Acad. Sci. USA* **1979**, *76*, 5269–5273. [CrossRef]
25. Van Hannen, E.J.; Zwart, G.; van Agterveld, M.P.; Gons, H.J.; Ebert, J.; Laanbroek, H.J. Changes in Bacterial and Eukaryotic Community Structure after Mass Lysis of Filamentous Cyanobacteria Associated with Viruses. *Appl. Environ. Microbiol.* **1999**, *65*, 795–801. [CrossRef]
26. Shannon, C.E. A Mathematical Theory of Communication. *Bell Syst. Tech. J.* **1948**, *27*, 379–423. [CrossRef]
27. Throbäck, I.N.; Enwall, K.; Jarvis, Ã.; Hallin, S. Reassessing PCR Primers Targeting NirS, NirK and NosZ Genes for Community Surveys of Denitrifying Bacteria with DGGE. *FEMS Microbiol. Ecol.* **2004**, *49*, 401–417. [CrossRef]
28. Hallin, S.; Lindgren, P.-E. PCR Detection of Genes Encoding Nitrite Reductase in Denitrifying Bacteria. *Appl. Environ. Microbiol.* **1999**, *65*, 1652–1657. [CrossRef]
29. Tamura, K.; Stecher, G.; Peterson, D.; Filipski, A.; Kumar, S. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Mol. Biol. Evol.* **2013**, *30*, 2725–2729. [CrossRef]
30. Thompson, J.D.; Higgins, D.G.; Gibson, T.J. CLUSTAL W: Improving the Sensitivity of Progressive Multiple Sequence Alignment through Sequence Weighting, Position-Specific Gap Penalties and Weight Matrix Choice. *Nucleic Acids Res.* **1994**, *22*, 4673–4680. [CrossRef]
31. Altschul, S.F.; Gish, W.; Miller, W.; Myers, E.W.; Lipman, D.J. Basic Local Alignment Search Tool. *J. Mol. Biol.* **1990**, *215*, 403–410. [CrossRef]
32. Saitou, N.; Nei, M. The Neighbor-Joining Method: A New Method for Reconstructing Phylogenetic Trees. *Mol. Biol. Evol.* **1987**, *4*, 406–425. [CrossRef]
33. Kim, E.; Shin, S.G.; Jannat, M.A.H.; Tongco, J.V.; Hwang, S. Use of Food Waste-Recycling Wastewater as an Alternative Carbon Source for Denitrification Process: A Full-Scale Study. *Bioresour. Technol.* **2017**, *245*, 1016–1021. [CrossRef]
34. Her, J.-J.; Huang, J.-S. Influences of Carbon Source and C/N Ratio on Nitrate/Nitrite Denitrification and Carbon Breakthrough. *Bioresour. Technol.* **1995**, *54*, 45–51. [CrossRef]
35. Peng, Y.; Ma, Y.; Wang, S. Denitrification Potential Enhancement by Addition of External Carbon Sources in a Pre-Denitrification Process. *J. Environ. Sci.* **2007**, *19*. [CrossRef]
36. Queiroz, L.M.; Aun, M.V.; Morita, D.M.; Alem Sobrinho, P. Biological Nitrogen Removal over Nitritation/Denitritation Using Phenol as Carbon Source. *Braz. J. Chem. Eng.* **2011**, *28*, 197–207. [CrossRef]

37. Gong, L.; Huo, M.; Yang, Q.; Li, J.; Ma, B.; Zhu, R.; Wang, S.; Peng, Y. Performance of Heterotrophic Partial Denitrification under Feast-Famine Condition of Electron Donor: A Case Study Using Acetate as External Carbon Source. *Bioresour. Technol.* **2013**, *133*, 263–269. [CrossRef]
38. Zhang, Y.; Wang, X.C.; Cheng, Z.; Li, Y.; Tang, J. Effect of Fermentation Liquid from Food Waste as a Carbon Source for Enhancing Denitrification in Wastewater Treatment. *Chemosphere* **2016**, *144*, 689–696. [CrossRef]
39. Wang, Q.; Chen, Q.; Chen, J. Optimizing External Carbon Source Addition in Domestics Wastewater Treatment Based on Online Sensoring Data and a Numerical Model. *Water Sci. Technol.* **2017**, *75*, 2716–2725. [CrossRef] [PubMed]
40. Philippot, L.; Bru, D.; Saby, N.P.A.; Čuhel, J.; Arrouays, D.; Šimek, M.; Hallin, S. Spatial Patterns of Bacterial Taxa in Nature Reflect Ecological Traits of Deep Branches of the 16S RRNA Bacterial Tree. *Environ. Microbiol.* **2009**, *11*, 3096–3104. [CrossRef]
41. Braker, G.; Zhou, J.; Wu, L.; Devol, A.H.; Tiedje, J.M. Nitrite Reductase Genes (*NirK* and *NirS*) as Functional Markers To Investigate Diversity of Denitrifying Bacteria in Pacific Northwest Marine Sediment Communities. *Appl. Environ. Microbiol.* **2000**, *66*, 2096–2104. [CrossRef]
42. Herold, M.B.; Giles, M.E.; Alexander, C.J.; Baggs, E.M.; Daniell, T.J. Variable Response of NirK and NirS Containing Denitrifier Communities to Long-Term PH Manipulation and Cultivation. *FEMS Microbiol. Lett.* **2018**, *365*, fny035. [CrossRef]
43. Shi, R.; Xu, S.; Qi, Z.; Huang, H.; Liang, Q. Seasonal Patterns and Environmental Drivers of NirS- and NirK-Encoding Denitrifiers in Sediments of Daya Bay, China. *Oceanologia* **2019**, *61*, 308–320. [CrossRef]
44. Che, Y.; Liang, P.; Gong, T.; Cao, X.; Zhao, Y.; Yang, C.; Song, C. Elucidation of Major Contributors Involved in Nitrogen Removal and Transcription Level of Nitrogen-Cycling Genes in Activated Sludge from WWTPs. *Sci. Rep.* **2017**, *7*, srep44728. [CrossRef] [PubMed]
45. Philippot, L.; Hallin, S. Finding the Missing Link between Diversity and Activity Using Denitrifying Bacteria as a Model Functional Community. *Curr. Opin. Microbiol.* **2005**, *8*, 234–239. [CrossRef]
46. Juretschko, S.; Loy, A.; Lehner, A.; Wagner, M. The Microbial Community Composition of a Nitrifying-Denitrifying Activated Sludge from an Industrial Sewage Treatment Plant Analyzed by the Full-Cycle RRNA Approach. *Syst. Appl. Microbiol.* **2002**, *25*, 84–99. [CrossRef]
47. Hagman, M.; Nielsen, J.L.; Nielsen, P.H.; Jansen, J.L.C. Mixed Carbon Sources for Nitrate Reduction in Activated Sludge-Identification of Bacteria and Process Activity Studies. *Water Res.* **2008**, *42*, 1539–1546. [CrossRef]
48. Thomsen, T.R.; Kong, Y.; Nielsen, P.H. Ecophysiology of Abundant Denitrifying Bacteria in Activated Sludge. *FEMS Microbiol. Ecol.* **2007**, *60*, 370–382. [CrossRef]
49. Heylen, K.; Vanparys, B.; Gevers, D.; Wittebolle, L.; Boon, N.; de Vos, P. Nitric Oxide Reductase (NorB) Gene Sequence Analysis Reveals Discrepancies with Nitrite Reductase (Nir) Gene Phylogeny in Cultivated Denitrifiers. *Environ. Microbiol.* **2007**, *9*, 1072–1077. [CrossRef]
50. Beaumont, H.J.E.; Lens, S.I.; Westerhoff, H.V.; van Spanning, R.J.M. Novel *NirK* Cluster Genes in *Nitrosomonas Europaea* Are Required for NirK-Dependent Tolerance to Nitrite. *J. Bacteriol.* **2005**, *187*, 6849–6851. [CrossRef] [PubMed]
51. Baldrian, P. The Known and the Unknown in Soil Microbial Ecology. *FEMS Microbiol. Ecol.* **2019**, *95*, fiz005. [CrossRef]
52. Taş, N.; de Jong, A.E.; Li, Y.; Trubl, G.; Xue, Y.; Dove, N.C. Metagenomic Tools in Microbial Ecology Research. *Curr. Opin. Biotechnol.* **2021**, *67*, 184–191. [CrossRef]
53. Chaudhary, D.K.; Khulan, A.; Kim, J. Development of a Novel Cultivation Technique for Uncultured Soil Bacteria. *Sci. Rep.* **2019**, *9*, 6666. [CrossRef] [PubMed]
54. Guo, J.; Ni, B.-J.; Han, X.; Chen, X.; Bond, P.; Peng, Y.; Yuan, Z. Data on Metagenomic Profiles of Activated Sludge from a Full-Scale Wastewater Treatment Plant. *Data Brief* **2017**, *15*, 833–839. [CrossRef] [PubMed]