Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biotechnology and Food Science

Elin Håberg

The impact of washing procedures on nitrifying capacity and bacterial community composition of Leca biofilms in nitrifying biofilters at VEAS

Master's thesis in Biotechnology
Supervisor: Ingrid Bakke
Co-supervisor: Olav Vadstein
May 2021

NTNU
Norwegian University of
Science and Technology

Elin Håberg

The impact of washing procedures on nitrifying capacity and bacterial community composition of Leca biofilms in nitrifying biofilters at VEAS

Master's thesis in Biotechnology
Supervisor: Ingrid Bakke
Co-supervisor: Olav Vadstein
May 2021

Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biotechnology and Food Science

NTNU
Norwegian University of
Science and Technology

Abstract

At Vestfjorden Avløpsselskap (VEAS) nitrification is performed in a fixed film process where lightweight expanded clay aggregate (Leca) is used as carrier material. Washing of these nitrifying biofilters are necessary to remove excess biomass and to prevent fast growing heterotrophic organisms to out-compete slow growing autotrophic nitrifiers, due to high concentrations of organic material in incoming wastewater. VEAS has experienced problems with loss of filter mass from the nitrifying biofilters during the process of washing. This has led to reduced nitrifying capacity and problems in different parts of the plant. To reduce the loss of filter mass VEAS has changed the original washing procedure. This new washing procedure has previously been found to reduce the loss of Leca biofilm carriers, but the effect on nitrification efficiency and bacterial community composition has not been investigated. This study set out to investigate the differences in bacterial community composition and activity of nitrifying and denitrifying bacteria in Leca biofilms exposed to the new and original washing procedure. The new washing procedure was implemented in a process hall with four nitrifying biofilters, while the original washing procedure was conducted in an equivalent process hall with four nitrifying biofilters at VEAS. In this study Leca biofilm samples from these nitrifying filters were used to compare the new and original washing procedure. This was done by small scale batch experiments to estimate nitrification capacity in Leca biofilm, analysis of the process of nitrification and denitrification in biofilm through monitoring of gas kinetics and characterisation of Leca biofilm communities with the use of Illumina sequencing of 16S rDNA amplicons.

The bacterial community composition in Leca biofilm at VEAS was dominated by Betaproteobacteria, Alphaproteobacteria, Nitrospira and Actinobacteria. The nitrifying bacterial community had the highest relative abundance of nitrite oxidizing bacteria (NOB) and was dominated by one Nitrospira-zOTU, which by phylogenetic analysis showed evolutionary relationship to *Nitrospira Salsa*. The relative abundance of ammonium oxidising bacteria was low in comparison with the abundance of NOB. This study has shown that the composition and diversity of Leca biofilm changed over time and that the washing procedures influenced the community structure. It appeared that the new washing procedure led to a biofilm community which was more susceptible to changes in ammonium concentration in incoming wastewater than Leca biofilm exposed to the original washing procedure. The immediate effect of washing on bacterial community composition was generally low, but there was a notable effect when the number of repetitions with draining followed by scouring with water and air in the filter was increased with the new washing procedure. This led to a decrease in relative abundance of nitrifiers, and small-scale batch experiments revealed reduced nitrification rate in these Leca biofilm communities. There was also observed an effect of the depth in nitrifying biofilters on Leca biofilm exposed to both washing procedures. The bacterial diversity was higher in samples from the top of the nitrifying filter compared with samples from the bottom of the filter.

Monitoring of gas kinetics in batch experiments with Leca biofilm samples from VEAS revealed that the process of denitrification was present in biofilm exposed to both washing procedures under aerobic conditions. The experiment further indicated that the process of denitrification could be driven by the high oxygen consumption from nitrification in the Leca biofilm. Leca biofilm exposed to the original washing procedure had a considerable increase in heterotrophic activity between two washing events and in addition a distinct increase in O_2 consumption and production of N_2O and N_2. Thus, in contrast to the results based on 16S rDNA amplicon sequencing, the robotized incubation experiment indicated that the original washing procedure led to a more unfavourable biofilm community in regards of nitrification.

Sammendrag

Hos Vestfjorden Avløpsselskap (VEAS) blir nitrifikasjon utført i ein "fast film prosess" der lett ekspandert leireaggregat (LECA) blir brukt som berermateriale for biofilm. Vask av desse nitrifiserande biofiltra er nødvendig for å fjerne overflødig biomasse, og for å forhindre at raskt veksande heterotrofiske organismar ut-konkurrera dei sakte voksande autotrofe nitrifiserande organismane på grunn av høge konsentrasjonar av organisk materiale i innkommande avløpsvatn. VEAS har opplevd utfordringar med tap av filtermasse ved vasking av dei nitrifiserande biofiltera. Tapet av Leca har ført til redusert nitrifikasjonskapasitet og problem i ulike delar i anlegget. For å reduserer tapet av filtermasse, har VEAS endra den originale vaskeprosedyra. Den nye vaskeprosedyra har tidlegare vist å minimera tapet av Leca biofilm berarar, men effekten på nitrifikasjonseffektiviteten og bakterielt samfunn er ikkje vorte undersøkt. Målet med denne studien var å undersøke forskjellane i samansetning av det bakterielle samfunnet og aktivitet av nitrifiserande og denitrifiserande bakteriar i Leca biofilm utsett for den nye og originale vaskeprosedyren. Den nye vasken vart implementert i ein prosesshall med fire nitrifiserande biofilter, medan den originale vaskeprosedyra var beheldt i ein ekvivalent prosesshall med fire nitrifiserande biofilter på VEAS. I denne studien vart Leca biofilm-prøvar frå desse nitrifiserande filtera brukt til å samanlikna den nye og original vaskeprosedyra. Dette vart gjennomført med små-skala batchforsøk for å estimerer nitrifikasjonskapasitet i Leca biofilm, analysere prosessen av nitrifikasjon og denitrifikasjon i biofilmen gjennom overvåking av gasskinetikk og karakterisering av Leca biofilm samfunn med Illumina sekvensering av 16S rDNA amplikoner.

Det bakterielle samfunnet i Leca biofilm ved VEAS var dominert av Beta-proteobakteriar, Alpha-proteobakteriar, Nitrospira og Aktinobakteriar. Det nitrifiserande samfunnet hadde høgast forkomst av nitritt oksiderande bakteriar (NOB) og var dominert av ein Nitrospira-zOTU, som ved fylogenetiske analysar viste evolusjonært forhold til *Nitrospira Salsa*. Den relative førekomsten av ammonium oksiderande var låg i forhold til førekomsten av NOB. I denne studien vart det vist at samansetninga og diversiteten i Leca biofilmen endra seg med tid og at vaskeprosedyrene påverka strukturen av samfunna i biofilmen. Det såg ut til at den nye vaskeprosedyra førte til eit biofilmsamfunn som var meir utsett for endringar i ammonium konsentrasjon i innkomande avløpsvatn enn biofilmen utsett for den originale vaskeprosedyra. Den umiddelbare effekten av vask på samansetning av det bakterielle samfunnet var generelt låg, men der var ein observerbar effekt når talet på repetisjonar av hurtigdrenering følgd av skuring med luft og vatn vart auka med den nye vaskeprosedyra. Dette førte til ein reduksjon i relativ førekomst av nitrifiserande bakteriar og små-skala batchforsøk viste at nitrifikasjonsraten i Leca biofilm samfunna vart redusert. Det var òg observert ein effekt av djupn i dei nitrifiserande biofiltera på Leca biofilm utsett for begge vaskeprosedyrene. Diversiteten i bakteriesamfunna var høgare i prøvar frå toppen av det nitrifiserande filteret samanlikna med prøvar frå botnen av filteret.

Overvaking av gasskinetikk i robotisert inkubasjonsforsøk med Leca biofilmprøver frå VEAS viste at prosessen med denitrifikasjon var til stades i biofilm utsett for begge vaskeprosedyrene under aerobe forhold. Forsøket indikerte vidare at prosessen med denitrifikasjon vart driven av det høge oksygenkonsumet frå nitrifikasjon i Leca biofilmen. Leca biofilmen utsett for den originale vaskeprosedyra hadde ein betydeleg auke i heterotrof aktivitet før vask og i O_2 konsum og produksjon av N_2O og N_2. Dermed, i motsetning til resultata basert på 16S rDNA amplikon-sekvenseringa, indikerte det robotiserte inkubasjonseksperimentet at den originale vaskeprosedyra førte til eit meir ugunstig biofilmsamfunn med omsyn til nitrifisering.

Preface

This master thesis was conducted in collaboration with Vestfjorden Avløpsselskap. I would like to thank Anne-Kari Marsteng for help and guidance at the plant and helping me understand the different treatment processes conducted at VEAS. I also want to thank Lars Bakken and Kjell Rune Jonassen for all the help and guidance, which made it possible to conduct the robotized incubation experiment at NMBU. I would also like to give a special thanks to my supervisor Ingrid Bakke; I am thankful for all the help and support.

I also want to thank to my friends who have made these five years in Trondheim memorable. Finally, I am grateful for my family and my boyfriend Haavard!

Trondheim, May 2021

Elin Håberg

Table of Contents

List of Figures

List of Tables

Abbreviations

Abbreviation	Explanation
HRT	Hydraulic retention time
EPS	Extracellular polymeric substance
AOB	Ammonia oxidating bacteria
AOA	Ammonia oxidating archaea
NOB	Nitrite oxidising bacteria
(C/N)	Organic carbon/inorganic nitrogen
PERMANOVA	Permutational multivariate analysis of variance
ANOVA	Analysis of variance
SED	Sedimentation tank
PHA	Process hall
NIT	Nitrifying filter
DEN	Denitrifying filter
PCR	Polymerase chain reaction
zOTU	Zero radius operational taxonomic unit
RDP	Ribosomal database project
PCoA	Principal Coordinate Analysis
E.g	For example
i.e	That is
BW	Before wash
AW	After wash
V	Velocity
V_{amo}	Velocity of ammonia oxidation

1 Introduction

1.1 Wastewater treatment

Municipal wastewater treatment is fundamental for protection and reduction of risk to human health and the natural environment against pollution. Wastewater contains multiple contaminants as organic matter, heavy metals, microorganisms, and excess nutrients as nitrogen and phosphorus [1, 2]. These contaminants must be reduced or removed to improve water quality, before wastewater can be returned to nature, incorporated to water cycle, or reused with minimum environmental impact.

The process of improving water quality of wastewater can consist of physical, biological, or chemical treatment, or a combination of these processes [3]. Physical treatment can include sedimentation, screening, aeration, and filtration, which use physical phenomena to improve wastewater quality [2]. Chemical treatment involves processes with chemical compounds which initiate reactions for removal of pollutants. Commonly used chemical processes involve chlorination, pH neutralisers, coagulants and flocculants [4]. Biological methods use microorganisms, mainly bacteria, to break down contaminants through biochemical processes. The availability of oxygen characterises the biological treatment as either an aerobic or anaerobic method [5].

There are numerous devices used in municipal wastewater treatment and these commonly combine physical, chemical, and biological methods as mentioned above. These methods can generally be grouped in six methods, preliminary treatment, primary treatment, secondary treatment, disinfection, sludge treatment and tertiary treatment [2, 6]. Disinfection can be applied at all stages in the treatment of wastewater and is therefore considered as a method by itself. Preliminary treatment removes or degrade large suspended and floating solids, and excess amount of grease. Commonly used devices are screens, grinders, and cutters. Primary treatment includes sedimentation by reduction of velocity of wastewater, where most of the settleable solids are separated or removed. Chemicals can be used with the primary sedimentation for the removal of colloidal solids [2].

Secondary treatment is primarily dependent on the biochemical decomposition of organic solids to inorganic or stable organic solids by aerobic organisms. Commonly used devices for secondary treatment are trickling filters, activated sludge, intermittent sand filters and stabilization ponds [2]. From secondary and primary treatment, it is necessary with the removal of solids referred to as sludge. Sludge treatment includes the removal of water to reduce the volume and also the decomposition of organic solids to more stable compounds or mineral solids before the sludge is disposed [7].

Tertiary treatment removes contaminants that secondary treatment was not able to remove. Stronger and more advance treatment systems are used to get a higher purification of wastewater effluent. Tertiary treatments are used as an extension of conventional secondary biological treatment to stabilize oxygen demands, remove excess nutrients and/or toxic materials [8]. When only secondary treatment is used a large amount of the nitrogen present in wastewater as ammonia is discharged with the effluent.

Nitrogen is a common ingredient in all fertilizers and excess amounts of nitrogen could lead to algae bloom which could be toxic to aquatic life and aesthetically unpleasing [2].

1.2 Suspended or attached growth in nitrifying bioreactors

As previously stated, excess input of nitrogen to the environment could lead to eutrophication in aquatic-ecosystems. Nitrogen can contaminate air and soil, which again can lead to misbalance of biodiversity in different ecosystems [9, 10]. In addition, the toxic nature of reactive nitrogen compounds (ammonia, nitrite, and nitrate) and nitrogen containing compounds can create serious threats to living organisms [11, 12]. Anthropogenic activity, such as discharge of industrial and domestic sewage, animal manure from farming industry and fertilizers in agriculture, has led to an over enrichment of nitrogen to the environment and as a result nitrogen is a key pollutant in wastewater[10, 13, 14]. Consequently, the need of an efficient and proper process of nitrification for the removal of nitrogen is crucial.

The biological process of nitrogen removal in wastewater treatment can be done by applying; suspended or attached microbial growth. In suspended growth systems microorganisms move freely, which provides direct contact between the bacterial cells and the liquid [15]. An example of suspended growth system is the activated sludge process, where microbes grow in flocs in the aqueous medium. These flocs are suspended in the aqueous phase where they multiply and make larger aggregates before they sediment at the bottom of the reactor as activated sludge. The settled sludge is continuously recycled to continue the oxidation of organic compounds and removal of excess nutrients in the reactor [16].

In an attached growth system, the bacterial cells grow in a biofilm attached to the surface of a solid support medium. This process is also called fixed film process. The solid medium can be rocks, sand or plastic dependent on the type of fixed film bioreactor [15]. These attached growth systems could be fixed-film trickling filter, rotating biological contactor or submerged attached growth bioreactors [2]. The biofilm in fixed film processes, consist of cells in an aggregated matrix of extracellular polymeric substances. The biofilm creates a protective environment against stress and unfavourable conditions [15] as extreme pH, high salinity, poor nutrient concentrations, high pressure and ultraviolet radiation [17]. Biofilms are found to enhance the bacterial community resistance against stress by a 1000 times [15].

There are several advantages with the attached growth system. In comparison with suspended growth systems the risk of washing out bacteria and loosing biomass is low [18]. This reduces the hydraulic retention time (HRT) of the rector and it is therefore possible to obtain a more effective process with less areal requirements. On the other hand, the low HRT makes the fixed film biofilters more subjected to sudden changes in pollution load and operating conditions. The short wastewater transit time leads to a decreased buffer capacity and the process must be monitored to prevent clogging [19].

1.3 Biofilm community in nitrifying bioreactors

The biological removal of nitrogen includes the aerobic process of nitrification and the anaerobic process of denitrification [20]. Nitrification is the microbial conversion of ammonia to nitrite, and nitrite to nitrate. Denitrification is the microbial reduction of nitrogen containing compounds (NO_3, NO_2, NO, N_2O) to elemental nitrogen gas (N_2) [21].

The process of ammonia oxidation in nitrifying biofilters are carried out by chemolithoautotrophic microbes; Ammonia-oxidising bacteria (AOB) consisting of the genera *Nitrosomonas*, *Nitrosospira* and *Nitrococcus,* and ammonia-oxidising archaea (AOA) named *Nitrososphaerea* and *Nitrosopumilus,* which oxidate ammonia to nitrite [22]. AOB were considered the only contributor to the oxidation of ammonia until AOA were discovered in Crenarchaeota in 2004 [23]. The conversion of nitrite to nitrate is carried out by nitrite-oxidising bacteria (NOB) and the main genera are *Nitrospira*, *Nitrobacter and Nitrotoga*. These different groups of AOB and NOB coexist in microbial ecosystems as nitrifying biofilms, where they cross feed each other [24].

In addition to AOB and NOB the process of nitrification is also found to be done by anammox bacteria, where ammonium and nitrite are oxidised under anaerobic conditions to nitrogen gas. The order Brocadiales (Planctomycetes) host the anammox bacteria [24]. Complete ammonia oxidisers called comammox microbes, can complete the whole process of nitrification. One single organism catalysis both ammonia oxidation and nitrite oxidation. Comammox microbes belongs to the genus *Nitrospira* and directly oxidise ammonia to nitrate. This process is more energetically favourable for the bacteria than the process of individual oxidation [24].

The process of denitrification is carried out by a large group of diverse bacteria. Several of these bacteria are heterotrophs, some utilize one-carbon compounds, while other grow autotrophically on hydrogen gas and carbon dioxide, or reduced sulphur compounds. The process of denitrification is the stepwise reduction of NO_3 to N_2 gas and most denitrifying organisms possess the reductase necessary for complete reduction [25]. The intermediate gaseous nitrogen oxide compounds include nitrite (NO_2), nitric oxide (NO) and nitrous oxide (N_2O). Some heterotrophic nitrifiers are oxygen-tolerant denitrifies and can reduce nitrate to nitrite if oxygen is present. This process is called aerobic denitrification [24]. However, the process of denitrification is generally favoured by low oxygen concentrations, high concentrations of organic carbon and sufficient concentrations of nitrate [26].

1.4 The processes of nitrogen removal in wastewater treatment

Biological nitrogen removal can be accomplished in a series of different approaches. The conventional method used in wastewater treatment plants are a direct line with the aerobic oxidation of organic material and nitrification as the first step, followed by the anaerobic process of denitrification. This process requires additional added organic matter, often methanol, as electron donor for the denitrification [27]. The addition of external organic matter and aeration leads to high energy costs. The process of nitrogen removal can also be done by anaerobic denitrification as the first step followed by aerobic organic and nitrogen oxidation. The oxidised nitrogen (NO_3 and NO_2) is recycled back to an anoxic reactor for the reduction to elemental nitrogen gas by the process of denitrification. This method eliminates the addition of external organic matter and is therefore more cost effective [20].

Due to high energy costs in the conventional methods for nitrogen removal mentioned in the section above, new methods have emerged. An example is the SHARON process (Single reactor system for High Ammonia Removal Over Nitrite), where ammonia is only oxidised to nitrite by AOB, i.e. partially nitrification, and then nitrite can be reduced to nitrogen gas by denitrification (Figure 1.1.) [28]. This process is beneficial compared with the conventional methods mentioned above, due to the reduction of aeration with partial nitrification. There is also a lower oxygen demand in the subsequent denitrification since only nitrite is reduced to nitrogen gas [29]. The SHARON process can be coupled to the process of anammox in two separate reactors called, SHARON-ANAMOX process (Figure 1.1.). The SHARON process can also be coupled to the process of anammox in one single reactor and is then called the CANON process (Completely Autotrophic Nitrogen removal Over Nitrite) [29].

In the CANON process, half of the ammonia is first oxidized to NO_2 by partial nitrification. This step is mainly controlled by the level of dissolved oxygen to prevent further oxidation of nitrite to nitrate by NOB [28]. Anammox bacteria use nitrite as electron acceptor under anaerobic conditions to oxidise the rest of the ammonia (e-donor) and initially converting both ammonia and nitrite to nitrogen gas [30]. This process further reduces the cost from the SHARON coupled denitrification process, due to the elimination of external added methanol for the reduction of nitrite to nitrogen gas (used to sustain denitrification) [20].

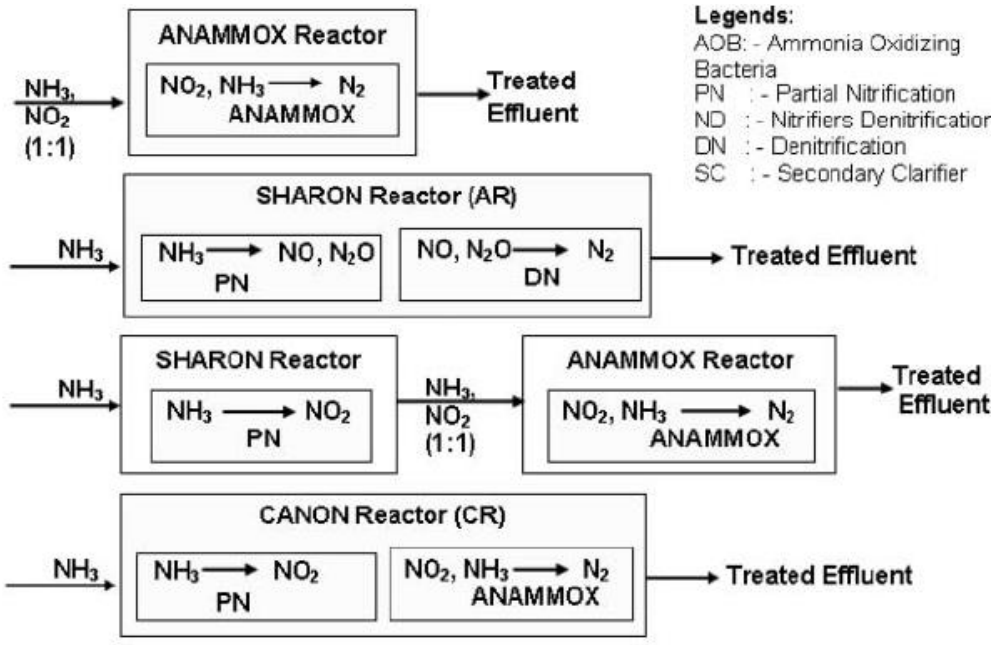


Figure 1.1. Schematic figure of biological nitrogen removal processes. The microbial principles used in anammox reactor, SHARON reactor, SHARON coupled anammox reactor and canon reactor. Figure from article by Bagchi et al. [28], "Critical Reviews in Environmental Science and Technology Autotrophic Ammonia Removal Processe".

1.5 Abiotic and biotic factors affecting biofilm composition

The biofilm on carrier material in nitrifying filters at wastewater treatment plants host a great diversity of organisms in addition to the autotrophic nitrifiers. Many of the additional microorganisms which constitutes the complex diversity are heterotrophs. Presumably some of the heterotrophic bacteria in the complex biofilm community are denitrifies which could potentially reduce significant fractions of nitrate in anoxic or oxygen limited parts of the biofilm [31].

AOB and NOB has a slow-growing nature in contrast to the heterotrophic bacteria [22]. As a consequence, heterotrophs can outcompete nitrifiers for space, oxygen and important nutrients [32]. If space and oxygen is limited a high organic carbon/inorganic nitrogen (C/N) ratio can lead to inhibition of the process of nitrification due to the competition from the heterotrophic bacteria [32, 33]. In addition, several heterotrophic bacteria can compete with nitrifiers in absence of organic matter, due to the release of organic metabolites and lysate from dead cells. These components can be used as carbon and energy source for the heterotrophic bacteria [24].

Thickness of the biofilm is believed to influence the structure of the community composition and the function of the biofilm. Thickness is linked to external factors as flow [34], nutrient [35], age of biofilm [36], C/N ratio[37] and temperature [38]. Selection is one of the major drivers for community composition in biofilms and is driven by abiotic and biotic conditions. The community composition is also driven by the ecological process of drift, which relies on birth and death events [39]. Biofilm thickness can lead to a concentration gradient due to limited diffusion though the matrix. This can create structured microenvironments with different electron donors and acceptors at different parts of the biofilm. The gradient of electron-donors and –acceptors are important in the process of selection in the biofilm communities and different populations can establish at different parts in the matrix [39]. It is found that AOB occupy outer layers near the oxygenated water and NOB in the deeper layers of the biofilm. Anammox and denitrifying bacteria can establish in deeper layers of the biofilm which could potentially be anoxic [39, 40].

Suarez et al. [39] made a mathematical model for one-dimensional biofilms which predicted that biofilm thickness of 50 µm could be fully oxygenated and in some scenarios, have anoxic regions. Biofilms of 400 µm contained a completely anoxic region in the deepest part for all scenarios in the predicted model [39]. These results indicated that there could be higher diversity in thicker biofilms, due to a steeper redox gradient. Thin biofilms without anoxic zones could inhibit the growth of obligate anaerobe bacteria like anammox. As a consequence, one could expect that the richness of the microbial community composition would be higher in thicker biofilms, due to the possibility of the establishment of aerobic and anaerobic populations [39].

In addition to stratification due to the redox potential, substrate gradients formed by biological activity could influence the pattern of microbial community composition. Other biotic factors are competition, quorum sensing, and predation which can influence and alter biofilm communities [39, 41]. Changes in environmental conditions as temperature and availability of nutrients can create differences in microbial community composition over time [42].

1.6 Vestfjorden Avløpsselskap

Vestfjorden Avløpsselskap (VEAS) is Norway's largest wastewater treatment plant and treats wastewater from more than 835 000 citizens. The plant has a capacity of 11 000 litres per second, which is equivalent to 347 million m^3 of wastewater in one year. The treatment processes at VEAS can be divided in three main steps: pre-treatment and chemical precipitation, sludge treatment, and tertiary treatment with biological removal of contaminants (Figure 1.2.) [43].

The different steps of pre-treatment involves mechanical screens for the removal of large solids from the incoming wastewater, before sand and grit are removed. The chemical step involves a two-point dosing of ferric and aluminium chloride for the aggregation of small flocs and removal of phosphorus. For the formation of larger flocs for precipitation, an anionic poly acryl-amide polymer is added to create higher density for sedimentation. The step of sedimentation removes suspended solids, total organic carbon, total Kjeldahl Nitrogen and approximately 33-43% of nitrite and nitrate [44]. In this step the suspended particles settle in a sedimentation tank and the solid now referred to as sludge is further treated. The sludge is first thickened before it is anaerobically digested under mesophilic conditions for biogas production. The treated sludge is further dewatered and sanitized [43].

After sedimentation the pre-treated wastewater is distributed from the sedimentation tank and into four aerated parallel stationary nitrifying biofilters. Wastewater from nitrifying filters are further entering the anaerobic denitrification filters for the final removal of nitrogen from the wastewater before the treated water is going into Oslofjorden. At VEAS the plant is divided in eight process halls and in each process hall there is four nitrifying and four denitrifying filters, in total 64 biofilters [43].

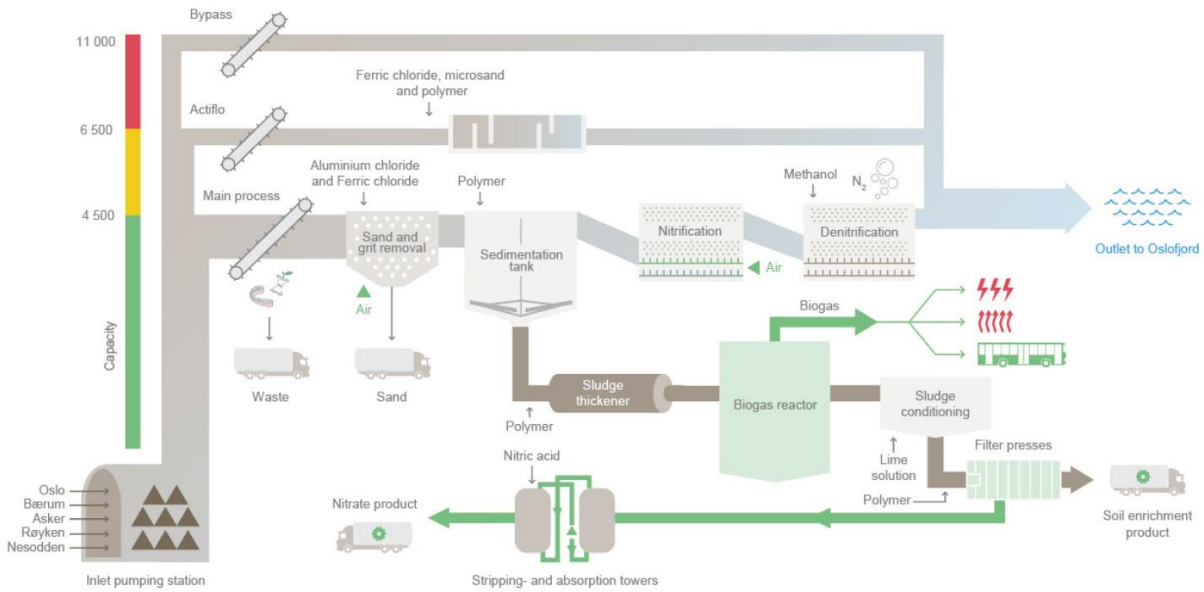


Figure 1.2. Schematic presentation of Vestfjorden Avløpsselskap treatment plant processes. The main treatment processes at VEAS is divided in three main steps: pre-treatment and chemical precipitation, tertiary treatment with biological removal of contaminants, and sludge treatment. Figure reprinted from catalogue, "From sewage treatment plant to biorefinery", by VEAS.

1.6.1 Nitrifying biofilters at VEAS

The process of nitrification at VEAS is conducted as a fixed film process in biological reactors for aerobic water treatment (BioFOR) filters (Figure 1.3.). Each nitrifying filter consist of a net base area of 87 m^2, with an approximately level of 4 meters of light expanded clay aggregates (Leca) which function as biofilm carriers. Leca are 3-5 mm in diameter and has a high density (1.24 gmL^{-1}) compared to normal Leca. Wastewater is introduced through the bottom of the granular media filter bed and the average residence time is 18 minutes [44].

Air for the aerobic reaction is provided through an up flow of process air co-current with the wastewater. Treated wastewater is leaving the reactor at the top. The average load of ammonia to the nitrifying biofilters are approximately 200 kg per day per filter, but with large variations in concentration. In average approximately 90% of the incoming ammonia is oxidised. In the VEAS treatment process there is no aerobe biological step for the removal of organic material before the process of nitrification. This leads to high concentrations of dissolved organic carbon entering the nitrifying filters. In the nitrifying biofilters approximately 50% of total organic carbon is removed, where 30% is removed by heterotrophic respiration and 20% by assimilation/adsorption [44].

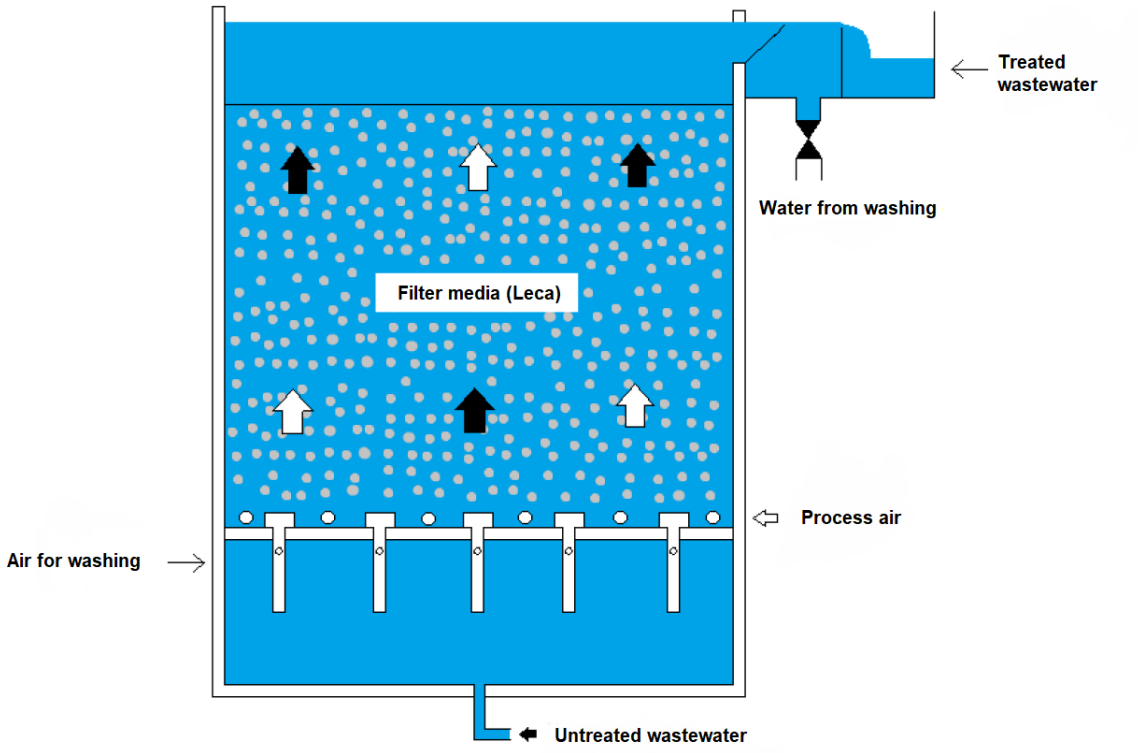


Figure 1.3. Cross section of nitrifying biofilter with Leca carriers at Vestfjorden Avløpsselskap. Untreated wastewater is introduced through the bottom of the filter and treated wastewater is leaving the reactor at the top. The blue colour indicate wastewater, grey dots indicate Leca carriers and arrows shows the direction of water and air introduced in the nitrifying reactor.

1.6.2 Washing procedures in nitrifying biofilters at VEAS

The nitrifying filters at VEAS are regularly washed with approximately ten to sixteen hours between each washing procedure. The washing procedure is regulated by head loss of filter, a pre-set time interval or manually initiated by signal from operator. Sufficient pressure to reach head loss of nitrifying filters are seldom reached and the interval between washing is normally regulated by the time interval. For the process of washing, high-rate aeration combined with high-rate water is introduced up flow from the bottom of the filter to initiate a scour effect.

During operation, biofilm will grow on the Leca carriers and produce an increasing matrix between two washing events. Over time, excess biomass and filtered particles will accumulate and can potentially lead to clogging of the biofilter. Clogging will reduce filtration and the efficiency of removal of contaminants. In addition, the nitrifying bacteria attached to the filter media must be maintained during the washing procedure, while heterotrophic bacteria should be removed. Thickness of the biofilm should be regulated, to minimise anoxic zones and to prevent out-competition of nitrifying autotrophs by heterotrophic organisms. Therefore, the procedure of washing is important to regulate the performance of nitrification.

The effect of washing and different washing procedures on biofilter performance and bacterial community composition is not well studied even though most plants conduct washing of biofilter as a daily routine. The over enrichment of nitrogen to the environment due to anthropogenic activity has made nitrogen to a key pollutant in wastewater. An appropriate washing routine for successful operation of a nitrifying biofilter could potentially increase the rates of nitrification and reduce the emission of toxic nitrogen compounds to the environment.

1.7 Aim of study and objectives

VEAS has experienced problems with loss of filter mass from the nitrifying biofilters due to the original washing procedure that they have conducted. This has led to reduced nitrifying capacity and problems in different parts of the plant. To reduce the loss of filter mass VEAS has changed the washing procedure in four parallel nitrifying filters in process hall seven (PHA7). The main objective of this study was to assess the effect of the new washing procedure compared to the original washing procedure on the nitrification efficiency and bacterial community composition at VEAS. The original washing procedure was maintained in four nitrifying filters in process hall eight (PHA8) equivalent to the once exposed to the new washing procedure in PHA7. The new washing procedure had previously been found to reduce the loss of Leca biofilm carriers, but the effect on nitrification efficiency and bacterial community composition had not been examined. More specifically the objectives of this study were to:

- Characterize the Leca biofilm bacterial communities from nitrifying filters in PHA7 (new washing procedure) and PHA8 (original washing procedure).
    - To assess temporal variability in bacterial community composition in Leca biofilm sampled from PHA7 and PHA8.
    - To examine the immediate effect of the washing procedures on bacterial Leca biofilm communities.
    - To investigate the bacterial community compositions at different depths in the nitrifying biofilters.
- Examine the activity of the bacterial community in Leca biofilm from nitrifying filters in PHA7 and PHA8 exposed to the new and original washing procedure, respectively.
    - Assess the immediate effects of the washing procedures on the nitrification efficiency in Leca biofilm communities.
    - Investigate the process of nitrification in Leca biofilm.
    - Examine the potential for the process of denitrification under aerobic conditions in Leca biofilms.
    - Elucidate the autotrophic and heterotrophic communities in the Leca biofilm communities.

2 Method

To investigate the impact of the new and original washing procedure on the process of nitrification in Leca biofilm at VEAS, different experiments and analysis were conducted. This was done by small-scale batch experiments to estimate nitrification capacity in Leca biofilm, analysis of microbial activities through monitoring of gas kinetics in Leca biofilm and Illumina sequencing of 16S rDNA amplicon library to characterise the biofilm communities.

2.1 Testing different washing procedures of nitrifying biofilters at VEAS

As previously stated, VEAS has experienced trouble with the loss of Leca from the nitrifying biofilters due to the washing procedure which they have conducted. This has led to reduced nitrifying capacity and problems in different parts of the plant. To solve the problem with loss of Leca from the nitrifying filters a new washing procedure has been tested at VEAS.

For comparison of the original and the new washing procedure, the new washing procedure was implemented in four parallel nitrifying filters in process hall seven (PHA7) and the original washing procedure was maintained in four parallel filters in process hall eight (PHA8) (Figure 2.1.). Wastewater entering the nitrifying biofilters in PHA7 and PHA8 are assumed to be similar since wastewater is entering from the same sand filter. In addition, there is a pipe for levelling wastewater between sedimentation pool seven and eight.

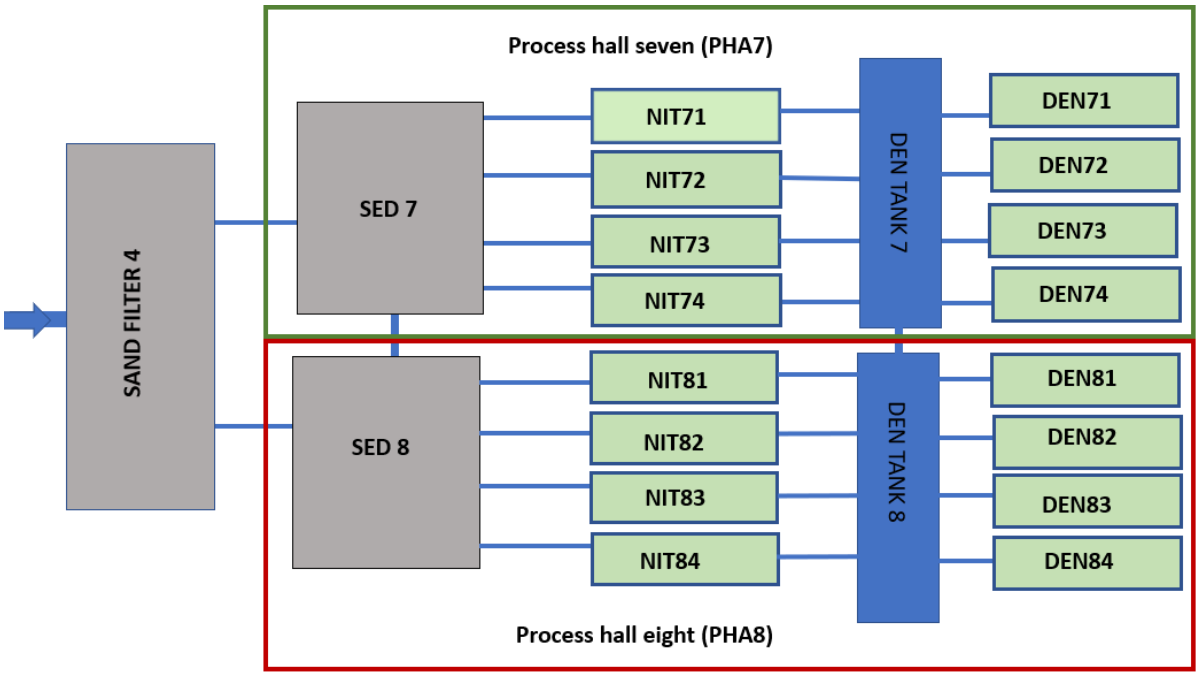


Figure 2.1. Overview of water treatment in process hall seven and eight at VEAS. Water is entering through sand filter 4 before it is distributed between sedimentation tank seven (SED 7) and eight (SED 8) in process hall seven (PHA7) and eight (PHA8), respectively. Water is further distributed between four nitrifying filters in PHA7, (NIT71, NIT72, NIT73, NIT74) and four nitrifying filters in PHA8 (NIT81, NIT82, NIT83, NIT84). Treated water is collected in a denitrification tank (DEN TANK 7 and DEN TANK 8) before it is pumped into four parallel denitrification filters (DEN) in each process hall. Blue lines indicate pipeline for distribution of wastewater.

2.1.1 Description of the original and new washing procedure of nitrifying filters at VEAS

As described in the section above the new washing procedure was conducted in nitrifying filters in PHA7 and the original washing procedure was conducted in the nitrifying filters in PHA8. Figure 2.2. shows a detailed cross section of a nitrifying bioreactor with direction and flow of water and air used for washing. Table 2.1. shows the different steps of the washing procedure for the original wash from step D1 to D10.

In the original washing procedure of nitrifying biofilters at VEAS, the valve for untreated water entering the filter through the bottom of the reactor is closed and filtration is stopped (D1). The filter is then drained approximately one meter above the top of the filter bed (D2). An air blanket is made underneath the double bottom of the reactor with compressed air for washing before the air is released into the nitrifying filter (D3). Compressed air is then pushed through the compact Leca where accumulated particles create a suspension (D4). Water for washing is then introduced up flow in the nitrifying filter to further expand the filter bed and carry the suspended particles and biomaterial through the Leca (D5-D6). Outlet valve for used water and particles from washing is then opened at the top of the filter and water for washing, excess biomaterial and particles are leaving at the top of the reactor and are sent to inlet pumping station and recycled within the plant. See Figure 1.2. for sketch of main wastewater treatment processes at VEAS.

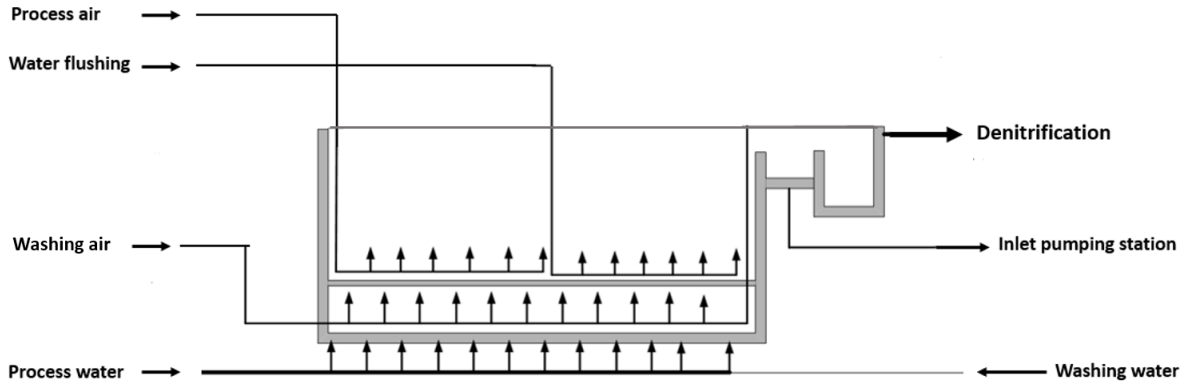


Figure 2.2. Cross section of nitrifying bioreactor at VEAS with direction and flow of water and air used for operational processes. Direction and flow of water and air are marked with arrows.

Water and air are still on when water is flowing over the edge of the reactor, this creates high turbulence and Leca is leaving the reactor with the water used for washing (D6). This Leca is transported to inlet pumping station and is further transported around in the plant causing troubles for machines and different wastewater treatment processes. For the finishing steps of the washing procedure the air blanket is removed (D7), which means that the air is turned off. Water is added in the step called "cleaning/purification" (D8) before the filter has a one-minute resting period (D9). In the last step, the filter is washed with process water (D10) before washing water is turned off and wastewater and process air is turned on again.

Table 2.1. Overview of the different steps in the original washing procedure of nitrifying biofilters at VEAS. Each step of the washing procedure is given in minutes (min) from start to stop. Steps are numbered from D1-D10.

	Steps	Time (min)
D1	Stop filtration	-
D2	Draining of filter	8
D3	Make air blanket	1
D4	Air on	3
D5	Air + water, low	4
D6	Air + water, normal	12
D7	Remove air blanket	1
D8	Cleaning/purification	2
D9	Resting period	1
D10	Finish cleaning/purification	2

The new washing procedure implemented in the nitrifying filters in PHA7 to reduce the loss of filter mass is described in Table 2.2. In this new washing procedure, the air for washing is stopped before the water reaches the top of the reactor to reduce turbulence and give Leca time to settle before water flows over the edge of the reactor (Figure 2.2.). This is done by removing step D5 and reducing the time for step D6 from 12 to 3.5 minutes. Step D2 to D6 can then be repeated, which consist of draining of the filter followed by scouring with water and air. The standard procedure at VEAS is to run the new washing procedure with three repetitions of step D2 to D6. In this study the nitrification filters in PHA7 were also exposed to the new washing procedure with one and five repetitions of step D2-D6 from mid-June to the start of July. This was done to investigate if the duration of the washing procedure and repeated cycles of draining and scouring could affect nitrification capacity and bacterial community composition. Step D1 to D4 and step D7 to D10 in the new washing procedure is identical to the original washing procedure.

Table 2.2. Overview of the different steps in the new washing procedure of the nitrifying biofilters at VEAS. Each step of the washing procedure is given in minutes (min) from start to stop. Steps are numbered from D1-D10.

	Sequence	Time (min)	
D1	Stop filtration	-	
D2	Draining of filter	8	
D3	Make air blanket	1	
D4	Air on	3	
D6	Air + water, normal	3.5	
D2	Draining of filter	8	Repeated 1-5 times
D3	Make air blanket	1	
D4	Air on	3	
D6	Air + water, normal	3.5	
D7	Remove air blanket	1	
D8	Cleaning/purification	2	
D9	Resting period	1	
D10	Finish cleaning/purification	2	

2.2 Small-scale batch experiments to assess nitrification capacity

Small-scale batch experiments were performed to assess the immediate effect of different washing procedures on the nitrifying capacity of the Leca biofilms from nitrifying filters at VEAS. Leca sampled for these experiments were exposed to the new and original washing procedure and were sampled from the nitrification filters directly prior to and after the process of washing. From mid-June to the start of July the new washing procedure was tested with one, three and five repetitions of step D2-D6 in PHA7 (Table 2.2.).

2.2.1 Experimental set-up and analysis of batch experiment

Batch experiments were conducted in a beaker (1.5 litre) with one litre of synthetic medium (Appendix A). Experimental temperature was 13 °C and was regulated by CORIO C Heating immersion circulator (JULABO) with added cooling elements. Circulation was generated by a magnetic stirrer and aeration was provided through an air stone (0.5 mL/sek). Galvanic dissolved oxygen sensor StirrOx®G (WTW) was used to monitor the oxygen concentration in the media during the experiments to prevent inhibition of nitrification due to oxygen limitations. A filter with sieves was inserted over the magnet and air stone to provide an aerobic environment for Leca which was placed on the top of the filter. This was also done to mimic the aeration used in full scale nitrifying filters at VEAS.

It was conducted a total of 13 experiments with Leca sampled from nitrifying filters directly prior to washing and 13 experiments directly after the procedure of washing. See Table 2.3. for detailed information of sampling of Leca biofilm carriers used in this experiment. Leca was sampled from the top of the filter bed in the nitrifying filters before it was firmly dried in a paper towel and transported to the lab in an open plastic bag. Leca was weighed prior to each experiment for calculation of nitrate production per 100-gram Leca, before it was added to the top of the sieved filter in the beaker.

Table 2.3. Detailed information on sampling of Leca biofilm carriers used in small scale batch experiments at VEAS and bacterial community analysis by Illumina sequencing to investigate the immediate effect of the washing procedures. Leca samples were sampled from the nitrifying filter (NIT) exposed to the original washing procedure (original wash) in process hall eight (PHA8) and the new washing procedure (New wash) with one (one rep), three (three rep) and five repetitions (five rep) of step D2-D6 of the washing procedure from process hall seven (PHA7). Samples were taken directly prior and after the washing procedures.

Date	Type wash	Filter	Process hall
10.06.2020	Original wash	NIT82	PHA8
26.06.2020	Original wash	NIT81	PHA8
29.06.2020	Original wash	NIT83	PHA8
17.06.2020	New wash - One rep	NIT71	PHA7
24.06.2020	New wash - One rep	NIT71	PHA7
18.06.2020	New wash - One rep	NIT71	PHA7
19.06.2020	New wash - One rep	NIT71	PHA7
12.06.2020	New wash - Three rep	NIT72	PHA7
25.06.2020	New wash - Three rep	NIT73	PHA7
30.06.2020	New wash - Three rep	NIT71	PHA7
01.07.2020	New wash - Five rep	NIT71	PHA7
02.07.2020	New wash - Five rep	NIT74	PHA7
03.07.2020	New wash - Five rep	NIT74	PHA7

All experiments were done in a period of 120 minutes with sampling and analysis of ammonia, nitrate, and nitrite every 30 minutes. Concentrations of total ammonia-nitrogen (TAN; i.e. $NH4^+$; $NH4^+$–N), nitrate-nitrogen ($NO3^-$; $NO3^-$–N) and nitrite-nitrogen (NO_2^-; NO_2^-–N) were determined spectrophotometrically on Hach DR 2800™ Portable Spectrophotometer (Hach-Lange). Standard cuvette tests (Hach-Lange) were used for each individual parameter. Nitrate-nitrogen (NO_3-N) concentrations were measured using LCK 339 cuvette test at 345 nm with a range of 1-60 mg/L. Ammonium-nitrogen ($NH4^+$-N) was measured by LCK 303 at 694 nm with a range of range 2.0-47.0 mg/L. Nitrite-nitrogen was measured by LCK 342 at 515 nm with a range of 2-20 mg/L.

2.3 Robotized incubation system monitoring gas kinetics in Leca biofilm

To further investigate the process of nitrification efficiency and in addition the potential of denitrification and the activity of microbial community in Leca biofilms exposed to the new and original washing procedure, a lab scaled robotized incubation system was used. Microbial community cultures in the Leca biofilms were monitored over time (~68 hours) in an automated incubating system located at the Norwegian University of Science and technology (NMBU).

2.3.1 Experimental setup of robotized incubation system

For this experiment it was made vials of 120 mL of glass which was compatible with the robot. Leca (two grams) was placed in netting hoses inside these vials with 50 mL media. Media was used as substrate for biofilm communities and was composed of sterile filtrated wastewater (0.22 µm, Sterivex) and two milli molar (mM) KNO_3 and two mM NH_4Cl.

After the addition of Leca carriers and media, the vials were sealed with rubber septa and aluminium caps. The air retained in the vials were then removed and replaced with helium prior to the experiment. This was done by five repeated cycles with evacuation and helium filling (180 seconds vacuum, 30 seconds helium). The overpressure in each vial were removed by a piston free syringe filled with ethanol to avoid contamination. Then 20 mL pure O_2 was added to each vial at the start of the experiment. After 25 hours from start, additionally two mM NH_4Cl was added to investigate if ammonia could be substrate limiting to the process of nitrification in the Leca biofilm. Temperature in vials were regulated by a water bath and experimental temperature was set to 20 °C with magnetic stirring (850 rpm).

All Leca biofilm samples were taken at the top of the filter bed. Leca biofilm exposed to the new washing procedure were sampled from the top of nitrifying filter in NIT74 before wash and in NIT73 after wash. The new washing procedure was conducted with three repetitions of step D2-D6 (Table 2.2). Samples from the original washing procedure in PHA8 was sampled in NIT83 before wash and in NIT84 after the process of washing. See Appendix H for detailed information of sampling. It was made five replicate vials for each treatment before and after wash for the new washing procedure, before and after wash for the original washing procedure, and five vials for experimental control. In total 25 vials. The experimental control was made with autoclaved and distilled water as media, and with thorough washed and scrubbed Leca (two grams) rinsed with distilled water. The

experimental control was used to detect the amount of nitrogen emitted from the system. Three vials with gas standards for O_2, N_2, N_2O, NO and CO_2 were used for calibration of gasses.

An autosampler took samples from headspace above media in each vial and sampled gas went through an injector of a gas chromatograph to analyse O_2, N_2, N_2O and CO_2 (Figure 2.3). The peristaltic pump of the autosampler was then reversed and sampled gas was replaced by helium after sampling. For detailed description of the system, see Molstad et al. [45]. The Excel spreadsheet, KINCALC, made by Lars Bakken was used for calculating gas kinetics for the experiment. Samples for analyses of NO_3 and NO_2 (10 µL) were taken manually from the liquid media in two replicates for each treatment. Samples were analysed by a reaction with NaI in acetic acid(~10 mg NaI mL-1 acetic acid) for measurements of nitrite and VCl_3 in 1 molar HCl (0.8g VCl3 til 100 mL 1 M HCl) for measurements of nitrate and nitrite. Both reactions produced NO-gas, which was measured by a NO-analyser.

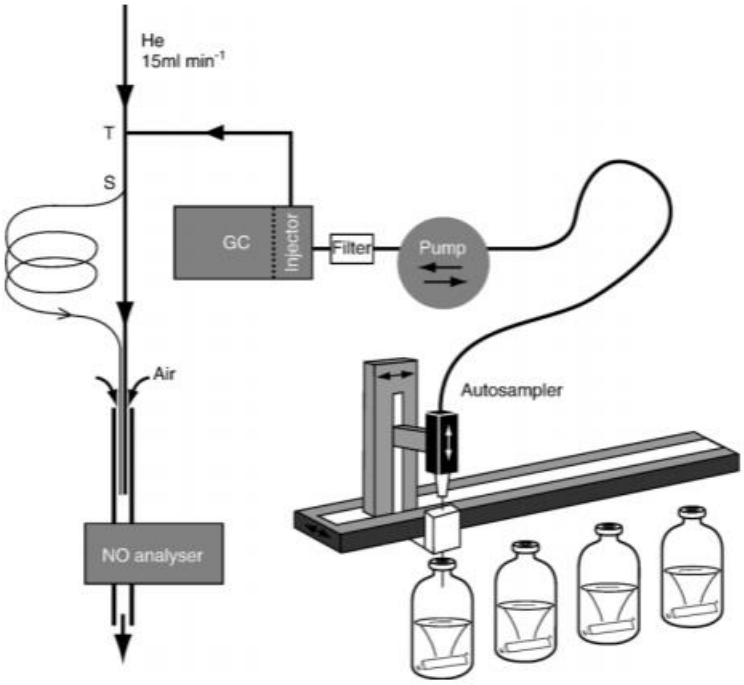


Figure 2.3. Sketch of sampling and gas analysis in the robotized incubation system monitoring gas kinetics at NMBU with Leca biofilm samples from VEAS. See section 2.3. for detailed description. Figure from article, "Robotized incubation system for monitoring gases (O_2, NO, N_2O N_2) in denitrifying cultures" , by Molstad et.al [46].

2.4 Characterization of Leca biofilm communities by Illumina sequencing of 16S rDNA amplicons

The bacterial biofilm community composition was investigated by Illumina sequencing of 16S rDNA amplicons. This was done to assess the immediate effect of biofilm communities exposed to the new and original washing procedure, temporal variations, variations between different nitrifying filters, and the effect of variations at different depths in the biofilter.

2.4.1 Sampling of Leca biofilm carriers for Illumina sequencing

Leca carriers were collected from the top of four nitrifying filters in PHA7 (NIT71, NIT72, NIT73, NIT74) exposed to the new washing procedure, and from four nitrifying filters in PHA8 (NIT81, NIT82, NIT83, NIT84) exposed to the original washing procedure. Samples were collected in April, May, June, August, and September to elucidate temporal variations and the effect of washing over time. In addition, Leca samples were collected from each batch experiment (Table 2.3.) for community analysis to investigate the short-term effect of washing. Samples were taken directly prior and after the process of washing. All the samples mentioned above were sampled from the top of the nitrifying filter with a kitchen strainer attached to a wooden shaft (three meter). For detailed information on sampling date, time and filters for all samples analysed by Illumina sequencing see Appendix B.

Leca samples were in addition collected at different depths in the nitrifying filters to examine if the bacterial composition in Leca biofilm could differ at different depths. Detailed information of filter, date and depths are given in Table 2.4. The nitrifying filters are filled with approximately four meters of Leca carriers and samples were taken at four different depths; From the top of the filter bed in nitrifying filter, from zero to minus two meters down in the filter (upper mid), from minus two meters to minus four meters down in the filter (lower mid) and the bottom of the filter (bottom). These samples were taken with a metal pole (eight meter) equipped with an integrated sampling box at the end which could be opened and closed at different depths.

Table 2.4. Overview of nitrifying filters, depth and dates for Leca biofilm samples used in analysis of the effect of depth on bacterial community. Depths are named accordingly: Top of the filter bed in nitrifying filter (top), from zero to minus two meters down in the filter (upper mid), from minus two meters to minus four meters down in the filter (lower mid) and the bottom of the filter (bottom)

Filter	Depth	Date
NIT73	Top	16.09
NIT73	Top	20.08
NIT73	Upper mid	16.09
NIT73	Upper mid	26.08
NIT73	Lower mid	26.08
NIT73	Lower mid	16.09
NIT73	Bottom	26.08
NIT74	Top	16.09
NIT74	Upper mid	26.08
NIT74	Lower mid	16.09
NIT74	Bottom	16.09
NIT81	Top	23.06
NIT81	Upper mid	23.06
NIT81	Lower mid	23.06
NIT81	Bottom	23.06

All Leca samples were carefully dried in a paper towel before storage in small zip lock plastic bags. Leca samples were stored in fridge at -20 °C before DNA extraction.

2.4.2 DNA extraction

Total DNA was extracted from the Leca biofilm samples using PowerSoil® DNA Isolation Kit (MO BIO) as described by the manufacturers' protocol (Appendix D). Leca samples were crushed in a ceramic mortar prior to DNA extraction. DNA extracts were stored in -20°C. A negative control, where DNA-free water was used as input was included in all rounds of DNA extraction to detect potential contaminants during the process of extraction. Concentration and purity of extracted DNA was analysed on NanoDrop™ 2000/2000c Spectrophotometer (Thermo Scientific™) prior to PCR amplification.

2.4.3 PCR amplification of 16S rDNA

PCR was used for amplification of one region of the bacterial 16S rRNA-gene which includes the variable regions V3 and V4 from the total DNA extracts. This was done by using primer Ill 338F (5'-cctacgggwggcagcag-3') and Ill 805R (5'-gactacnvgggtatctaakcc-3') (Sigma-Aldrich) with attached Illumina sequence adapters. Each PCR reaction contained a final concentration of 0.3 mM of each primer (Sigma-Aldrich), 1 µL template (1/50 dilution of the total DNA extract), 200 µM dNTP, 0.5 mM MgCl2, 0.02 $U\mu L^{-1}$ Phusion Hot Start DNA polymerase, 1x Phusion buffer HF from Thermo Scientific™. Reactions were runed on T100™ Thermal Cycler (BIO-RAD) with denaturation at 98°C for 1 min followed by 35 cycles of following steps; 98°C for 15 sek, 53°C for 20 sek, 72°C for 20 sek, and at the end an elongation step at 72°C for 5 min. In addition to the kit-blank from the DNA-

extraction, it was included a negative non-template and one positive control for all runs of PCR.

2.4.4 Verification of PCR amplification of 16S rDNA

Agarose gel electrophorese was conducted to evaluate quality and quantity of PCR products. It was made a 1% agarose gel (1%) with 1 gram Lonza™ SeaKem™ LE Agarose (Thermo Scientific™) per 100 mL 1 x Tris-acetate-EDTA (TAE) buffer (Appendix C). The solution was heated until all agarose was dissolved. Solution was cooled down to ~65C before 5 µL GelRed® (Qiagen) per 100 mL agarose gel was added. Then 5 µL PCR product was mixed with 1 µL 6x DNA loading dye (Thermo Scientific™) before the samples were loaded to the wells. To determine approximately size of DNA fragments 6 µL GeneRuler™ 1 kb Plus DNA Ladder (Thermo Scientific™) was used. Agarose gel electrophoresis was runed for one hour with Voltage 110, current 220 mA, power 20 w. Pictures of agarose gel were taken under UV light in G:box (Syngene) with GeneSnap (Syngene) to confirm PCR amplification of 16S rDNA.

2.4.5 Amplicon library preparation

For preparation of the amplicon library for Illumina sequencing, PCR products were purified and normalised using SequalPrep™ Normalization plate (96) Kit (Invitrogen™) following manufactures protocol (Appendix E). Multiplexing of the normalized PCR products were done prior to pooling of the samples. The samples were indexed with individual sequence barcodes using set A and set B, TG Nextera® XT Index kit v2 (Illumina), since sample amount exceeded 96 samples. PCR reactions were runed in T100™ Thermal Cycler (BIO-RAD) with denaturation at 98°C for 2 min followed by 11 cycles of following steps; 98°C for 15 sek, 50°C for 20 sek, 72°C for 20 sek, and at the end an elongation step of 72°C for 5 min followed by cooling in 4°C for 1 min. Each PCR reaction had a final reaction volume of 25 µL containing 5.0 µL 5xPhusion buffer HF, 0.5 µL dNTP (10 mM), 0.2 µL Phusion Hot start DNA polymerase from Thermo Scientific™ and 2.5 µL of each indexing primer (TG Nextera® XT Index kit v2, Illumina) and 2.5 µL template for each reaction. To evaluate the indexing PCR, PCR product were run on agarose gel electrophorese as described above in section 2.4.4.

After verification of the Indexed PCR products, a second normalisation and purification were done with SequalPrep™ Normalization plate (Invitrogen™) following manufactures protocol (Appendix E). After this step all samples were pooled into one tube prior to up-concentration using Amicon® Ultra-0.5 centrifugal Filter Device (Merck Millipore) following manufacturer's protocol (Appendix F). Up-concentration of pooled samples were repeated two times following same procedure as described above. The concentrated and pooled sample was sent to the Norwegian Sequencing Centre (NCS) for sequencing on an Illumina MiSeq run (Illumina) with V3 reagents (Illumina).

2.4.6 Processing of DNA Sequence reads for bacterial community analysis

Sequence data was stored as fastq.gz file and processed using the USEARCH pipeline (version 11; https://www.drive5.com/usearch/). The command fastq_mergepairs was used to merge forward and reverse reads by aligning, remove primer sequences (demultiplexing) and filter out reads shorter than 390 base pairs. Maximum numbers of mismatches in the overlapping/merged regions were set to 20. Merged reads were further quality filtered and converted to fasta file with associated sample labels with the command fastq_filter (Error threshold was set to 1). Sequences were further sorted by size and singleton reads were removed (fastx_uniques_sortbysize).

A zero radius Operational Taxonomic Unit (zOTU) table was made by denoising with the aim to identify all biological sequences by the unoise3 command. The zOTUs with a lower abundance than eight in all samples were removed, as recommended in the USEARCH documentation. Taxonomy was assigned to zOTUs using the command Sintax with Ribosomal Database Project (RDP) 16 s rRNA training set v18, and a confidence threshold of 0.8. The RDP Classifier [47] was used for subsequent classification of zOTU6 with the RDP 16s rRNA training set v18 as a reference dataset, and a confidence threshold of 0.80. For the identification of zOTUs representing nitrifiers, MiDAS 3 16S rRNA reference data set based on full length 16S rRNA gene amplicons from activated sludge and anaerobic digester systems was also used to classify the zOTUs [48].The zOTU table was manually inspected in Excel and zOTUs which was more abundant in non-template control and negative control for DNA extraction than in the Leca biofilm samples were removed. The zOTU table was normalised to 23200 reads by multiplying the fraction of each community profile by 23200 and rounding numbers to integers. The USEARCH command Taxa summary was then used to generate a taxa summary at various taxonomic level (genus, family, order and class) (Sintax_summary).

2.4.7 Statistical analysis

PAST version 4.05 was used for multivariate statistical analysis [49]. Ordination by Principal coordinates (PCoA) based on Bray Curtis similarities were used to visualise similarity/dissimilarity in bacterial composition in different samples (beta-diversity). Average Bray Curtis similarities were calculated within and between sample groups to evaluate differences/similarities. One-way Permutational multivariate analysis of variance (PERMANOVA) based on Bray Curtis similarities were used to test if differences in zOTU profiles between groups of samples were statistically significant ($p < 0.05$). Bonferroni correction was used when more than two groups were compared in the One-way PERMANOVA test. To further investigate observed differences in Bra Curtis similarities a SIMPER analysis was conducted with PAST. This was done to assess the contribution of each zOTU to the dissimilarity between sample groups. The USEARCH command alpha_div was used to calculate alfa diversity metrices. Alfa diversity metrices were exported to Microsoft excel for further analysis.

The data analysis tool in Microsoft Excel (www.microsoft.com) was used for statistical analysis. To test if differences in alfa diversity metrices between sample groups were significant, a One-way analysis of variance (ANOVA) was conducted between sample groups. The One-way ANOVA test was also used to test for statistically significance between Leca biofilm exposed to the different washing procedures in the robotized batch

experiment monitoring gas kinetics, for O_2 consumption and production of different N-compounds. Results were considered significant if $p < 0.05$.

2.4.8 Estimation of evolutionary relationships for Nitrospira-zOTUs

There was a high relative abundance of zOTUs classified to *Nitrospira* compared to zOTUs classified to ammonia oxidising bacteria. The estimated evolutionary relationship between known *Nitrospira* sequences and the zOTU sequences from the dataset were therefore investigated to assess if the sequences could be related to comammox bacteria. Molecular Evolutionary Genetics Analysis (MEGA-X, version 10.2.4) software was used to generate a phylogenetic tree with maximum likelihood method and Tamura-Nei model [50, 51]. FASTA file used in analysis was made with sequences classified to *Nitrospira* with a confidence level above 80 % from zOTU table and 16S rDNA-sequences from earlier described *Nitrospira* strains, including comammox bacteria. In addition, type strain for two *Nitrospira*-genera, *Thermodesulfovibrio* and *Leptospirillum,* of the family *Nitrospiraceae* were used. The 16S rRNA genes were compared by ClustalW alignment using default settings. *Thermodesulfovibrio* was used as an outgroup to root the phylogenetic tree, since it has been showed to represent the oldest genus in the family of *Nitrospiraceae* [52]. Sequences were retrieved from RDP database, and accession number for each sequence is given in the results. The maximum likelihood tree was constructed by default settings, and the analysis was conducted with a bootstrap replication of 1000. The resulting tree was condensed to 50% bootstrap cut off value.

3 Results

3.1 Characterisation of bacterial biofilm communities in Leca biofilm from nitrifying filters at VEAS

Biofilm community composition was characterised to investigate the effect of the new and original washing procedure, the effect of different depths in the bioreactors and if the microbial community showed temporal variations. A total of 7137 zOTUs were identified in the zOTU-table for biofilm Leca samples. The Bacterial community composition on the class level was determined for all samples (Figure 3.1.). The most abundant classes for samples were Betaproteobacteria, Alphaproteobacteria, Nitrospira and Actinobacteria with average relative abundances of 18.66, 17.49, 15.37, and 9.78 % of the total reads, respectively. Generally, these taxa were relatively equally represented in all samples except from samples taken in August (20.08) from NIT71, NIT73 and NIT74, where Nitrospira was less common (relative abundance of 2.33 to 4.32 %).

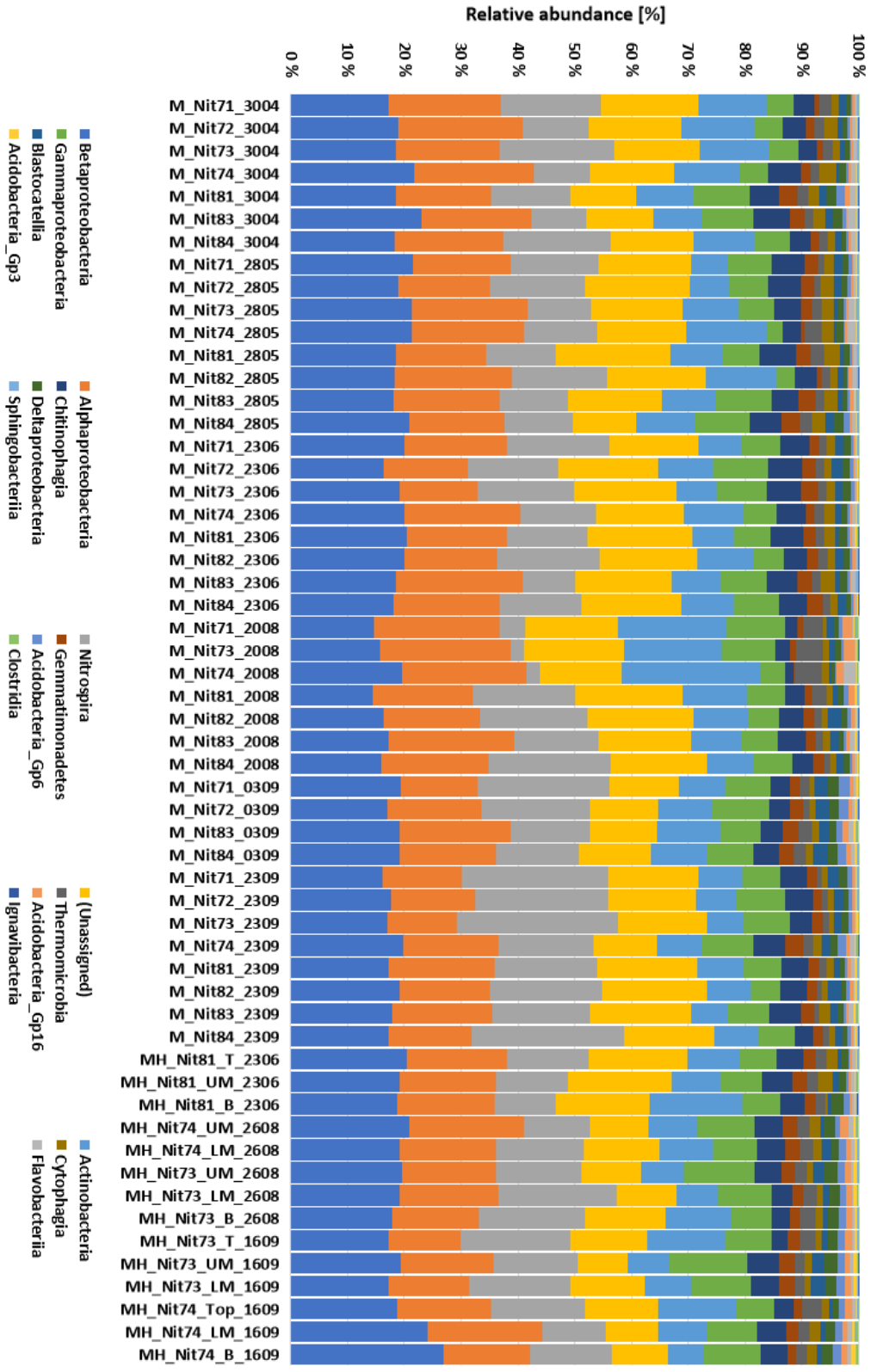


Figure 3.1. Relative abundance (%) of bacterial classes for samples of Leca biofilm from nitrifying filters at VEAS. Each bar represents the relative class distribution in one sample. Samples are named with M for monthly samples taken at the top of the filter, and MH for samples taken from different depths in the filter. The different depths are named; T for Top, UM for upper mid, LM for lower mid and B for bottom. Samples from process hall seven are written NIT71, NIT72, NIT73, NIT74, and from process hall eight, NIT81, NIT82, NIT83, NIT84. Date for sampling is given at the end of each sample name with first number indicating date and last numbers indicating month (e.g. 30th of September is written, 3004). All samples were taken in 2020. Classes with relative abundance over 0.1 % are included in the graph.

3.1.1 The effect of washing procedures and temporal variations in the community structure of Leca biofilms

To investigate the differences in bacterial community composition between samples of Leca biofilm exposed to the new- (PHA7) and the original washing procedure (PHA8) a Principal coordinate analysis (PCoA) based on Bray Curtis similarities was performed (Figure 3.2.). It was observed larger differences in community composition between samples from different dates, than between samples from PHA7 and PHA8 in bacterial community composition (Figure 3.2.A.). These results indicated a larger difference du to temporal variations, compared to differences between the new and original washing procedure on bacterial community composition. PCoA plot indicated no distinct differences in biofilm community composition between samples taken from different nitrifying filters in PHA7 and PHA8 (Figure 3.2. B. and C.). This was confirmed by a one-way PERMANOVA test based on Bray Curtis similarities which showed no significant difference between the different nitrifying filters within each process hall ($p > 0.05$).

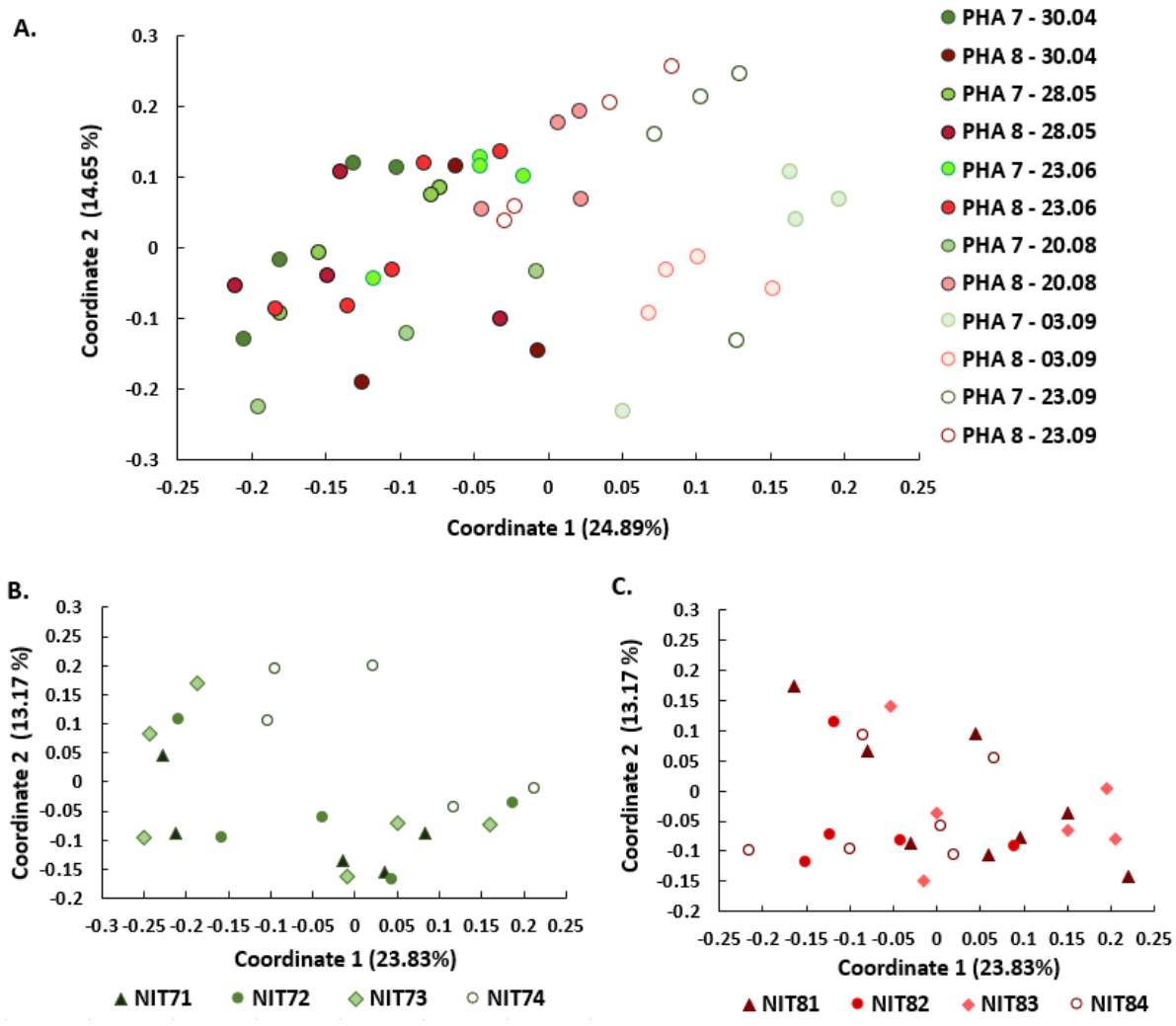


Figure 3.2. PCoA based on Bray Curtis similarities of bacterial biofilm communities from Leca biofilm sampled from April (30.04) to September (23.09). Samples were taken in process hall 7 (PHA7) from four parallel nitrifying filters (NIT71, NIT72, NIT73, NIT74) exposed to the new washing procedure and process hall 8 (PHA8) from four nitrifying filters (NIT81, NIT82, NIT83, NIT84) with the original washing procedure. All samples were taken from the top of the filter. Percentages in parentheses on the axis titles refer to variation explained by ordination axis. (A.) Variation in bacterial community composition from samples taken from PHA7 and PHA8. Samples are named with sampling date (B.) Variation in microbial community composition between nitrifying filter 71, 72, 73 and 74 in PHA7. (C.) Variation in bacterial community composition between nitrifying filter 81, 82, 83 and 84 in PHA8.

To further investigate the effect of the washing procedures on community structure a One-way PERMANOVA test based on Bray Curtis dissimilarities were calculated for all monthly samples (April to September) grouped by PHA7 (new wash) and PHA8 (original wash). The differences were seen to be significant ($p = 0.018$) which suggest that the washing procedures led to changes in bacterial community composition. To identify which zOTUs contributed most to the differences in bacterial community composition between PHA7 and PHA8, a Simper analysis based on Bray Curtis dissimilarities was conducted. There were three zOTUs that contributed to a cumulative difference of 9 %. zOTU1 (*Nitrospira*) contributed to 7 % of the observed difference and was slightly more abundant in PHA7 than PHA8. Further, zOTU6 (*Sphaerobacteraceae*) and zOTU14 (*Rhodobacteraceae*) contributed to 2.36 % of the observed differences.

To investigate temporal variations in Leca biofilm average Bray Curtis similarities were calculated for each separate date (Figure 3.3.A.) in PHA7 and PHA8. These results indicated that the observed differences in Leca biofilm communities were generally similar between PHA7 and PHA8 as within each process hall. There was seen a larger difference in Bray Curtis similarities between PHA7 and PHA8 in August (20.08) compared to the other dates. The increased differences could be explained by the low abundance of Nitrospira in biofilm samples from PHA7 (Figure 3.1.).

To further investigate the temporal variations in Leca biofilm, average Bray Curtis similarities were calculated between samples taken on subsequent dates from nitrifying filters in the same process hall (Figure 3.3.B.). Similarities in bacterial community composition was generally lower between samples from same process hall between different dates (Figure 3.3.B.), than between samples from same date (Figure 3.3.A.) Biofilm communities seemed to change from April to June and August to September. One-way PERMANOVA test was used to test the differences in community profiles between all combination of dates. This showed that samples from September (03.09 and 23.09) had a significantly different bacterial community composition than samples taken on other dates ($p < 0.005$). In addition, samples taken in August (20.08) was significantly different from samples taken in May (28.05) ($p < 0.005$). These results indicated a shift in bacterial community composition from August to September.

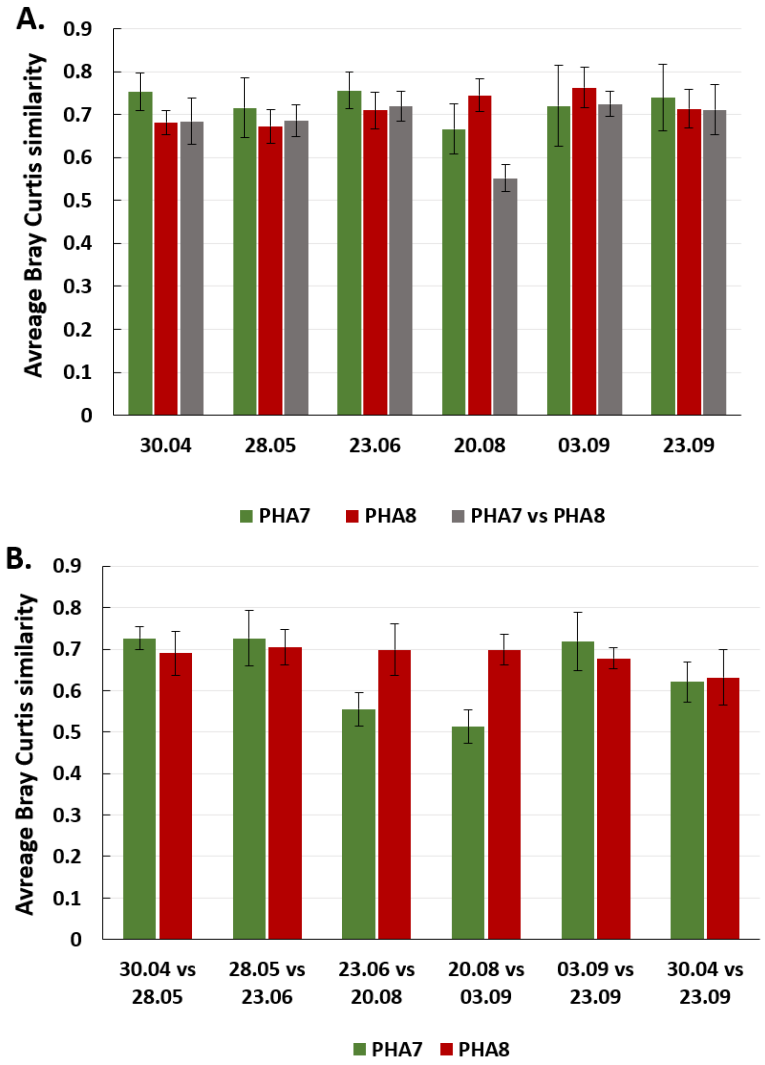


Figure 3.3. Average Bray Curtis similarities for nitrifying filters in PHA7 and PHA8 at VEAS within and between dates. Average was based on samples from four nitrifying filters (NIT71, NIT72, NIT73, NIT74.) in process hall seven (PHA7), except on 20.08 where the average was calculated from three samples (missing sample NIT72). For process hall eight (PHA8) average was calculated from four nitrifying filters (NIT81, NIT82, NIT83, NIT84) for all dates, except 30.04 which was calculated from only three samples (NIT82 is missing). Error bars represent standard deviation. (A.) Average Bray Curtis similarities within dates in PHA7 and PHA8, and between PHA7 and PHA8 (PHA7 vs PHA8) at the same date. (B.) Average Bray Curtis similarities between dates for PHA7 and PHA8.

For further investigation of the differences in bacterial community composition, average alpha diversity metrices were calculated for Leca biofilm sampled in PHA7 and PHA8 for each sampling date (Figure 3.4.). The observed zOTU richness were generally similar between samples from PHA7 and PHA8 on the same dates. Comparison of the observed richness to the estimated zOTU richness (Chao1), showed an average of 73 and 69 % sequencing coverage for PHA7 and PHA8, respectively. Exponential Shannon's index increased for samples from PHA8 from April to June and decreased from June to August by 44 %. This could be explained by the observed decrease in richness and evenness in the community composition from August to September, which might be explained by temporal variations. In August (20.08) samples from PHA7 had an exponential Shannon's index which was 53 % higher compared to PHA8. The high exponential Shannon's index in PHA7

on this day is probably explained by a slightly higher evenness in PHA7 due to the low relative abundance of zOTU1 (Nitrospira) in these samples (Figure 3.1.).

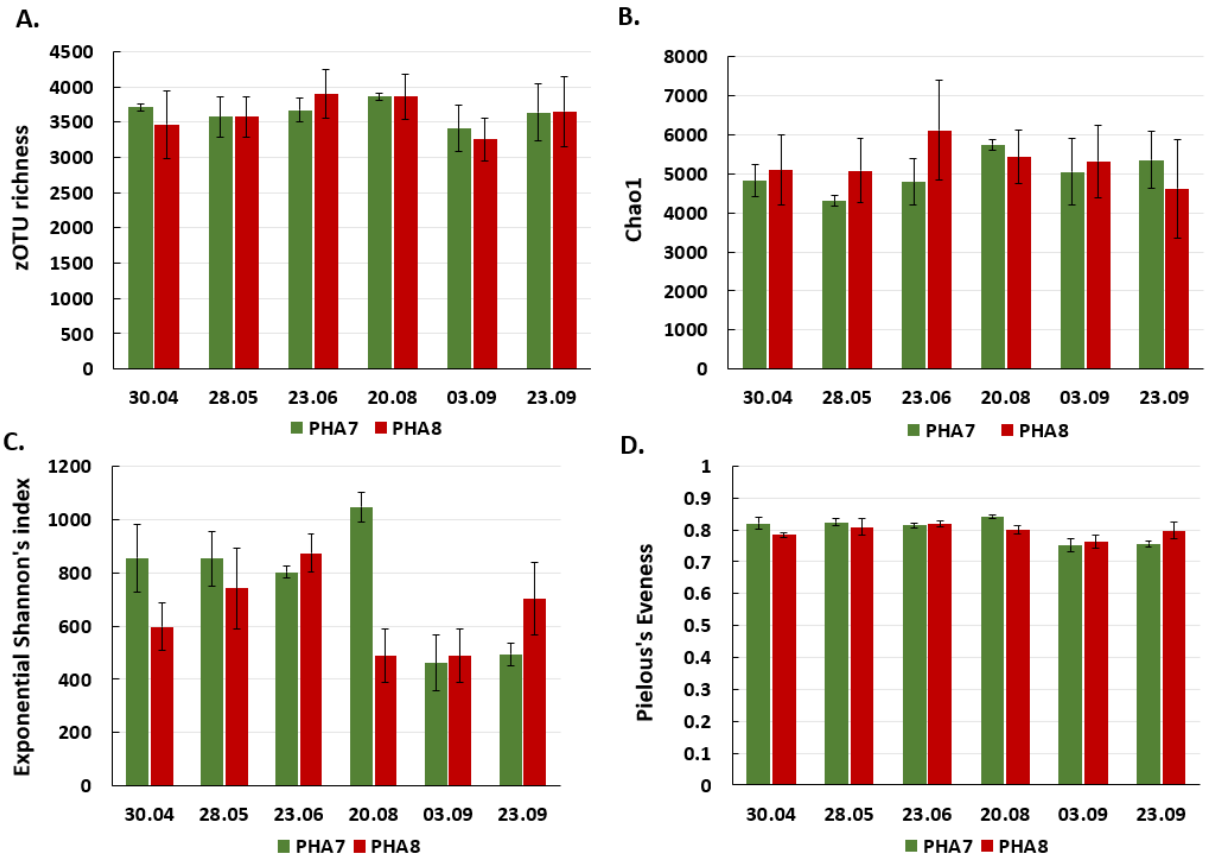


Figure 3.4. Average alfa diversity metrices for bacterial communities in Leca biofilm samples from nitrifying biofilter at VEAS at all sampling dates. Calculated average was based on samples from four nitrifying filters (NIT71, NIT72, NIT73, NIT74) in process hall seven (PHA7), except on 20.08 where the average was calculated from three samples (missing sample NIT72). For process hall eight (PHA8) average was calculated from four nitrifying filters (NIT81, NIT82, NIT83, NIT84) for all dates, except 30.04 which was calculated from only three samples (NIT82 is missing). Error bars represent standard deviation. (A.) Observed zOTU richness. (B.) Chao1, theoretical zOTU richness. (C.) Exponential Shannon's index. (D.) Pielou's evenness.

3.1.2 The effect of depth in nitrifying biofilters on bacterial community composition

To investigate differences in bacterial community composition at different depths in the nitrifying filters it was taken samples from four different depths in PHA7 (NIT73, NIT74) and in PHA8 (NIT81). See section 2.4, Table 2.4 for detailed description of sampling. PCoA based on Bray Curtis similarity index showed that samples tended to cluster closer based on filter than on depth (Figure 3.5.). These results indicate that differences were larger in biofilm communities between different nitrifying filters, than between different depths within one filter.

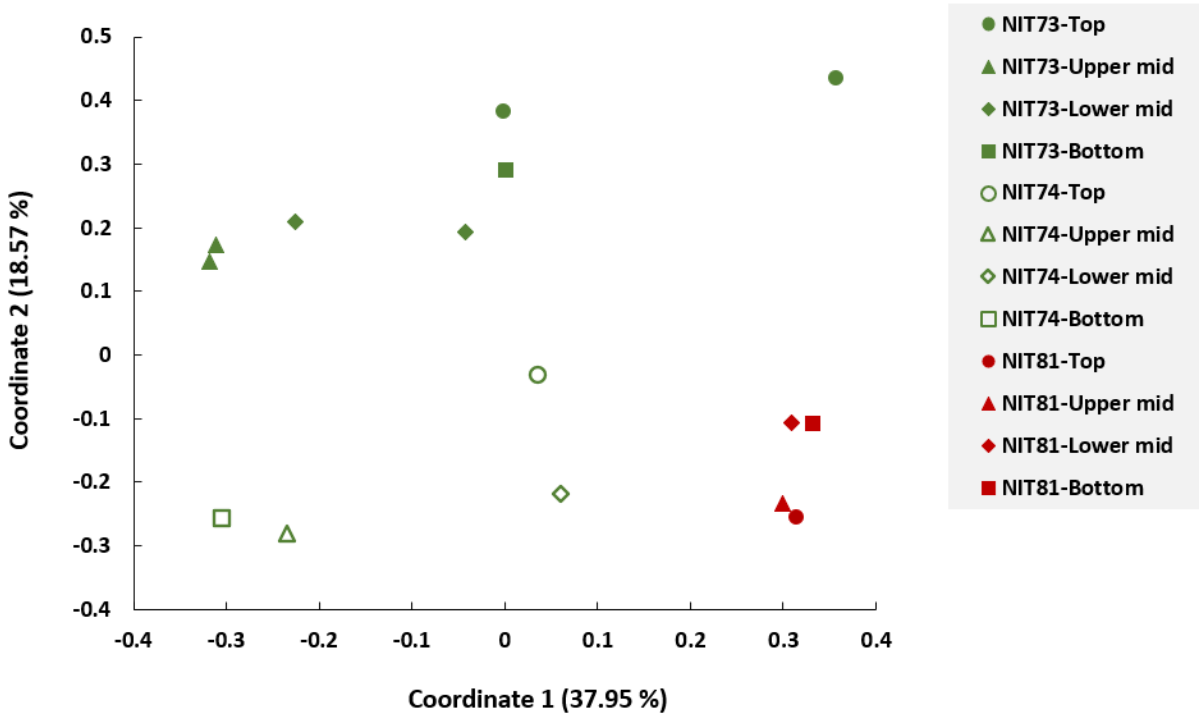


Figure 3.5. PCoA based on Bray Curtis similarity index for Leca biofilm samples taken at four different depths in nitrifying biofilters at VEAS. Samples were taken from four different depths in PHA7 (two NIT73, one NIT74) and in PHA8 (NIT81); from the top of the filter bed in nitrifying filter (top), from zero to minus two meters down in the filter (upper mid), from minus two meters to minus four meters (lower mid) and the bottom of the filter (bottom). Samples from NIT73 were sampled 16.09 and 26.08. Samples from NIT74 were sampled 26.08 and samples from NIT81 were sampled 23.06. Percentages in parentheses on the axis titles refer to variation explained by ordination axis.

Alfa diversity metrices were calculated for biofilm samples to further investigate differences in bacterial diversity at different depths (Figure 3.6.). Generally, all diversity metrices were higher in PHA8 (original washing procedure) than in PHA7 (new washing procedure). Since there were only sampled one height profile in PHA8 it is hard to draw conclusions based on differences observed between PHA7 and PHA8. One the other hand, it was quite interesting that the observed richness, evenness, and consequently exponential Shannon's index was lower at deeper depths than at the top in both PHA7 and PHA8. These results indicate a higher abundance of dominant zOTUs at lower depths. In addition, estimated zOTU richness (Chao1) was increasing down to Lower mid in both PHA7 and PHA8, which indicates more low abundance zOTUs at this depths.

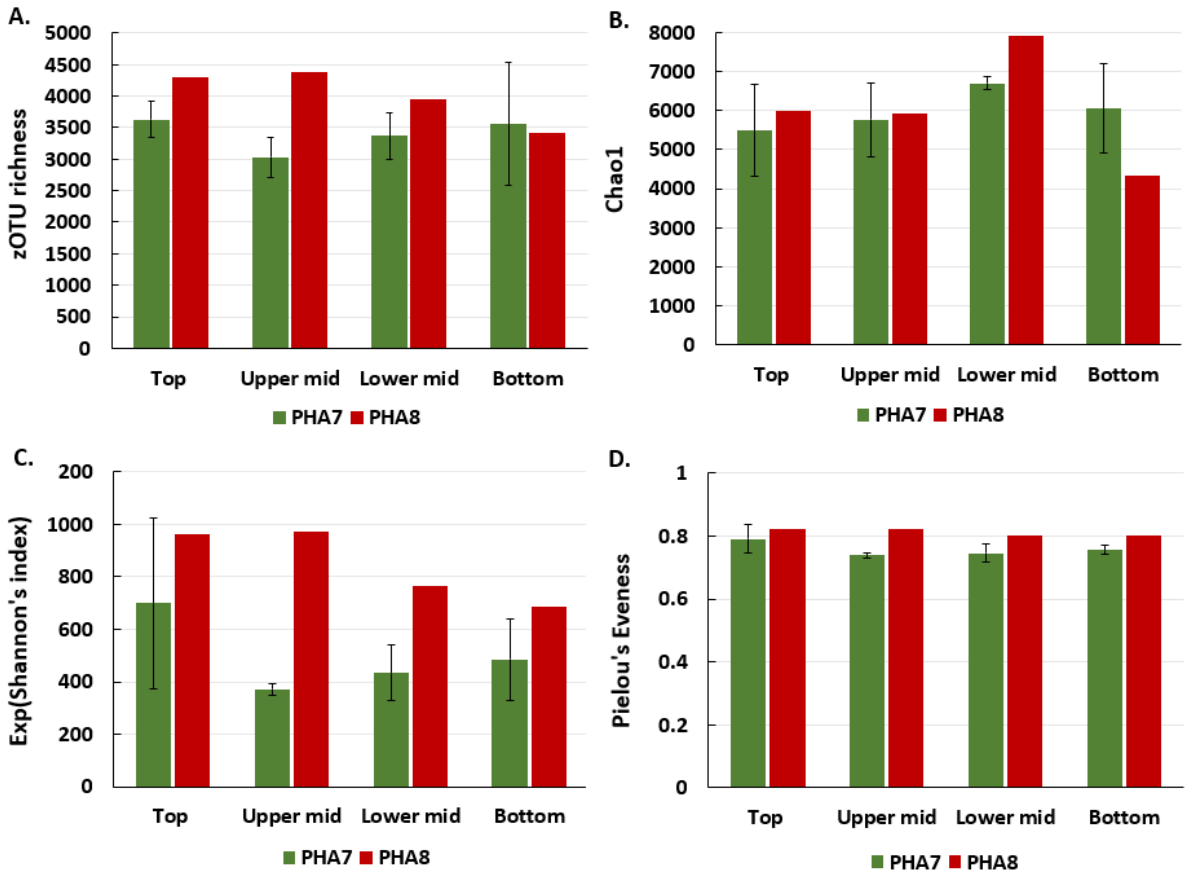


Figure 3.6. Alfa diversity metrices based on Leca biofilm samples from different depths in nitrifying filters at VEAS. Samples from NIT73 were sampled 16.09 and 26.08. Samples from NIT74 were sampled 26.08 and samples from NIT81 were sampled 23.06. Average was calculated from three samples at all depths in PHA7 except from Bottom where only two samples were used. Alfa diversity at different depths in PHA8 were based on single samples. Error bars represent standard deviation for calculated average. A) Observed zOTU richness. B) Chao1, theoretical zOTU richness. C) Exponential Shannon's index. D) Pielou's evenness.

3.1.3 The immediate effects of washing on Leca biofilm communities

To investigate the immediate effect on the Leca biofilm communities of the different washing procedures, samples of Leca biofilm from nitrifying biofilters were taken within one hour before and after washing at VEAS. The new washing procedure was conducted with one, three and five repetitions of step D2-D6 (Table 2.2.), to see if changes in repetitions could affect biofilm community composition. Original wash followed standard procedure (Table 2.1.). Average relative abundance of different bacterial classes was determined for Leca biofilm samples and no distinct effect of the washing procedures on composition on class level were observed (Appendix G). PCoA based on Bray Curtis similarities was conducted to investigate if the washing procedures influenced biofilm community composition (Figure 3.7.). Leca biofilm samples did not cluster based on washing procedure or if samples were taken directly prior or after the procedure of washing.

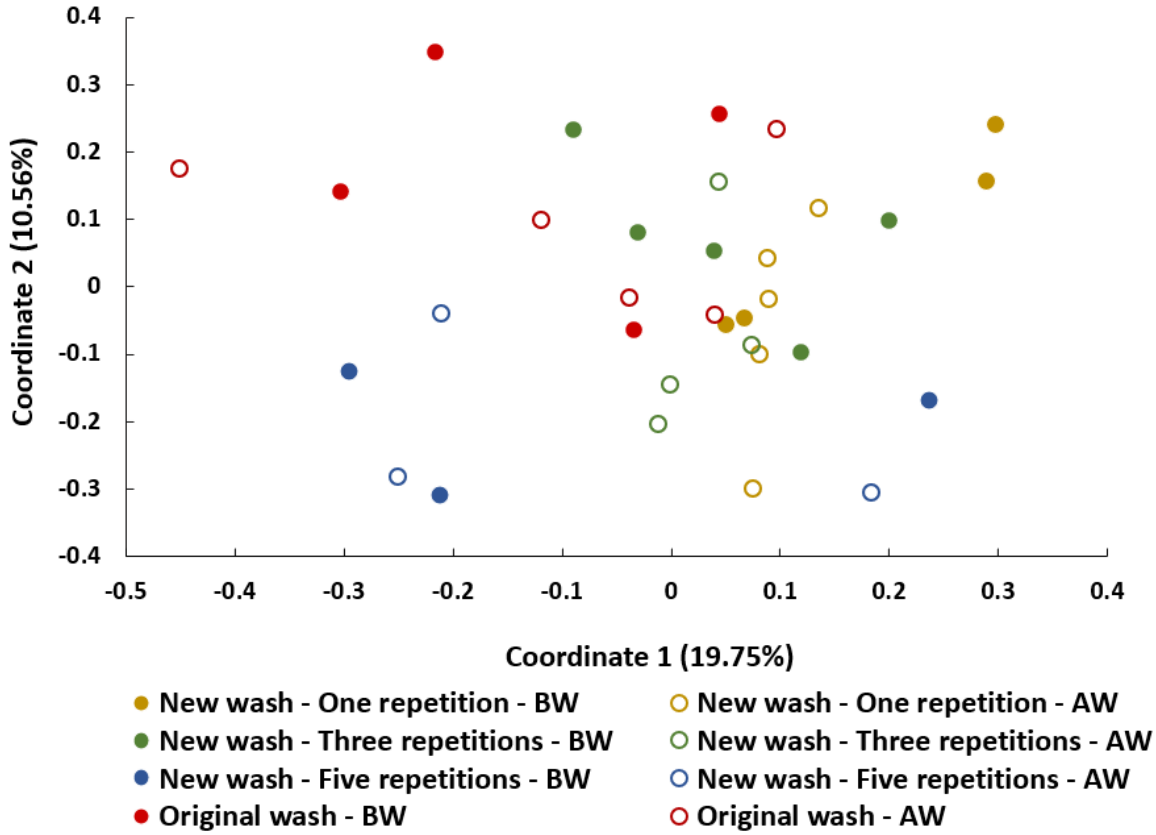


Figure 3.7. PCoA based on Bray Curtis similarity index for Leca biofilm samples from nitrifying filter taken directly before- (BW) and after the washing (AW) procedure at VEAS. The new washing procedure was conducted with one, three and five repetitions of step D2-D6 and the original wash followed standard procedure (Table 2.1. and Table 2.2.). Variation explained by coordinates are represented as percentages in brackets on axis titles.

Average Bray Curtis similarity was calculated for samples exposed to the different washing procedures (APPENDIX F). Leca biofilm samples exposed to different washing procedures showed similar bacterial community composition before and after wash and between different washing procedures. There was no significant difference in Bray Curtis similarities between the Leca biofilm exposed different washing procedures, or between before and after washing (One-way PERMANOVA, $p > 0.05$). This indicated that the general effect of the different washing procedures on variation in bacterial community composition was small.

Despite the small differences in beta-diversity, average alfa diversity metrices were calculated to assess if larger differences could be observed in Leca biofilm communities. Average alfa diversity metrices were calculated for samples taken before and after each washing procedure (Figure 3.8.). Exponential Shannon's index showed that there was a higher diversity in Leca biofilm after the procedure of washing for the new wash with one and three repetitions, due to a slightly higher evenness in these samples. This indicates a decrease in evenness in biofilm communities between two washing events. The highest exponential Shannon's index was observed in Leca biofilm exposed to the new washing procedure with five repetitions, due to a somewhat higher evenness both before and after washing. The lowest exponential Shannon's index was observed in Leca biofilm before wash

exposed to the new washing procedure with one repetition of step D2-D6. This washing procedure led to the largest difference between before and after washing in Leca biofilm samples. This suggests that the Leca biofilm communities get more dominated by a few bacterial populations between two washing events with this washing procedure.

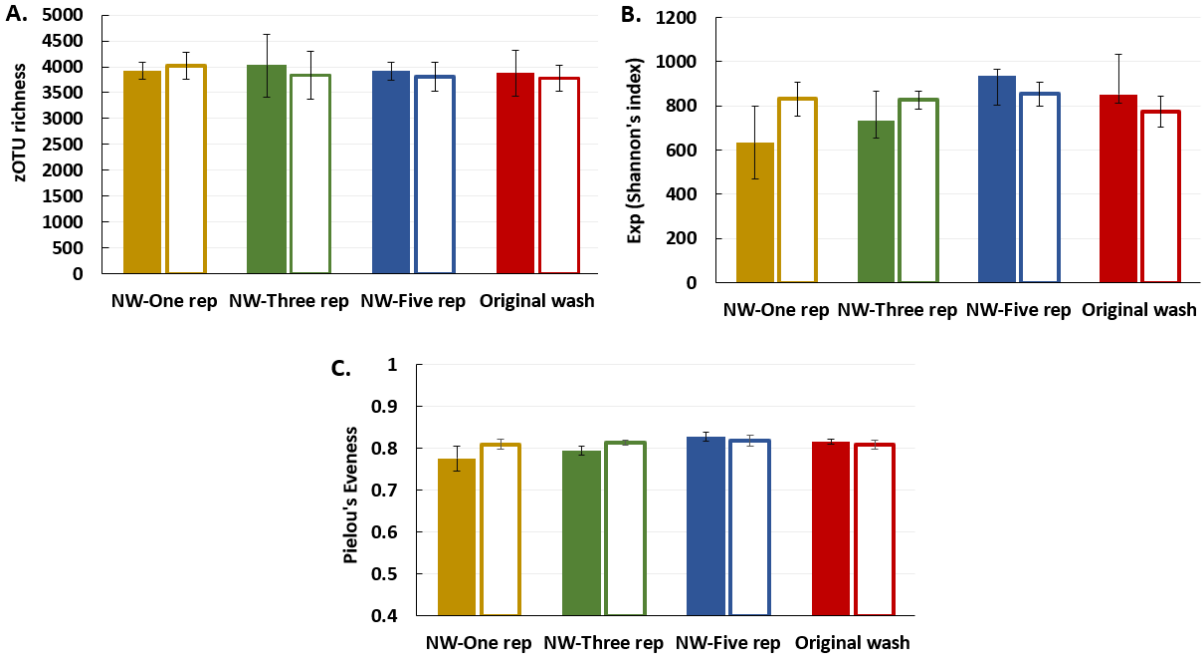


Figure 3.8. Calculated average alfa diversity metrices for bacterial communities in Leca biofilm from nitrifying filters sampled directly before (BW) and after (AW) wash. The new washing procedure was conducted with one, three and five repetitions of step D2-D6 and the original washing procedure followed standard procedure (Table 2.1 and Table 2.2.). Bars with fill colour represent samples before wash and "empty" bars represent after wash. Average was calculated from three replicates for all calculations except the new washing procedure with one repetition AW, which had four replicates and the new wash with three repetitions AW, which had two replicates. Error bars represent standard error. (A.) Observed zOTU richness (B.) Exponential Shannon index. (C.) Pielou's evenness.

3.1.4 zOTUs representing nitrifying bacteria from nitrifying filters at VEAS

To investigate if there were differences in relative abundance of nitrifiers, the fifteen most abundant zOTUs classified to genus level as previously described NOBs or AOBs were identified (Figure 3.9.). Their abundance was variable between samples. There was no significant difference in the relative abundance between PHA7 (new wash) and PHA8 (original wash) samples (One-way ANOVA, $p = 0.80$), or between dates when samples from PHA7 and PHA8 were merged (One-way ANOVA, $p = 0.37$). However, there was observed a decrease by 77% of nitrifiers from June to August in PHA7, which suggests that the nitrifying community exposed to the new washing procedure might be more susceptible to temporal changes.

The abundance of AOB-zOTUs was very low compared to the abundance of NOB-zOTUs. The most abundant NOBs were represented by five zOTUs classified to *Nitrospira* (zOTU1, zOTU29, zOTU78, zOTU123, zOTU140) and two zOTUs classified to *Nitrotoga* (zOTU55,

zOTU154). zOTU1 was most abundant with an average abundance of 12 % of total sample and dominated the nitrifying biofilm community. zOTUs representing *Nitrobacter* was not found. The most abundant AOB was represented by six zOTUs classified as *Nitrosomonas* (zOTU24, zOTU166, zOTU394, zOTU480, zOTU545, zOTU560) and two zOTU classified to *Nitrosospira* (zOTU38, zOTU583). zOTU24 was classified to *Nitrosomonas* and was the most abundant AOB-zOTU with an average relative abundance of 0.5 % of total sample.

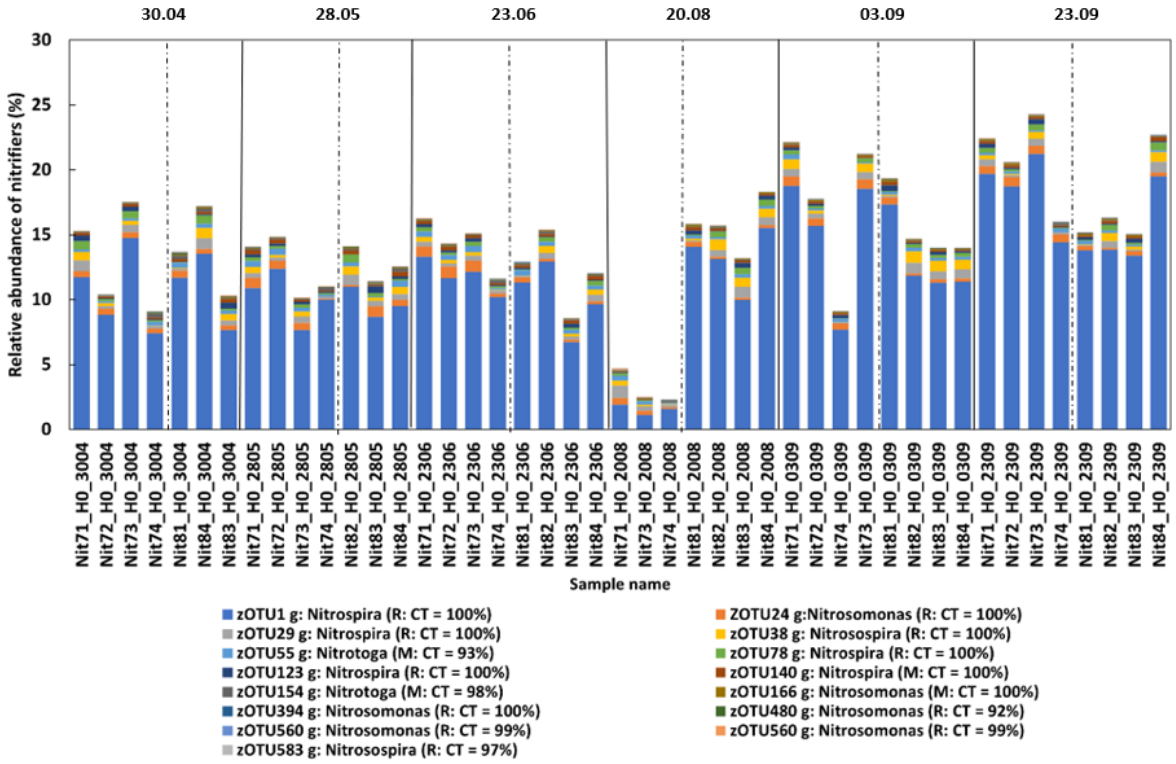


Figure 3.9. Relative abundance (%) of zOTUs classified to nitrifiers at genus level (g) for Leca biofilm samples taken at different dates at VEAS. Solid line separates date, while dashed line separate samples taken from process hall seven (new wash) and eight (original wash) at the same date. R = classified with RDP, M = classified with MiDAS, CT = confidence threshold. Each bar represents the relative distribution of nitrifiers in one sample. Samples from process hall seven are named NIT71, NIT72, NIT73, NIT74, and from process hall eight, NIT81, NIT82, NIT83, NIT84. All samples were taken at the top of the filter bed and are marked H0. The date for sampling is written at the end of each sample name with first number indicating date and last numbers indicating month (e.g., 30th of September is written, 3004).

To further investigate the immediate effect of the new and original wash, relative abundance of nitrifiers was calculated in Leca biofilm samples taken directly before and after the washing procedure from mid-June to the start of July (Figure 3.10.). The relative abundance of nitrifiers were generally somewhat higher in samples taken before the washing procedure than after, except from two Leca biofilm samples exposed to the new wash with five repetitions and two Leca biofilm samples exposed to the original washing procedure. This indicate that the washing procedures could reduce the relative abundance of nitrifiers in the Leca biofilm.

Leca biofilm exposed to the new washing procedure with one repetition of step D2-D6 had the highest number of nitrifiers before wash with an average relative abundance of 20 % (SD ± 5.3 %) of total sample. Leca biofilm exposed to the new washing procedure with

five repetitions had the lowest average relative abundance of nitrifiers before and after the procedure of washing (11 % SD ± 2.2 % and 12 % SD ± 1.7 %, respectively). This suggest that increasing the repetitions of step D2-D6 in the new washing procedure leads to a higher removal of nitrifiers in the Leca biofilm.

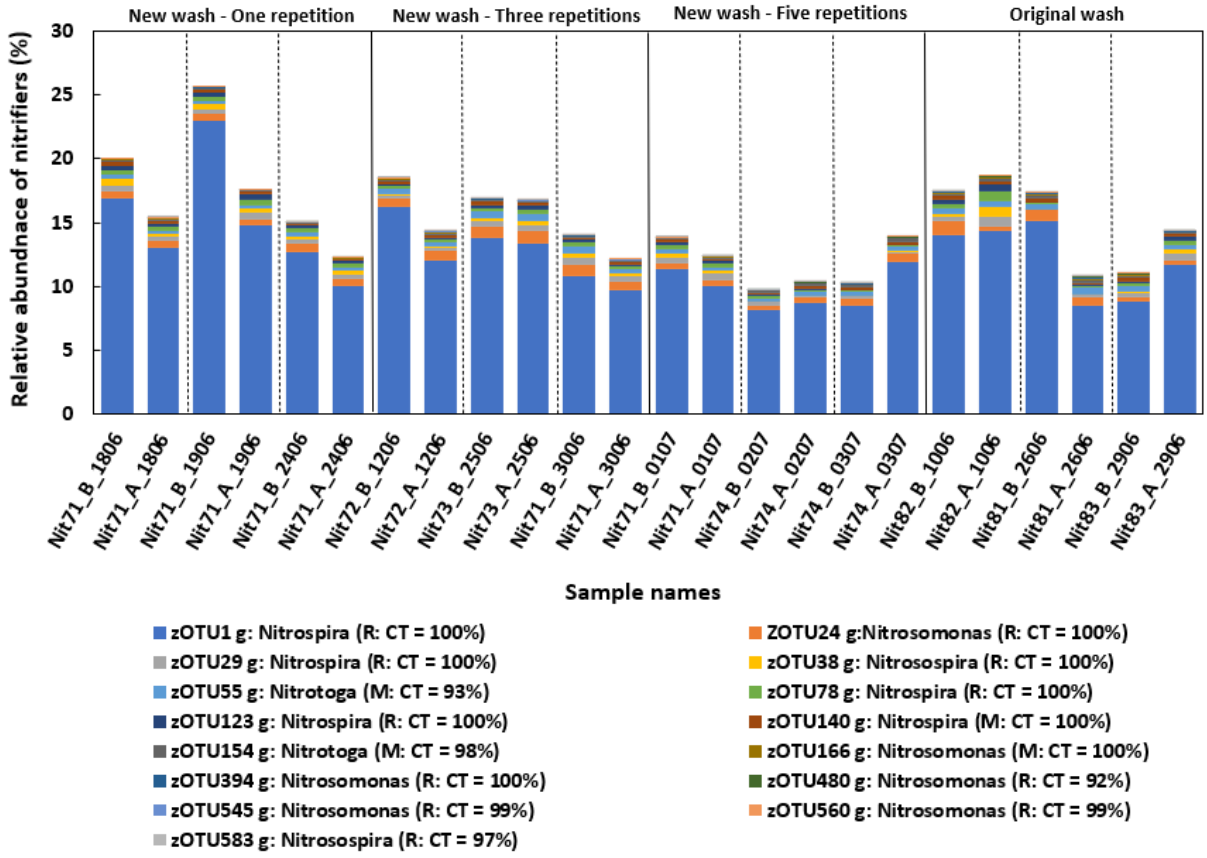


Figure 3.10. Relative abundance (%) of zOTUs classified to nitrifiers at genus level (g) for samples taken directly before and after the different washing procedures of nitrifying filters at VEAS. The new washing procedure was conducted with one, three and five repetitions of step D2-D6 and the original washing procedure followed standard procedure (Table 2.1 and Table 2.2.). Each bar represents the relative distribution of nitrifiers in one sample. Leca samples were taken from the conducted batch experiments directly before and after the washing procedures from the top of the filter bed (Table 2.3.). Samples were named according to nitrifying filter in PHA7 (NIT71, NIT72, NIT73, NIT74) and PHA8 (NIT81, NIT82, NIT83, NIT84), if the samples were taken before (B) or after (A) the procedure of washing, and date for sampling. All samples were taken in 2020. Solid line separates washing procedures, while dashed line separate samples taken on different dates. R = classified with RDP, M = classified with MiDAS, CT = confidence threshold.

A phylogenetic analysis was performed to investigate if the low abundance of AOB could be explained by zOTU1 (*Nitrospira*) being a comammox bacteria (Figure 3.11.). This was done by analysing the evolutionary relationship between the *Nitrospira* sequences retrieved from Leca biofilm at VEAS and 16S rRNA sequences from previously described *Nitrospira* strains. Maximum likelihood analysis showed that several of the zOTUs clustered together except zOTU1, which clustered to *Nitrospira salsa*. None of the zOTUs showed relations to previously described comammox bacteria (*Candidatus N. nitrificans, Candidatus N. inopinata*).

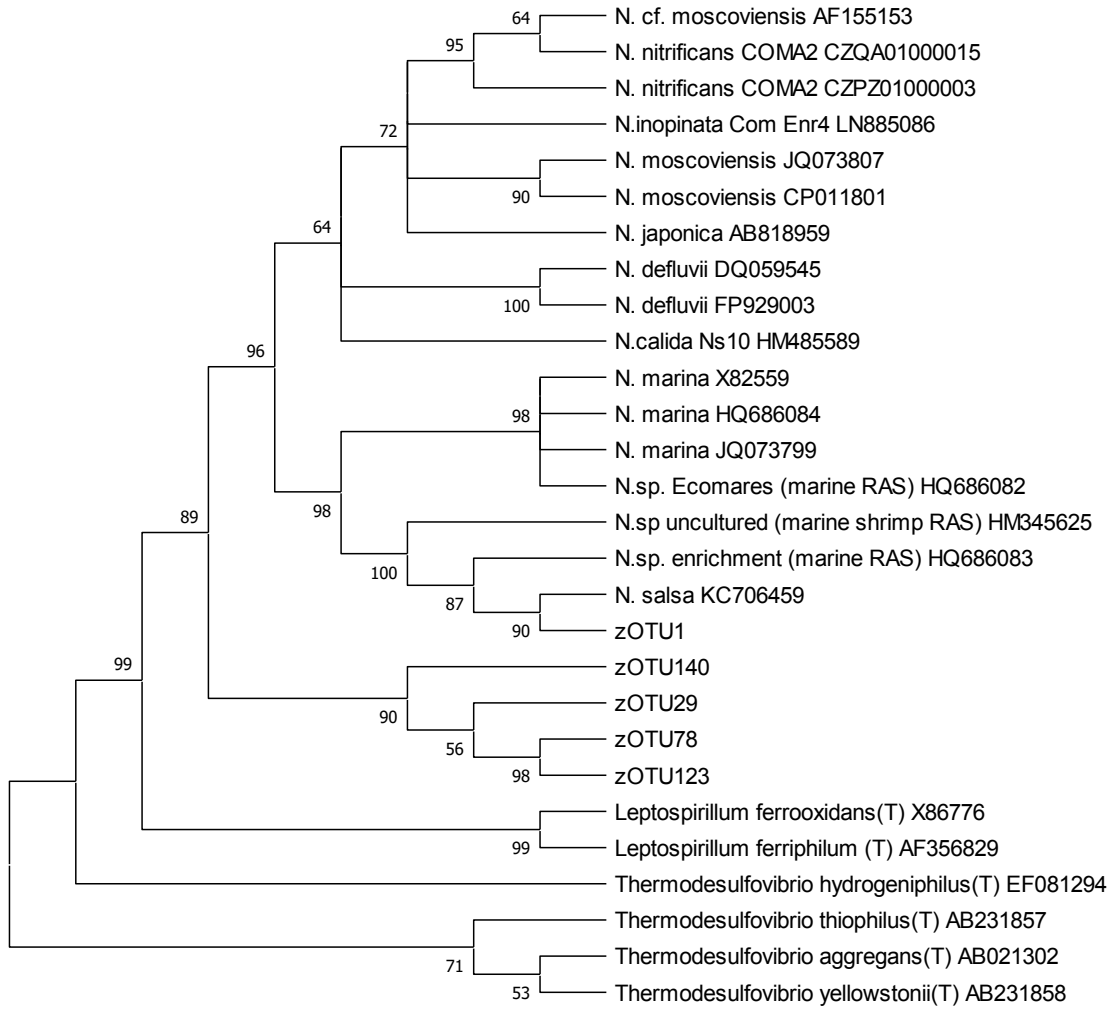


Figure 3.11. Maximum likelihood tree for evolutionary analysis of zOTUs classified to *Nitrospira (N.)* at genus level and 16S rRNA gene sequences from previously described Nitrospira. Sequences for previously described *Nitrospira* were retrieved from RDB database, and accession numbers are specified for with the species names. Type strains are indicated by a (T). The analysis was performed in MEGA X version 10.2.4. First, the sequences were aligned using ClustalW with the default parameters. Next, a maximum likelihood analysis was performed with 1000 bootstrap replicates and the Tamura-Nei model for sequence evolution. The tree was condensed with 50 % cut-off value, and bootstrap support values are shown at the nodes, and indicate reliability of clusters descending from that node. The tree includes representatives for the other genera included in the *Nitrospiraceae* family (*Thermodesulfovibrio* and *Leptospirillum*) and the tree is rooted at the *Thermodesulfovibrio* node.

3.2 Small-scale batch experiments monitoring nitrification capacity

To investigate the immediate effect of different washing procedures on nitrification capacity, batch experiments with Leca biofilm from nitrifying biofilters at VEAS was conducted. Nitrate production rate was calculated for Leca biofilm sampled before and after the new and original washing procedure (Figure 3.12.). The new wash was conducted with one, three and five repetitions of step D2-D6, and the original wash followed the standard procedure (Table 2.1 and Table 2.2.). Concentrations of nitrite (NO_2), nitrate (NO3) and ammonia (NH_4) were measured for all batch-experiments every 30 minutes for a total of 120 minutes. NO_2 concentrations were under the detection limit during the whole experiment. Measurements of concentrations of ammonia are not included in the results, due to problems with the Hach-Lange cuvettes which resulted in high uncertainty in these measurements. See Appendix I for measured ammonia concentrations and turbidity during the period where the small-scale batch experiments were conducted at VEAS.

Leca biofilm exposed to the new washing procedure with five repetitions of step D2-D6 had the lowest nitrate-production rate for all three experiments before washing compared to the other washing procedures. This suggest that an increasing number of repetitions of step D2-D6 led to lower nitrification capacity in Leca biofilm between two washing events. Leca biofilm exposed to the original washing procedure was more similar before and after wash than Leca biofilm exposed to the other washing procedures. This could indicate that the nitrate production by NOB was more stable between washing with the original procedure.

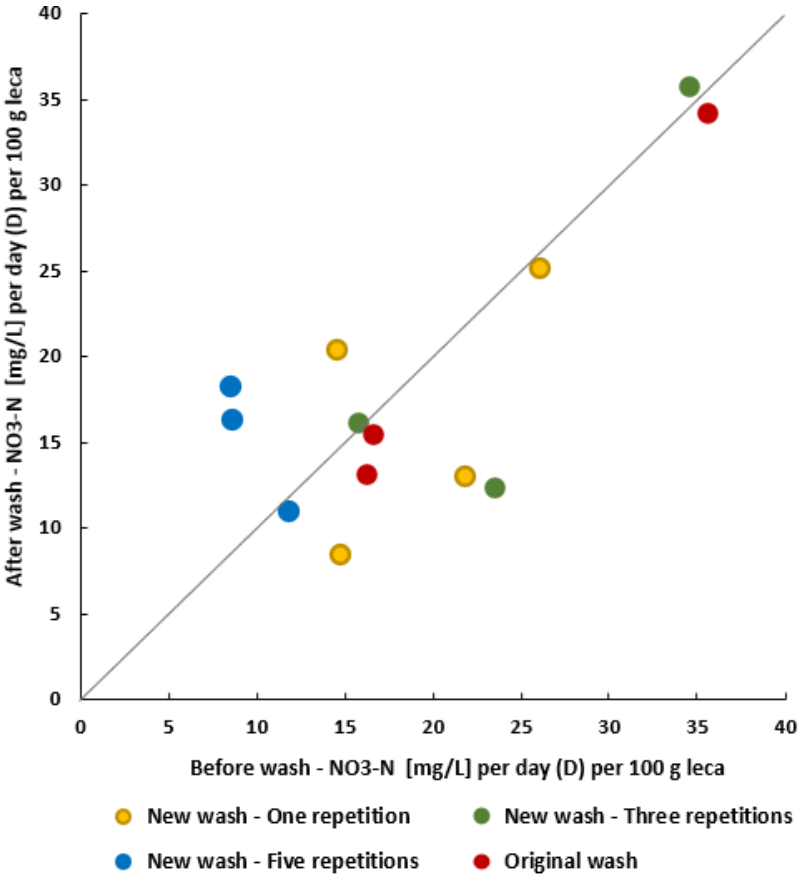


Figure 3.12. Total NO_3-N production in mg/L per day per 100-gram (g) Leca as calculated from small-scale batch experiments with Leca samples taken directly before (x-axis) and after (y-axis) the exposure to different washing procedures. Each point is based on a total NO_3-N production for small-scale batch experiments during a period of 120 minutes (converted to per day, 24 hours) with 100 g Leca biofilm sampled before and after the washing procedure. For Leca biofilm exposed to the original and new washing procedure with three and five repetitions of step D2 to D6 it was conducted three experiments before the washing procedure and three experiment after the washing procedure. For the new washing procedure with one repetition of step D2-D6 it was conducted four experiments with Leca biofilm sampled before wash and four experiments with Leca biofilm sampled after wash. See section 2.2, table 2.3. for details of sampling. 45-degree line indicate equability in nitrate production for biofilm samples taken before and after the washing procedure.

3.3 Robotized incubation system for measurements of gas kinetics in Leca biofilm from nitrifying filters at VEAS

The effect of the new and original washing procedure on nitrification capacity and microbial community in Leca biofilm was further investigated. This was done by batch experiments in a robotized incubation system with automated monitoring of O_2, N_2, N_2O and CO_2, with Leca biofilm sampled before and after the washing procedure. The concentrations of NO_3 and NO_2 were measured manually.

3.3.1 Nitrification efficiency in Leca biofilm exposed to the new and original washing procedure

To investigate the nitrification efficiency in Leca biofilm, the concentrations of nitrate and nitrite was measured in two replicate vials for each treatment (Figure 3.13.). Concentration of nitrate in the experimental control was below 0.12mM during the whole experiment for all replicates and are not shown. The concentrations of nitrite in the replicate controls were below the detection limit.

During the first 20 hours of the experiment NO_3 concentrations were increasing for Leca biofilm exposed to both the new and original washing procedure (Figure 3.13.A.). From 20 to 35 hours there was done eight measurements which gave fluctuation in nitrate concentration. At 25 hours all replicate vials were spiked with 2 mM ammonia, but no increase in the concentration of NO_3 was observed. Last measurements were done at 50 hours and the measured concentrations were approximately the same as measured at 20 hours. This indicated that nitrification stopped after 20 hours, and that the nitrification was not limited of low ammonia concentrations but possibly by the acidification from the process of nitrifaction.

NO_2 concentrations were decreasing in all Leca biofilm communities exposed to both the new and original washing procedure from five to 20 hours (Figure 3.13.B.). At 20 hours all treatments except from Leca biofilm exposed to the original wash before washing, which had a concentration of 3 µM, reached concentrations below 1 µM NO_2. This indicated that the activity of NOB could be limited due to substrate limitations, contradictory to the activity of AOB.

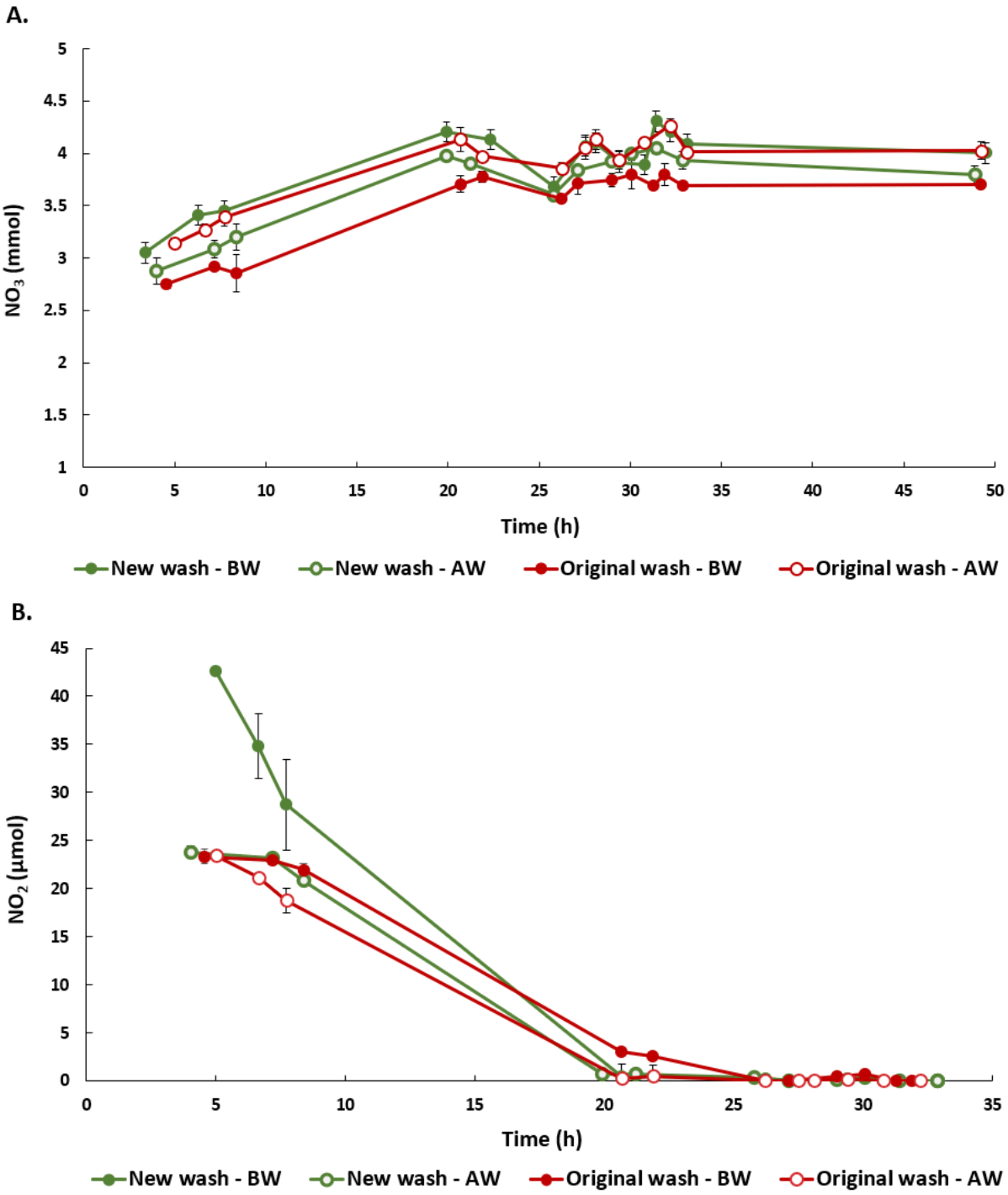


Figure 3.13. Measured NO_2 and NO_3 concentrations against time from robotized incubation system at NMBU with Leca biofilm from nitrifying filters at VEAS. Average was calculated from measurements of two replicate vials for each treatment; Leca biofilm sampled before and after the new washing procedure and Leca biofilm sampled before and after the original washing procedure. Leca samples exposed to the new wash were collected in PHA7 and Leca samples exposed to the original wash was collected in PHA8. See Appendix H for detailed information of sampling of Leca biofilm. Error bars represent standard error. (A.) Average NO_3 concentrations (mmol) against time (h). (B.) Average NO_2 concentrations (µmol) against time (h) for Leca biofilm.

A linear regression was done for the different treatments for the first five to 20 hours for measurements of NO_3 and NO_2 against time (Table 3.1.). The slopes were used to estimate the velocity of ammonia oxidation in Leca biofilm. Nitrification rates were found to be higher for Leca biofilm exposed to the new washing procedure both before and after wash, compared to the original washing procedure. The nitrification rates were however not significantly different between the washing procedures (One-way ANOVA, $p > 0.05$), but the results indicate a more effective process of nitrification with the new washing procedure.

Table 3.1. Slopes determined by linear regressions of changes in concentration of NO_3 µM and NO_2 µM against time for first four measurements (4-20 hours) in robotized incubation experiment with Leca biofilm from nitrifying filters at VEAS. Samples taken before wash are named (BW) and after wash (AW). Standard error is represented for slope. R^2 represent explained variance. Estimated velocity of ammonia oxidation (V_{amo}) are calculated from the slopes of NO_3 and NO_2.

	NO_3		NO_2		Estimated V_{amo} (µM h^{-1})	V_{amo} (µmol $vial^{-1}$ h^{-1})
	Slope (µMh^{-1})	R^2	Slope (µMh^{-1})	R^2		
New wash - BW	66±6.84	0.978	-2.52 ±0.29	0.975	63.5	3.18
New wash - AW	69±1.38	0.999	-1.49±0.17	0.999	67.5	3.38
Original wash - BW	60±6.18	0.979	-1.36±0.16	0.970	58.6	2.93
Original wash - AW	62±3.33	0.994	-1.47±0.035	0.998	60.5	3.03

3.3.2 Respiration in Leca biofilm from nitrifying filters

To measure microbial community respiration, oxygen (O_2) and carbon dioxide (CO_2) was measured during the robotized incubation experiment with monitoring of gas kinetics (Figure 3.14. A. and B.). Leca biofilm exposed to the original washing procedure had a higher cumulative oxygen consumption before wash compared to biofilm communities from the three other procedures. Leca biofilm exposed to the original wash had a final CO_2 production and O_2 consumption which was respectively, 37- and 40 % higher, before wash than after wash. Leca biofilm exposed to the new washing procedure showed a 20 % higher O_2 consumption and 7 % higher CO_2 production after wash than before, contrary to the original washing procedure. This indicates that Leca biofilm exposed to the original washing procedure gets a distinctly higher oxygen consumption between two washing events, wheras the oxygen consumption in biofilm exposed to the new washing procedure decreases between the process of washing. One-way ANOVA test based on the data shown in Figure 3.14. A. showed that there was a significant difference in the oxygen consumption between Leca biofilms exposed to both washing procedures and before and after wash in O_2 consumption ($p < 0.005$).

As earlier described, it was added 2 mM NH_4Cl at 25 hours to investigate if ammonium was the limiting factor for nitrification. No response was observed in O_2 consumption after ammonia was added, which indicated that ammonia was not limiting the respiration after 25 hours (Figure 3.14.A.). At the end of the experiment there were still oxygen left in all replicate vials for Leca biofilm exposed to both washing procedures and both before and after washing. The concentration of O_2 was above a 100 µmol O_2 per vial which is equivalent

to 3.35 vol % O_2 in the headspace and 42 µmol O_2 in the liquid, for all replicates except from two replicates with the original washing procedure which was less than 10 µmol.

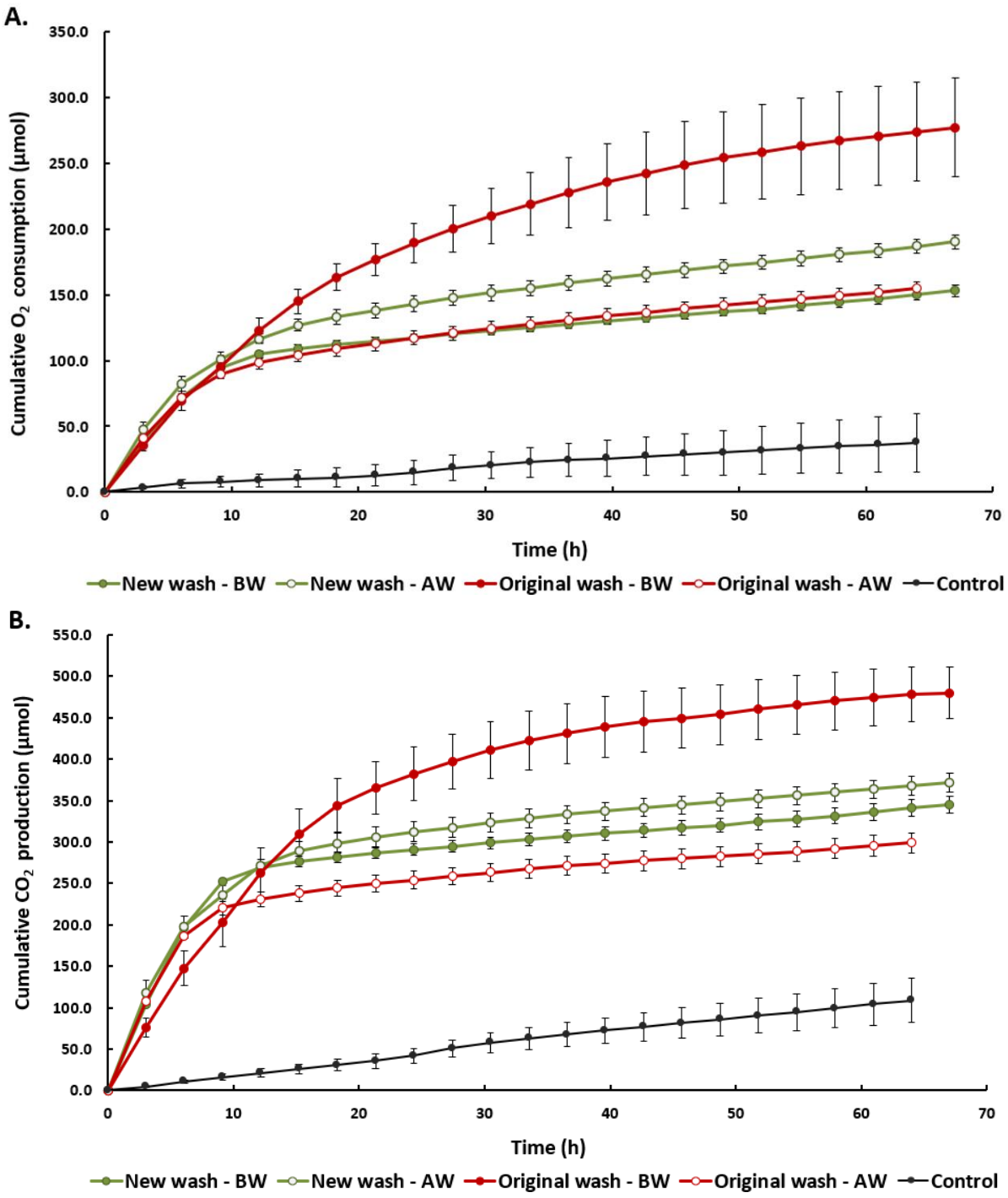


Figure 3.14. Average cumulative O_2 consumption (A.) and CO_2 production (B.) against time for robotized incubation experiment with Leca biofilm from nitrifying filters at VEAS. Time zero equals start of the experiment with a start concentration of 20 mL O_2. There were run five replicate vials for Leca biofilm sampled before (BW) and five replicate vials for Leca biofilm sampled after (AW) wash from both PHA7 (new washing procedure) and PHA8 (original washing procedure). There was also used five replicate vials for control with scrubbed Leca and autoclaved and distilled water. Average was calculated from five replicate vials for each treatment. Error bars represent standard error.

To assess the amount of oxygen used for nitrification for the first 20 hours of the experiment, the estimated velocity of ammonium oxidation was used to calculate the theoretical oxygen demand for the oxidation of ammonia. Oxygen used for the oxidation of ammonia was found to be higher in Leca biofilm exposed to the new washing procedure both before and after wash (Table 3.2.). Leca biofilm exposed the original washing procedure, sampled prior to the process of washing, had the lowest estimated velocity for ammonia oxidation which represented 66 % of the oxygen consumption. This indicates that Leca biofilm from PHA8, exposed to the original washing procedure had a higher heterotrophic activity between two washing events than Leca biofilm exposed to the new washing procedure in PHA7.

Table 3.2. Velocity (V) of O_2 and estimated velocity of ammonia oxidation (V_{amo}) for the first 20 hours of robotized incubation experiment with Leca biofilm from nitrifying filters at VEAS. Amount of O_2 estimated for the process of nitrification is given in percent (%) of the observed VO_2. Leca biofilm exposed to the new and original washing procedure were sampled before and after the process of washing.

	New wash		**Original wash**	
	Before	**After**	**Before**	**After**
VO_2 (μmol O_2 $vial^{-1}$ h^{-1})	5.74	6.91	8.85	5.63
V_{amo} (μmol NH_4-N $vial^{-1}$ h^{-1})	3.18	3.38	2.93	3.03
Estimated *VO_2_amo* (μmol O_2 $vial^{-1}$ h^{-1})	6.36	6.76	5.86	6.06
Estimated O_2 for amo (%)	100	98	66	100

Considering most of the oxygen was used in the process of nitrification, the production of CO_2 for all treatments were too high in comparison with the oxygen consumption (Figure 3.15.). The calculations for CO_2 production in all replicates were based on a stable pH of 7, whereas a decrease in pH would lead to an overestimation of the CO_2 concentrations. A decrease in pH could also explain the halt of nitrification after 20 hours. The pH was unfortunately not measured at the end of the experiment, but the assumptions are seen as reasonable. The CO_2 production for the first 20 hours could therefore not be used to estimate the heterotrophic activity.

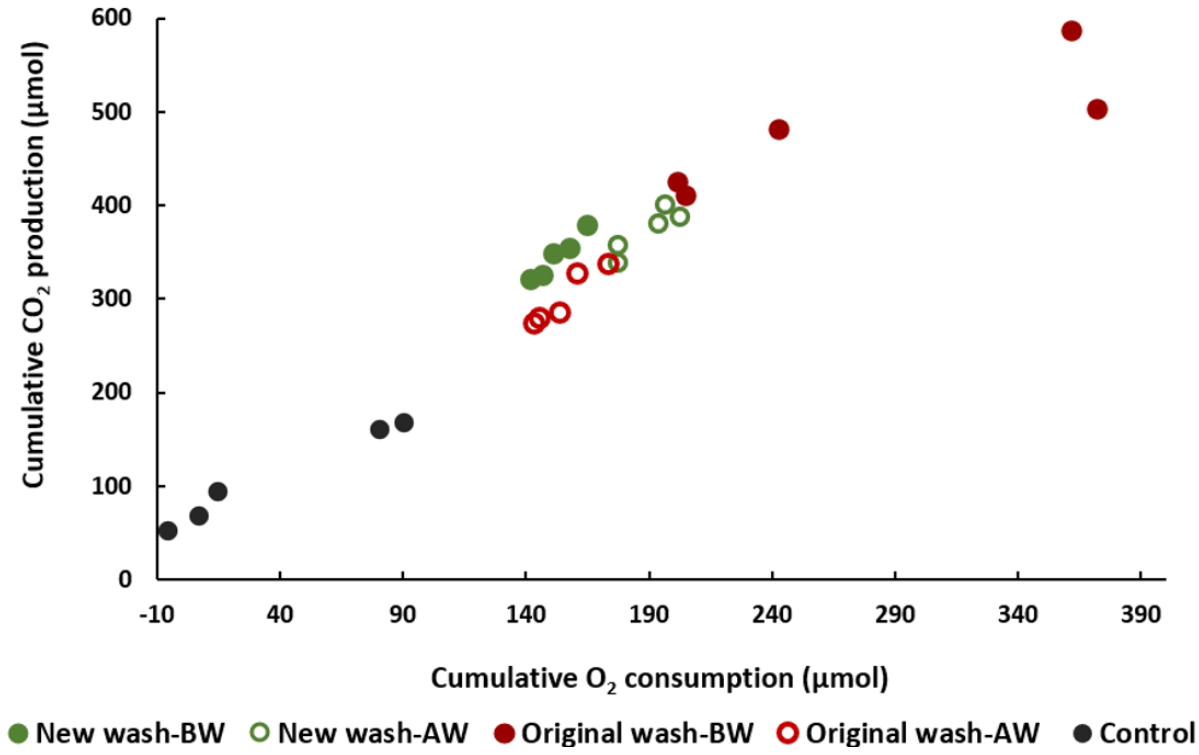


Figure 3.15. Final cumulative concentrations (µmol) of CO_2 production against O_2 consumption from robotized incubation experiment with Leca biofilm from nitrifying filters at VEAS. There were run five replicate vials for Leca biofilm sampled before (BW) and five replicate vials for Leca biofilm sampled after (AW) wash from both PHA7 (new washing procedure) and PHA8 (original washing procedure). There was also used five replicate vials for control with scrubbed Leca and autoclaved and distilled water. Final cumulative concentrations for all replicates are shown.

Leca biofilm exposed to the new washing procedure after the process of washing showed the highest oxygen consumption rate for the first five hours, which could be explained by the high nitrification rate (Figure 3.16.). The oxygen consumption rate peaked during the first five hours of the experiment for all treatments and decreased for the next 20 hours. The decrease was similar for all treatments except from Leca biofilm exposed to the original washing procedure sampled prior to washing, which showed a higher oxygen consumption rate from ten hours and throughout the rest of the experiment. These results indicate a higher heterotrophic activity in these Leca biofilm samples. After 20 hours the oxygen consumption in Leca biofilm exposed to the new washing procedure (both before and after wash) and the original washing procedure after the process of washing, converged towards the oxygen consumption rate observed in the experimental control. The control showed low biological activity by the consumption of oxygen. The Leca stones in the control were only scrubbed and not sterile and could explain the low O_2 consumption rate.

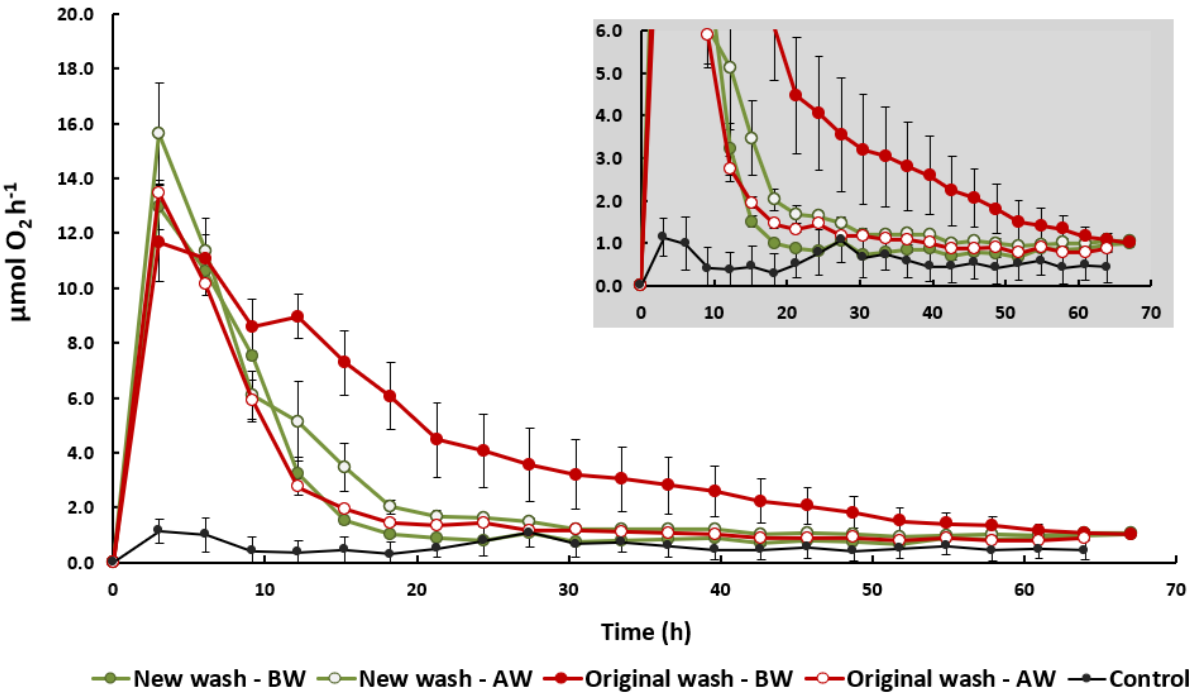


Figure 3.16 Oxygen consumption rate (µmol h^{-1}) against time (h) from the robotized incubation experiment with Leca biofilm from nitrifying filters at VEAS. Time zero equals start of the experiment with a start concentration of 20 mL O_2. There were run five replicate vials for Leca biofilm sampled before (BW) and five replicate vials for Leca biofilm sampled after (AW) wash from both PHA7 (new washing procedure) and PHA8 (original washing procedure). There was also used five replicate vials for control with scrubbed Leca and autoclaved and distilled water. Average was calculated from five replicate vials for each treatment. Error bars represent standard error.

After 20 hours the CO_2 production and O_2 consumption rate declined and converged against similar values for all treatments (Figure 3.17.). As earlier mentioned, Leca biofilm exposed to the original washing procedure before wash showed higher heterotrophic activity compared to the other treatments. Heterotrophic activity has an oxidation of carbohydrates with 1:1 molar ratios of O_2 : CO_2, which could explain the activity seen after 20 hours. The results indicate solely heterotrophic activity after 20 hours for Leca biofilm exposed to both washing procedures.

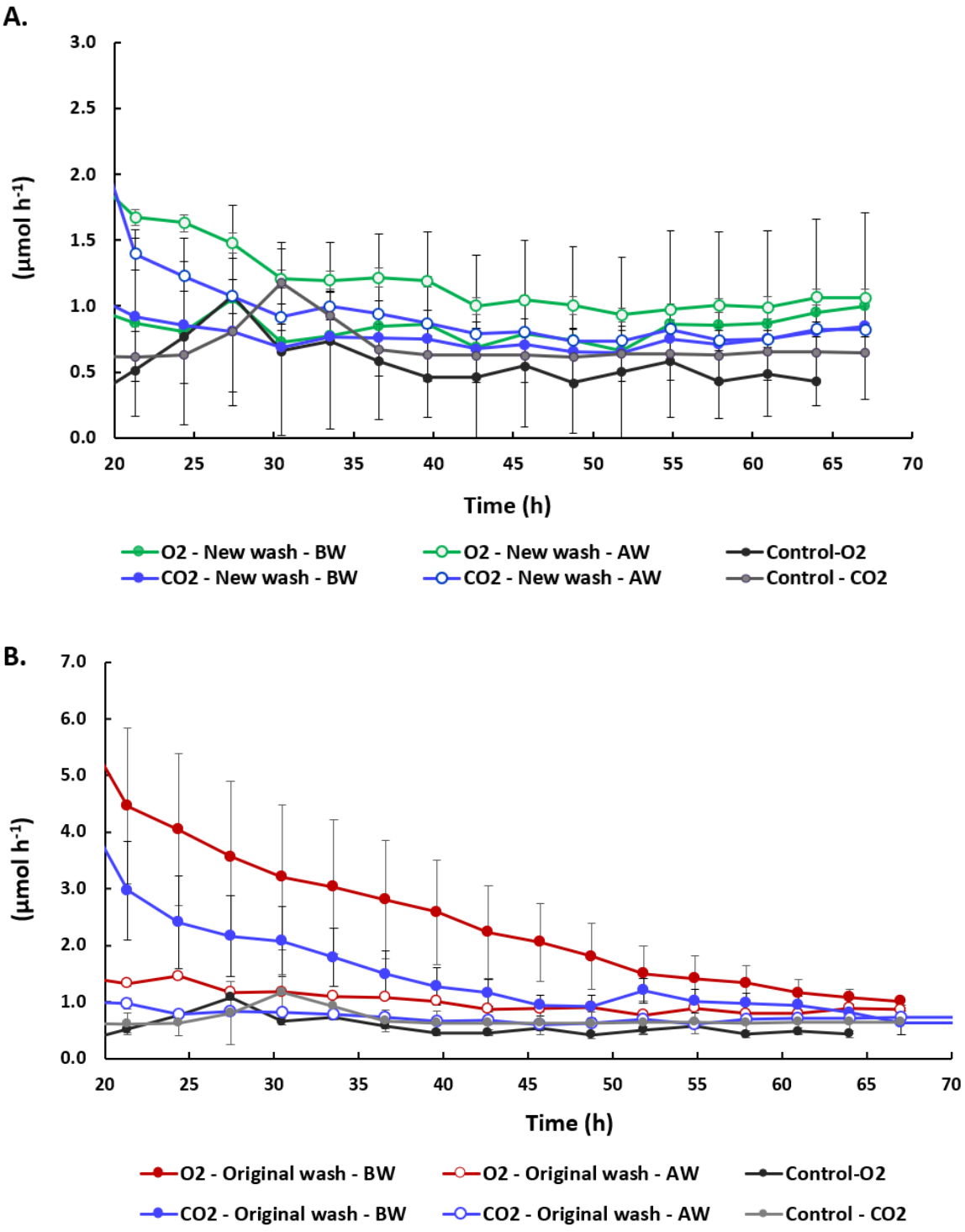


Figure 3.17 O_2 and CO_2 consumption rate (µmol h^{-1}) against time for robotized incubation experiment with Leca biofilm from nitrifying filters at VEAS from time 20 h after start of the experiment. There were run five replicate vials for Leca biofilm sampled before (BW) and five replicate vials for Leca biofilm sampled after (AW) wash from both PHA7 (new washing procedure) and PHA8 (original washing procedure). There was also used five replicate vials for control with scrubbed Leca and autoclaved and distilled water. Average was calculated from five replicate vials for each treatment. Error bars represent standard error. (A.) Leca biofilm samples exposed to the new washing procedure before (BW) and after (AW) wash from PHA7. (B.) Leca biofilm exposed to the original washing procedure BW and AW from PHA8.

3.3.3 The potential of denitrification under aerobic conditions in Leca biofilm from nitrifying filters at VEAS

To quantify the potential of denitrification under aerobic conditions in Leca biofilm from nitrifying filters at VEAS, concentrations of N_2 and N_2O gas was measured during the robotized incubation experiment (Figure 3.18.). Measurements done after 40 hours are not included due to a leakage of nitrogen into the system, which made these measurements unreliable.

Average concentrations of N_2-N and N_2O-N in Leca biofilm before washing exposed to the original washing procedure was respectively 61- and 86 % higher at 40 hours compared with the other treatments. The high N_2O-N concentrations indicated partial denitrification in Leca biofilm exposed to the original washing procedure when sampled before wash (Figure 3.18.A.). Large standard errors in these measurements of N_2O-N concentrations resulted in unsignificant difference between Leca biofilm samples exposed to the new and original washing procedure (One-way ANOVA, $p = 0.344$). The production of N_2-N was seen significantly higher in Leca biofilm exposed original wash before the washing procedure compared with Leca biofilm from the other treatments (One-way ANOVA, $p = 0.040$) (Figure 3.18.B.). These results confirmed that it occurred denitrification throughout the experiment in Leca biofilm communities exposed to the original wash before the process of washing.

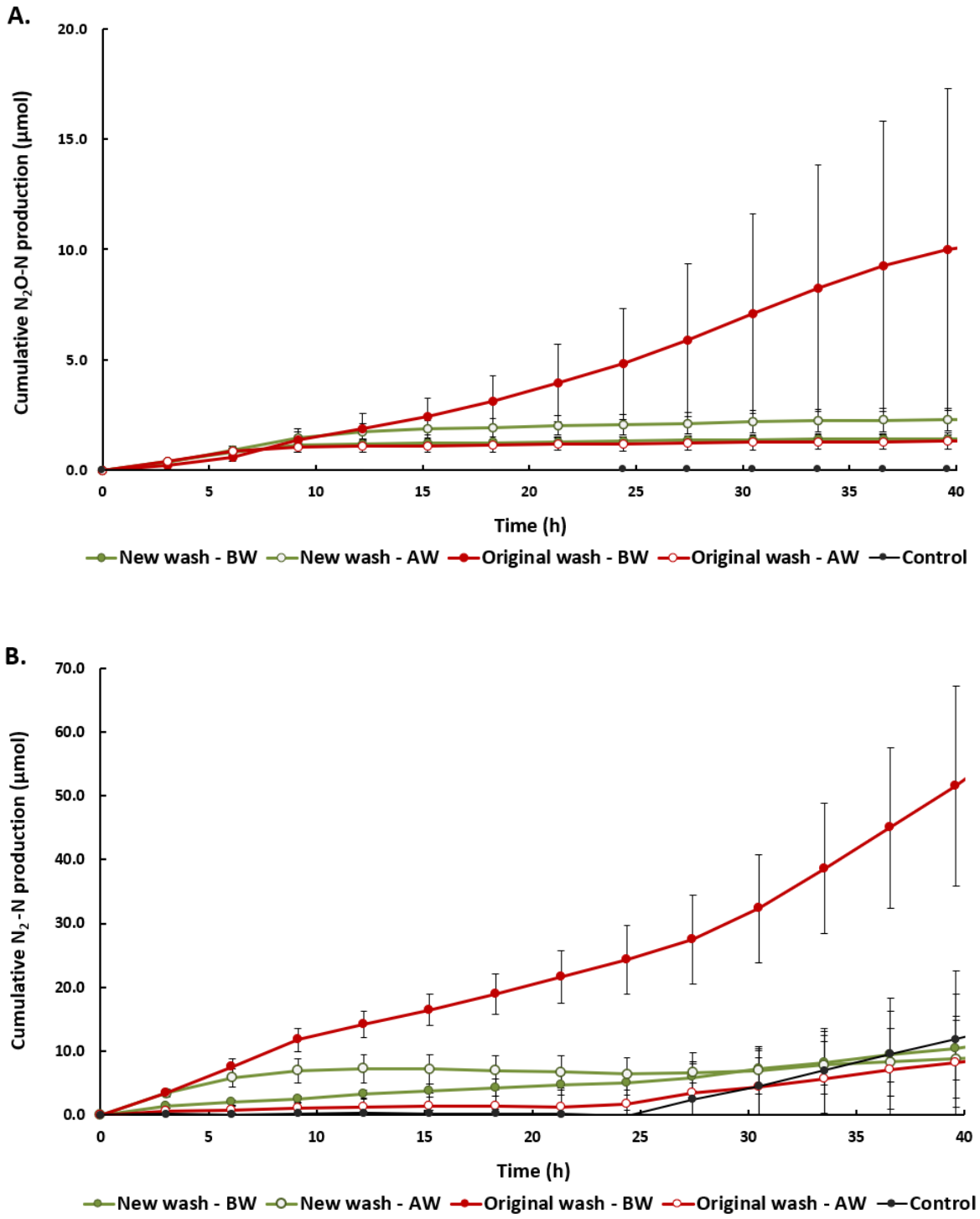


Figure 3.18. Average cumulative concentrations of N_2-N and N_2O-N production against time (h) from robotized incubation experiment with Leca biofilm from nitrifying filters at VEAS. Time zero equals start of the experiment. There were run five replicate vials for Leca biofilm sampled before (BW) and five replicate vials for Leca biofilm sampled after (AW) wash from both PHA7 (new washing procedure) and PHA8 (original washing procedure). There was also used five replicate vials for control with scrubbed Leca and autoclaved and distilled water. Average was calculated from five replicate vials for each treatment. Error bars represent standard error. Measurements after 40 hours are not included due to leakage of nitrogen into the system, which made these measurements unreliable. (A.) Average cumulative N_2O-N production (µmol) against time (h). (B.) Average cumulative N_2-N production against time (h).

At the start of the robotized incubation experiment there was observed a peak in N_2 and N_2O production from Leca biofilm exposed to both the new and original washing procedure (Figure 3.19.). This could be explained by anoxic conditions in parts of the biofilm due to the high oxygen consumption rate of nitrifying bacteria for the first ten hours. The N_2 and N_2O production continued in the Leca biofilm sampled prior to washing with the original washing procedure after the first five to 15 hours, while it decreased in the other treatments. This suggests zones with oxygen limitations in the Leca biofilm exposed to the original washing procedure before washing throughout the experiment, which led to the initiation of the process of denitrification.

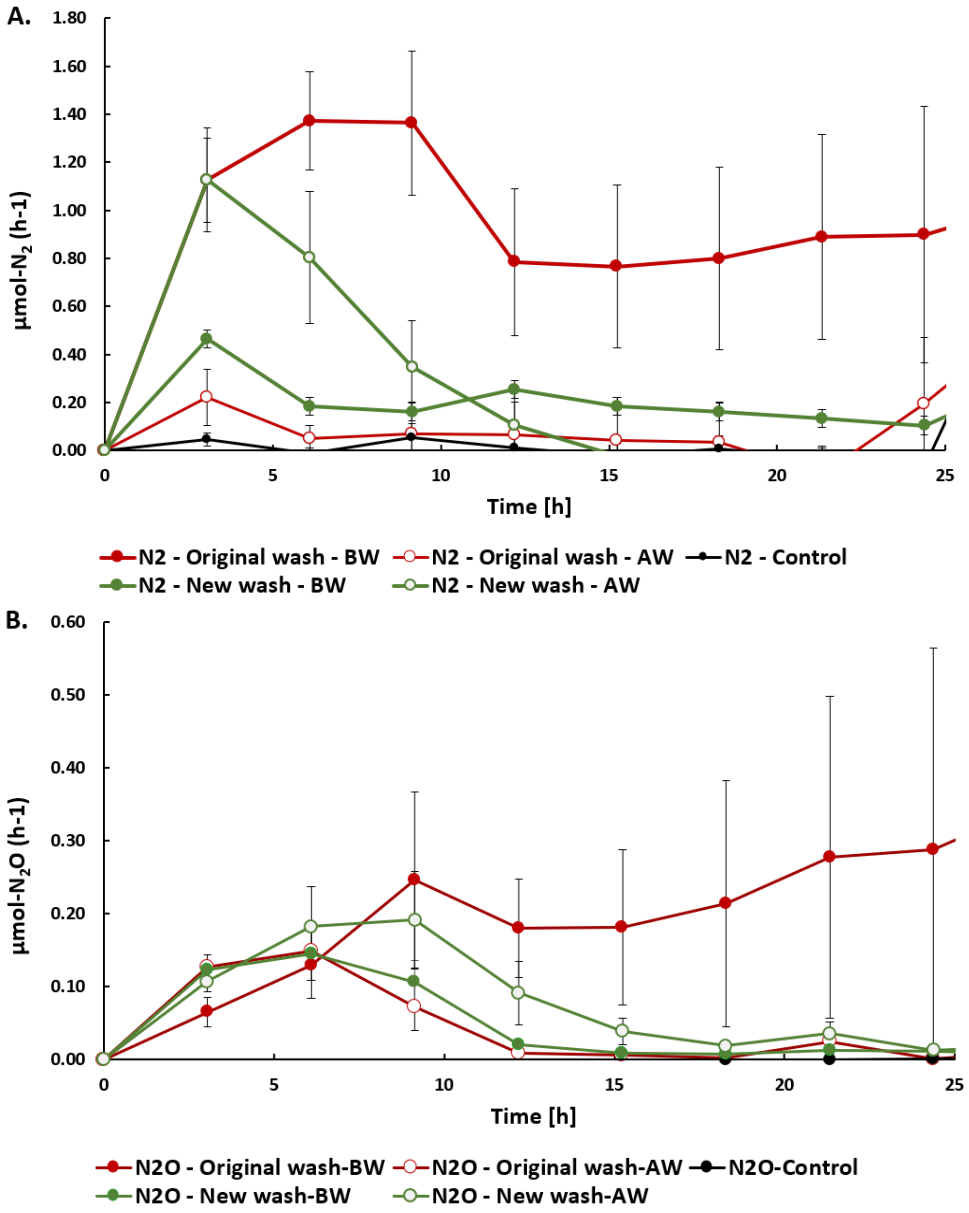


Figure 3.19. Production rate of N_2-N and N_2O-N against time for the first 25 hours of the robotized incubation experiment with Leca biofilm from VEAS. Time zero equals start of the experiment. There were run five replicate vials for Leca biofilm sampled before- (BW) and five replicate vials for Leca biofilm sampled after (AW) wash from PHA7 (new wash) and PHA8 (original wash) and five replicate vials for control with scrubbed Leca, and autoclaved and distilled water. Average was calculated from five replicate vials for each treatment. Error bars represent standard error. (A.) Production rate of N_2 against time. (B.) Production rate of N_2O against time.

The production of N_2O was present for the first 20 hours in Leca biofilm exposed to both the new and original washing procedure as seen above in Figure 3.19. To investigate how much of the N_2O production that could be explained by the activity of AOB, the yield of N_2O was calculated (Table 3.3.). The rule of thumb is that 0.1-1 % of N_2O production could be explained by the oxidation of AOB. All calculated yields were above 1%, which suggest that the concentration of produced N_2O was due to both the process of nitrification and denitrification for the first 20 hours of the robotized incubation experiment. The production yield of N_2O was increasing by 72% in Leca biofilm between two washing events with the original washing procedure. By contrast, Leca biofilm exposed to the new washing procedure had a decrease by 31 % of N_2O production between two washing events. This indicates that the new washing procedure leads to an increase in N_2O production in biofilm community after washing, whereas the original washing procedure has a gradual increase in production of nitrous oxide between two events of washing.

Table 3.3. The production rate of N_2O-N per vial per hour (µmol N_2O-N vial $^{-1}$ h^{-1}) and the yield (Y) of N_2O-N from the process of ammonia oxidation for the first 20 hours of the robotized incubation experiment with Leca biofilms.

	New wash		Original wash	
	Before	**After**	**Before**	**After**
N_2O-N (µmol N_2O-N vial $^{-1}$ h^{-1})	0.064	0.1	0.197	0.06
Y N_2O-N (%)	2.0	2.9	6.7	1.9

4 Discussion

4.1 The effect of washing procedures and temporal variations in bacterial community composition

The main aim of this study was to investigate the effect of the new and original washing procedure on Leca biofilm communities in nitrifying filters at VEAS, and additionally assess other factors that may affect the Leca biofilm. Beta diversity metrices indicated that the biofilm changed over time and that the different washing procedures had an effect on the bacterial community composition in Leca biofilms. A PERMANOVA test showed that the differences in Bray Curtis dissimilarity was significant between Leca biofilm communities in PHA7 (new washing procedure) and PHA8 (original washing procedure). This suggest that the new and original washing procedure led to differences in bacterial community composition in the Leca biofilms. zOTU1 which was classified to *Nitrospira* explained 7% of the observed differences in Leca biofilm exposed to the new and original washing procedure. zOTU1 was generally slightly more abundant in samples exposed to the new wash and indicate that the original washing procedure may reduce the relative abundance of NOB in the biofilm.

In addition to the effect of the washing procedures, the bacterial community composition in Leca biofilm showed temporal variations both in PHA7 and PHA8. Temporal changes were shown to be largest from April to August and August to September (Figure 3.2. and Figure 3.3.). The observed changes in community composition were most evident for the PHA7 samples. The alfa diversity metrices also indicated a shift in bacterial community composition in PHA8 from June to August and in PHA7 from August to September. The shift in bacterial community composition was accompanied by a decrease in evenness for both PHA7 and PHA8, due to an increase in relative abundance of zOTU1 (*Nitrospira*).

Jose et al. [53] showed that there was a decrease in ammonium mass loadings by 37% from June to July in wastewater entering VEAS in 2016. This decrease was explained by a reduction of inhabitants connected to the wastewater plant by 35 % due to the summer holiday. The ammonium concentrations were seen to gradually increase from the middle of July until the start of September [53]. A similar situation was observed for the wastewater treated at VEAS during the summer in 2020 (Appendix J) and may explain the shift in bacterial community composition seen from April to August and August to September. Changes in composition of incoming wastewater might have caused the selective pressure to change in the Leca biofilm communities. This suggest that the observed decrease in diversity is explained by a competitive advantage for some bacteria, which consequently dominated the biofilm community.

To further investigate the temporal shift in bacterial community composition the average relative abundance of nitrifiers were calculated for all samples taken monthly (Figure 3.9.). Nitrifiers in the Leca biofilm exposed to both the new and original washing procedure gradually increased from April to September, except in all Leca biofilm samples from PHA7 taken in August (20.08). The average abundance of nitrifiers were five times lower in these samples from PHA7 (new wash) in August compared with the samples taken from PHA8 (original wash). For all other dates, the relative abundance of nitrifiers were slightly higher in samples from PHA7 than in PHA8 (Figure 3.9.). It was assumed that the wastewater entering PHA7 and PHA8 was similar, and the only known difference between the nitrifying

filters in these two process halls were the washing procedures. It is therefore reasonable that the observed differences in Leca biofilms are related to the washing procedures. This implicate that the new washing procedure in PHA7 led to a biofilm community more vulnerable for out-competition of nitrifiers by heterotrophic bacteria during low ammonia concentrations from July to August.

4.2 The immediate effect of washing on Leca biofilm communities

The short-term effect of washing on Leca biofilm communities was investigated during June and the start of July, and in this period different variants of the new washing procedure was tested. This was done by exposing Leca biofilms to the original washing procedure and the new washing procedure with one, three and five repetitions of step D2 to D6 (Table 2.1. and Table 2.2.) and taking samples for community analysis directly prior and after the conducted washing procedure.

PCoA, calculations of average Bray Curtis similarities and alfa diversity metrices indicated that the immediate effect of the different washing procedures were small on the Leca biofilms (Figure 3.7., Figure 3.8. and Figure 3.9.). However, it was observed that the alfa diversity was higher for the Leca biofilm exposed to the new wash with five repetitions of step D2-D6 compared with the other washing procedures. The lowest exponential Shannon's index was observed in Leca biofilm exposed the new washing procedure with one repetition before wash. The results indicate that longer washing procedures with multiple repetitions of draining followed by scouring with water and air led to a higher diversity in Leca biofilms. This might be explained by a lower evenness in the outer layers of the biofilm, which was removed by washing.

The relative abundance of zOTUs representing nitrifying bacteria was calculated to investigate if the observed differences in both alfa- and beta diversity measurements could be related to the abundance of nitrifying bacteria in the Leca biofilm (Figure 3.10). The relative abundance of nitrifying-zOTUs in Leca biofilm was generally lower directly after wash than before wash. The relative abundance was highest in the Leca biofilm exposed to the new washing procedure with one repetition of step D2-D6 and lowest in the new washing procedure with five repetitions. The exponential Shannon's index and evenness was highest in the samples exposed to the new washing procedure with five repetitions (Figure 3.8.) and could be explained by the low abundance of *Nitrospira* in these samples. This suggest that it was mainly the abundance of *Nitrospira* which was reduced due to this washing procedure. These findings might explain the small differences observed in alfa diversity between the different washing procedures. The results also suggest that the different washing procedures probably removed nitrifying bacteria from the Leca biofilm during the process of washing. It also indicates that longer washing procedures with multiple repetitions of draining followed by scoring with water and air might lead to higher loss of nitrifying bacteria. The removal of nitrifying bacteria can potentially lead to a reduction of nitrifying capacity, which would not be beneficial to the water treatment process.

The nitrate production rate was assessed in small batch experiments at VEAS for the Leca biofilm communities exposed to the original washing procedure and the new washing procedure with one, three and five repetitions of step D2 to D6 in the procedure of washing. Leca biofilm used for the experiment were sampled directly prior and after the process of

washing to examine the short-term effect of washing. In the Leca biofilm sampled directly prior and after the washing procedure it was observed large variations in nitrate production rate (Figure 3.12.). The results showed highest variability between the new washing procedure with one and three repetitions of step D2-D6 and the original washing procedure.

The Leca biofilm exposed to the new washing procedure with five repetitions had the lowest nitrate production rate and the lowest variation in production rate. These measurements are in accordance with the low average relative abundance of nitrifiers in Leca biofilm exposed to this washing procedure (Figure 3.10.). This indicates that the new washing procedure with five repetitions of sequence D2-D6 might lead to a reduction of nitrifying biomass and loss of nitrifying capacity. These results are beneficial in an operational point of view since this washing procedure would have led to the highest energy consumption and highest cost, due to the amount of air and water needed. The new washing procedure with one repetition did not have a significantly lower nitrate production compared to the new wash with three repetitions or the original washing procedure (Figure 3.12.). These results in addition to the high average relative abundance of nitrifiers in biofilm exposed to the new washing procedure with one repetition indicates that a low number of repetitions of sequence D2-D6 should be the preferred washing procedure.

4.3 The effect of depth in nitrifying filters on Leca biofilm communities

To assess the effect of different depths in the nitrifying filters on Leca biofilm communities, the Leca biofilm was sampled at four depths in the nitrifying reactors at VEAS. PCoA based on Bray Curtis similarities indicated that bacterial community composition in Leca biofilm samples were more similar at different depths from the same nitrifying filter than at the same depth in different nitrifying filters (Figure 3.5.). The observed differences in community composition in biofilm were smallest between different depths for samples taken in PHA8 exposed to the original washing procedure. Still, an interesting finding was that samples taken at the top of the filter in both PHA7 (new wash) and PHA8 (original wash) had the highest exponential Shannon's index (Figure 3.6.). Exponential Shannon's index was decreasing for each depth further down in the nitrifying filter in PHA8.

Untreated wastewater and air are introduced through the bottom of the filter and flow upward through the Leca carriers. Therefore, it was assumed that the deeper layers of the biofilter would be subjected to higher concentrations of ammonia, organic carbon, and oxygen than the top layer which would affect the biofilm community. High substrate concentrations promotes bacterial growth, which may potentially lead to thicker biofilms [54]. These thick biofilms might contain larger variations of microbial populations compared to thinner biofilms, due to steeper concentration and redox gradients within the biofilm community [55]. It was therefore believed that there would be an increase in biodiversity at lower depths in the nitrifying biofilters due to a thicker biofilm on the Leca carriers. The contradictory results with lower exponential Shannon's index at lower depths, hence lower diversity, might be explained by the increasing concentrations of organic carbon at these depths. This could have led to the dominance of a few rapid-growing heterotrophic species in theses biofilm communities. A biofilm community dominated by a few species would lead to a decreased evenness and therefore a lower diversity in Leca biofilm at lower depths in the nitrifying filters.

4.4 zOTUs representing nitrifiers in the Leca biofilm samples

The most abundant zOTU in the dataset based on Leca biofilm samples was zOTU1 which was classified to the genus *Nitrospira*. Several zOTUs were classified to the genus *Nitrospira* and represented an average relative abundance of 13% in samples taken monthly from the nitrifying biofilters. Two zOTUs were classified to *Nitrotoga* and this was the only additional NOB genera identified in the dataset. These *Nitrotoga*-zOTUs had a lower abundance than *Nitrospira,* and *Nitrospira* was represented by an average of 38 times more reads than *Nitrotoga. Nitrotoga* is seen to have a competitive advantage with high nitrite concentrations and neutral pH [56, 57], which makes it reasonable to assume low nitrite concentrations in the nitrifying filters at VEAS. zOTUs representing *Nitrobacter* was not found in the dataset. This agrees with the assumption of low NO_2 concentrations in filter due to high *Nitrospira*/*Nitrotoga* ratio since *Nitrobacter* do not thrive at low nitrite concentrations [58]. The high abundance of *Nitrospira* is in accordance with previous observations in municipal wastewater treatments done by Daims et al. [59] and Spasov et al. [60].

Ammonium oxidising bacteria were represented by zOTUs classified to *Nitrosomonas* and *Nitrosospira*. *Nitrosomonas* was the most abundant AOB. This could be explained by the high optimum growth rate of *Nitrosomonas* of 0.088 h^{-1} compared to the maximum growth rate of 0.035h^{-1} of *Nitrosospira* [61]. The abundance of NOB zOTUs was 13 times higher than AOB zOTUs in the Leca biofilm communities. This is contradictory with the theoretical NOB/AOB ratio which can be calculated to approximately 0.5 since the biomass yield of NOB is expected to be around two times lower than AOB per unit of nitrogen. There are generated two electrons by the oxidation of nitrite to nitrate by NOB. This is three times less than electrons generated by the oxidation of ammonium to nitrite by AOB [62].

An explanation for the high NOB/AOB ratio might be that the ammonium oxidization was performed by ammonium oxidising archaea in the Leca biofilm. The primer sequences used for targeting 16s rRNA in this analysis were not suitable for amplification of archaeal sequences. In addition, reads less than 390 base pairs were sorted out in the DNA processing (Section 2.6), which would have discarded potential coamplified archaeal sequences. Mao et al. [31] attempted to clone archaeal *amoA* sequences from Leca biofilm at VEAS, but the results indicated that the abundance of previously known AOA was insignificant in the Leca biofilms from the nitrifying filters. This suggests a low probability of a high abundance of AOA in the Leca biofilms from VEAS. By contrast, if the low NOB/AOB ratio is explained by the presence of AOA in the Leca biofilm these archaea might not have been previously described.

Another explanation for the high NOB/AOB ratio may be a so cold nitrite loop, where a nitrite oxidation/nitrate reduction loop takes place [63, 64]. Incomplete denitrification from nitrate to nitrite can lead to the accumulation of nitrite, which can be reused by NOB and to some extent uncouple the growth of NOB from AOB [63]. A third theory is that the NOB in the nitrifying biofilters possess a higher biomass yield due to mixotrophic metabolism [65] that might lead to an increased NOB/AOB ratio as well.

Another interesting explanation for the high NOB/AOB ratio might be the process of complete ammonia oxidation by *Nitrospira*. Daims et al [66] discovered and cultivated the first completely nitrifying bacteria from the genus *Nitrospira*. To assess the relationships between *Nitrospira* sequences retrieved from Leca biofilm communities and already described *Nitrospira* strains a maximum likelihood phylogenetic tree was built. None of the

zOTUs from Leca biofilm at VEAS clustered with the already known comammox bacteria (*Candidatus N. nitrificans, Candidatus N. inopinata*). This indicated that the zOTUs were not related to comammox bacteria and most likely do not have the ability of complete ammonia oxidation. The phylogenetic analysis revealed that the highly abundant zOTU1 (*Nitrospira*) was found to be related to *Nitrospira (N.) Salsa*. *N. Salsa* is a marine nitrite oxidising *Nitrospira* species isolated from sea water [67]. zOTU1 was also grouped in the same clade as *N. marina. N. marina* is known to reach optimal grow in mixotrophic media [68], which is interesting in relation with the hypothesis about mixotrophic metabolism in NOB.

4.5 The effect of the new and original washing procedure on microbial activity with analysis of gas kinetics

To investigate differences in nitrification efficiency, the potential of denitrification and activity of microbial community in Leca biofilm exposed to the new and original washing procedure, a robotized incubation system was used. This robotized system monitored and analysed the composition of gasses emitted from the Leca biofilm during a period of 68 hours. The rate of nitrification was highest in Leca biofilm exposed to the new washing procedure both before and after the process of washing, compared to the Leca biofilm exposed to the original washing procedure (Figure 3.13. and Table 3.1.). This is in accordance with the results from the small-scale batch experiments (Figure 3.12.) where the nitrate-production was slightly higher in Leca biofilm exposed to the new washing procedure (three repetitions of step D2-D6). In Leca biofilm exposed to the original washing procedure the rate of nitrification was lowest before the process of washing, which is contrary to the results from the small-batch experiment were the nitrate-production rate in Leca biofilm was slightly higher prior to washing.

NO_2 concentrations reached approximately zero for all treatments after 20 hours (Figure 3.13.). This indicate that the oxidation of ammonia stopped and therefore the process of nitrite oxidation to nitrate got substrate limited. The addition of ammonia at 25 hours to the vials did not affect the concentration of nitrite and indicated that the process of ammonia oxidation by AOB was not substrate limited. It is reasonable to assume that the process of nitrification was inhibited by a drop in pH. Nitrification consumes alkalinity [69] and since the media used in this experiment was not pH buffered, it is reasonable that a drop in pH would have occurred. Unfortunately, the pH was not measured in the media before or after the experiment, so this hypothesis cannot be confirmed.

Princic et al. [70] found that the process of nitrification was inhibited at pH below 5.8 in a culture of nitrifiers from wastewater, while Ruiz et al. [71] found that pH lower than 6.45 completely inhibited the processes of both AOB and NOB. A pH of 7 was assumed at the start of the experiment and a drop in pH to below 6.45 would be expected based on the amount of oxidised ammonia in the experiment. Another possible explanation is that the wastewater entering VEAS has a pH of 7.4 [44] and the process of nitrification at VEAS is therefore conducted at the limits of optimum nitrification which is showed to be at pH 7.3-8.0 [72]. This can potentially lead to a selection of nitrifying bacteria which are sensitive to changes in pH and as a result small changes can inhibit the process of nitrification.

A decrease of pH in the media is also substantiated by the low ratio of O_2 consumption against CO_2 production (Figure 3.15). The oxygen consumption for the first 20 hours were

solely used in the process of nitrification (Table 3.1.) and the CO_2 production was therefore expected to be much lower. The high concentrations can probably be explained by the lowering in pH which would have caused acidification of the medium. The CO_2 was calculated assuming constant pH of 7 and would therefore lead to an overestimation. The concentration of CO_2 and carbonate are calculated as a function of temperature and pH [46] and is therefore dependent on stable chemistry.

The estimated velocity of ammonium oxidation (*Vamo*) was used to calculate the expected oxygen consumption for the process of ammonium oxidation (VO_2_*amo*) during the first 20 hours (Table 3.2.). Approximately all the consumed oxygen was found to be accounted for by the process of nitrification in Leca biofilm exposed to the new washing procedure both before and after the process of washing. Likewise, the Leca biofilm sampled after the process of washing with the original washing procedure, all oxygen was used for the oxidation of ammonium during the first 20 hours. The observed oxygen consumption was distinctly higher in the Leca biofilm exposed to the original washing procedure before wash, but only 66 % of the oxygen was used for the oxidation of ammonia. These results suggests that the unwashed biofilm exposed to the original washing procedure had higher heterotrophic activity compared to Leca biofilm from the other treatments.

The rate of oxygen consumption and carbon dioxide production converged against similar values after 15 to 20 hours (Figure 3.17.). This might be explained by heterotrophic respiration with an oxidation step of one, which corresponds to the oxidation of organic material (CH_2O) with one mole O_2 consumed per one mole CO_2 produced [73] for all Leca biofilm samples after 20 hours. As stated in the section above, the heterotrophic activity was found to be higher in Leca biofilm from the original washing procedure before wash. This indicates that the original washing procedure may lead to a higher abundance of heterotrophic bacteria over time than Leca biofilm exposed to the new washing procedure. This is not favourable since the growth of heterotrophic bacteria could outcompete the autotrophic nitrifiers, leading to a poorer process of nitrification [74, 75]. This is especially important at VEAS where there is no aerobic oxidation of organic carbon prior to the nitrifying filters. This leads to high concentrations of organic carbon entering the nitrification filters, which can increase the competitive advantages for heterotrophic growth.

4.5.1 The potential of denitrification in Leca biofilm under aerobic conditions

In the robotized incubation experiment monitoring gas kinetics, the production of N_2 and N_2O was 61- and 86 % higher at 40 hours in Leca biofilm samples from PHA8 (original wash) before wash compared to the other treatments (Figure 3.18.). N_2O concentrations were not significantly higher in the original washing procedure before wash compared to the other treatments. However, the nitrous oxide concentrations were distinctly higher than what would be expected from N_2O production by AOB alone and may be explained by the process of denitrification. The measured concentrations of N_2 were significantly higher in Leca biofilm from the original washing procedure before wash and indicates that the process of denitrification took place during the whole experiment. This suggest that the original washing procedure led to the development of thicker Leca biofilm between two washing events compared to the new washing procedure. Thicker biofilms can contain anoxic microenvironments that the denitrifying bacteria can occupy [39].

Leca biofilm exposed to both washing procedures had a peak in production rate of N_2-N for the first five hours (Figure 3.19.), which suggests that denitrification was driven by the process of nitrification. There was a high oxygen consumption at the start of the experiment (Figure 3.16) which was calculated to be solely from the process of nitrification based on calculations of V_{amo} (Table 3.2.). This probably led to anoxic microenvironments in other parts of the biofilm where denitrification as a result took place. In Wang et al. [76] this was also found to be the case in a moving bed sequencing reactor where an increasing rate of nitrification sustained the anoxic microenvironment in the inner parts of the biofilm were denitrification took place. Production of N_2 stopped between 10 and 15 hours in samples from the new washing procedure, which substantiate the theory of nitrification driven denitrification since the activity of NOB and AOB were highest for the first 20 hours.

A rule of thumb is that 0.1-1 % of N_2O production might be explained by the ammonia oxidation by AOB [77]. To investigate how much of the N_2O production that could be explained by AOB, the expected yield of N_2O was calculated (Table 3.3.). All calculated yields were above 1%, which suggest that the process of denitrification took place in all biofilm communities at the start of the experiment independently of the washing procedures. N_2O production yield was higher for Leca biofilm samples taken before wash than after from PHA7 (new washing procedure). Mao et al. [31] found that Leca particles with intact biofilm accumulated NO_2 and NO at one to two orders lower compered to dispersed biofilm. They speculated that the low emission of NO_2 an NO lies in the compound's induction of the expression of the process of denitrification in adjacent part of the biofilm compared to a dispersed biofilm. These findings may explain the elevated N_2O production ratios in the washed Leca biofilm compared with the unwashed biofilm; suggesting that the new washing procedure decreased the robustness and architecture of the biofilm directly after washing. The decreased integrity of the biofilm might have led to a weaker induction of denitrification in the washed Leca biofilm and therefore an increased production of N_2O after washing.

In summary, Leca biofilm exposed to the new washing procedure generally showed a higher stability between two washing events in O_2 consumption, CO_2 production and the rate of nitrification. The process of denitrification was present in Leca biofilm exposed to both washing procedures, due to the high yield of N_2O which could not solely be explained as a by-product from the process of nitrification. The distinctly higher CO_2 production, O_2 consumption, production of N_2O and N_2, in Leca biofilm between two washing events indicate a less efficient washing procedure with the original washing. Elevated oxygen demands in Leca biofilm can lead to higher capital, energy and operational costs and is therefore not favourable in an environmental or operational perspective [20]. The results substantiate the importance of a well-functioning washing procedure of nitrifying biofilters, that preserves the integrity and robustness of the biofilm during washing. This can potentially enhance the removal of ammonia from wastewater and in addition lower the N_2O emission.

4.6 Further work

The impact of different washing procedures on Leca biofilm communities should be further investigated in a long-term perspective. In further studies there should be taken samples over a longer period than what was presented in this study to assess the effect of washing procedures and seasonal variations on bacterial community composition. The monthly samples were sampled once a month only from April to September, lacking samples from July, and the autumn and winter months, which creates an uncertainty to the reproducibility of the observed temporal variations in Leca biofilm. It will also be beneficial to use specific marker genes for nitrifiers to get a higher phylogenetic resolution on strain and species level than what was obtained in this study with the 16S rRNA gene as target gene. Ammonia oxidisers can be quantified using *amoA,* encoding the alpha subunit of ammonia monooxygenase, as a marker gene [78]. The genes *nxrA* or *nxrB,* encoding alpa or beta subunit of the nitrite oxidoreductase, cold be used for the detection and quantification of NOB [78, 79]. This may potentially give a clearer answer to the high NOB/AOB ratio observed in the Leca biofilm communities.

Nitrous oxide (N_2O) has a greenhouse gas potential which is 300 times higher than carbon dioxide and is shown to be a large contributor to the carbon footprint of wastewater plants [80]. At VEAS the nitrification reactors are proved to be the most potent source of N_2O to the environment [44]. The elevated concentrations of N_2O production in Leca biofilm after the process of washing with the new washing procedure, is therefore an interesting aspect and could be further investigated if this washing procedure is implemented in all the nitrifying filters at VEAS.

As stated earlier, optimalisation of the washing procedure on nitrifying filters can be favourable in a financial and environmental perspective. The kinetic experiments indicated that the washing procedure might lead to a bacterial community with high nitrification rate and low oxygen consumption. This is an interesting finding if the washing procedure may lead to a biofilm community with lower oxygen demand. However, there should be taken precaution when extrapolating the results obtained in this study due to the small sample size used in the robotise incubation experiment. To strengthen the results and assess the reproducibility, the robotized incubation experiments should be repeated over several times during the year. This to further assess the observed temporal variability in Leca biofilms exposed to the new washing procedure and the effect of washing over a longer period. Further research should be undertaken to investigate if the washing procedures can be further optimised for the formation of Leca biofilm with low heterotrophic activity and high nitrification efficiency. This can potentially reduce the amount of air needed for an optimal process of nitrification.

Conclusions

This study set out to assess the effects of the new and original washing procedure of nitrifying biofilters at VEAS on bacterial community composition and activity of nitrifying and denitrifying bacteria in Leca biofilm. The bacterial community composition on class level was dominated by Betaproteobacteria, Alphaproteobacteria, Nitrospira and Actinobacteria. The community of nitrifying bacteria had a high abundance of nitrite oxidising bacteria and a low abundance of ammonium oxidising bacteria. The most abundant zOTU representing AOBs was classified to *Nitrosomonas* and the most abundant zOTU representing NOBs was classified to *Nitrospira*. This Nitrospira-zOTU dominated the bacterial community and was by phylogenetic analysis showed to be related to *Nitrospira salsa.*

The results demonstrated that the bacterial community composition changed over time and that the washing procedures affected the composition and diversity of bacterial communities in the biofilm. The analysis of Leca biofilms sampled monthly from March to September showed a very low abundance of nitrifiers in PHA7 (new washing procedure) in August, after a period of low ammonium concentrations in the incoming wastewater. This suggest that the Leca biofilm exposed to the new washing procedure was more susceptible to changes in properties of the incoming wastewater than biofilm exposed to the original washing procedure.

The effect of different repetitions of draining followed by scouring with water and air (step D2-D6) with the new wash showed that there was a notable effect when the number of repetitions was increased; Five repetitions of this step led to a reduced relative abundance of nitrifying bacteria in Leca biofilm compared to a lower number of repetitions. Small-scale batch experiments showed that the reduction of nitrifying bacteria in the biofilm communities led to a reduced nitrification rate between two washing events. These results suggest that it is beneficial with maximum three repetitions of draining followed by scouring with water and air with the new washing procedure on nitrifying filters at VEAS.

The characterisation of bacterial biofilm communities of samples from different depths of the nitrifying filters showed that the sapling depth affected the communities, and that the bacterial composition was more diverse at the top of the filters. The reduced diversity at lower depths might be explained by higher concentrations of organic carbon that probably led to the dominance of a few rapid-growing heterotrophic populations.

The robotized incubation experiment monitoring gas kinetics in Leca biofilm showed that the rate of nitrification was highest in Leca biofilm exposed to the new washing procedure. The process of denitrification was present under aerobic conditions in Leca biofilm exposed to both washing procedures and was driven by the high oxygen consumption of nitrification at the start of the experiment. However, the process of denitrification was significantly higher in Leca biofilm exposed to the original washing procedure in samples taken directly prior to washing and was present independently of the process of nitrification. These results suggest a thicker Leca biofilm community with anoxic zones throughout the experiment. The results of this study indicate that the original washing procedure led to increased heterotrophic activity and reduced nitrification efficiency between two washing events and could therefore be considered less efficient. An implication of these findings is that the new washing procedure potentially led to a more favourable nitrifying biofilm community, with higher rate of nitrification and lower oxygen consumption of heterotrophic bacteria, that may reduce energy consumption and operational costs.

References

1. Jiang, X. and M. Li, *Chapter 5 - Ecological safety hazards of wastewater*, in *High-Risk Pollutants in Wastewater*, H. Ren and X. Zhang, Editors. 2020, Elsevier. p. 101-123.
2. Mareddy, A.R., *12 - Technology in EIA*, in *Environmental Impact Assessment*, A.R. Mareddy, Editor. 2017, Butterworth-Heinemann. p. 421-490.
3. Englande, A.J., Jr., P. Krenkel, and J. Shamas, *Wastewater Treatment &Water Reclamation.* Reference Module in Earth Systems and Environmental Sciences, 2015: p. B978-0-12-409548-9.09508-7.
4. Lee, C.S., J. Robinson, and M.F. Chong, *A review on application of flocculants in wastewater treatment.* Process safety and environmental protection, 2014. **92**(6): p. 489-508.
5. Samer, M., *Biological and chemical wastewater treatment processes.* Wastewater treatment engineering, 2015. **150**.
6. Kim, Y.K., et al., *The capacity of wastewater treatment plants drives bacterial community structure and its assembly.* Scientific Reports, 2019. **9**(1): p. 14809.
7. Krüger, O., A. Grabner, and C. Adam, *Complete Survey of German Sewage Sludge Ash.* Environmental Science & Technology, 2014. **48**(20): p. 11811-11818.
8. Mudge, S.M. and A.S. Ball, *3 - Sewage*, in *Environmental Forensics*, R.D. Morrison and B.L. Murphy, Editors. 1964, Academic Press: Burlington. p. 35-53.
9. Ngatia, L., et al., *Nitrogen and Phosphorus Eutrophication in Marine Ecosystems*. 2019.
10. Thakur, I.S. and K. Medhi, *Nitrification and denitrification processes for mitigation of nitrous oxide from waste water treatment plants for biovalorization: Challenges and opportunities.* Bioresource Technology, 2019. **282**: p. 502-513.
11. Ghafari, S., M. Hasan, and M.K. Aroua, *Bio-electrochemical removal of nitrate from water and wastewater—A review.* Bioresource Technology, 2008. **99**(10): p. 3965-3974.
12. Capodaglio, A., P. Hlavinek, and M. Raboni, *Physico-chemical technologies for nitrogen removal from wastewaters: A review.* Ambiente & Água - An Interdisciplinary Journal of Applied Science, 2015. **Vol 10**.
13. Peña-Haro, S., et al., *Fertilizer standards for controlling groundwater nitrate pollution from agriculture: El Salobral-Los Llanos case study, Spain.* Journal of Hydrology, 2010. **392**(3-4): p. 174-187.
14. Infascelli, R., R. Pelorosso, and L. Boccia, *Spatial assessment of animal manure spreading and groundwater nitrate pollution.* Geospatial health, 2009. **4**(1): p. 27-38.
15. Dubey, N., et al., *The Survivors of the Extreme: Bacterial Biofilms*, in *Microbial Factories: Biodiversity, Biopolymers, Bioactive Molecules: Volume 2*, V.C. Kalia, Editor. 2015, Springer India: New Delhi. p. 161-182.
16. Pal, P., *Chapter 6 - Industry-Specific Water Treatment: Case Studies*, in *Industrial Water Treatment Process Technology*, P. Pal, Editor. 2017, Butterworth-Heinemann. p. 243-511.
17. Yin, W., et al., *Biofilms: The Microbial "Protective Clothing" in Extreme Environments.* International journal of molecular sciences, 2019. **20**(14): p. 3423.
18. van Niel, E.W.J., et al., *Competition between heterotrophic and autotrophic nitrifiers for ammonia in chemostat cultures.* FEMS Microbiology Ecology, 1993. **11**(2): p. 109-118.
19. Rocher, V., et al., *Clogging limitation of nitrifying biofilters: BiostyrDuo® process study.* Water Practice and Technology, 2019. **14**(1): p. 43-54.
20. McCarty, P.L., *What is the Best Biological Process for Nitrogen Removal: When and Why?* Environmental Science & Technology, 2018. **52**(7): p. 3835-3841.
21. Jokela, J.P.Y., et al., *Biological nitrogen removal from municipal landfill leachate: low-cost nitrification in biofilters and laboratory scale in-situ denitrification.* Water Research, 2002. **36**(16): p. 4079-4087.

22. Baskaran, V., et al., *Microbial community profiling of ammonia and nitrite oxidizing bacterial enrichments from brackishwater ecosystems for mitigating nitrogen species.* Scientific Reports, 2020. **10**(1): p. 5201.
23. Könneke, M., et al., *Isolation of an autotrophic ammonia-oxidizing marine archaeon.* Nature, 2005. **437**(7058): p. 543-546.
24. Vijayan, A., et al., *Nitrospira as versatile nitrifiers: Taxonomy, ecophysiology, genome characteristics, growth, and metabolic diversity.* Journal of Basic Microbiology, 2021. **61**(2): p. 88-109.
25. Knowles, R., *Denitrification.* Microbiological reviews, 1982. **46**(1): p. 43-70.
26. Martens, D.A., *DENITRIFICATION*, in *Encyclopedia of Soils in the Environment*, D. Hillel, Editor. 2005, Elsevier: Oxford. p. 378-382.
27. Rittmann, B.E. and P.L. McCarty, *Environmental biotechnology: principles and applications*. 2001: McGraw-Hill Education.
28. Bagchi, S., R. Biswas, and T. Nandy, *Critical Reviews in Environmental Science and Technology Autotrophic Ammonia Removal Processes: Ecology to Technology.* Critical Reviews in Environmental Science and Technology, 2012. **42**: p. 1353–1418.
29. Schmidt, I., et al., *New concepts of microbial treatment processes for the nitrogen removal in wastewater.* FEMS microbiology reviews, 2003. **27**(4): p. 481-492.
30. Wang, X., et al., *One-step start-up and subsequent operation of CANON process in a fixed-bed reactor by inoculating mixture of partial nitrification and Anammox sludge.* Chemosphere, 2021. **275**: p. 130075.
31. Mao, Y., et al., *Functional robustness and gene pools of a wastewater nitrification reactor: comparison of dispersed and intact biofilms when stressed by low oxygen and low pH.* FEMS Microbiology Ecology, 2008. **66**(1): p. 167-180.
32. Zhu, S. and S. Chen, *Effects of organic carbon on nitrification rate in fixed film biofilters.* Aquacultural Engineering, 2001. **25**(1): p. 1-11.
33. Michaud, L., et al., *Effect of particulate organic carbon on heterotrophic bacterial populations and nitrification efficiency in biological filters.* Aquacultural Engineering, 2006. **34**(3): p. 224-233.
34. Flemming, H.-C., et al., *Biofilms: an emergent form of bacterial life.* Nature Reviews Microbiology, 2016. **14**(9): p. 563.
35. Lydmark, P., et al., *Vertical distribution of nitrifying populations in bacterial biofilms from a full-scale nitrifying trickling filter.* Environmental Microbiology, 2006. **8**(11): p. 2036-2049.
36. Liu, T., X. Quan, and D. Li, *Evaluations of biofilm thickness and dissolved oxygen on single stage anammox process in an up-flow biological aerated filter.* Biochemical Engineering Journal, 2017. **119**: p. 20-26.
37. Okabe, S., et al., *Spatial microbial distributions of nitrifiers and heterotrophs in mixed-population biofilms.* Biotechnology and bioengineering, 1996. **50**(1): p. 24-35.
38. He, J., et al., *Community diversity and biofilm characteristic response to low temperature and low C/N ratio in a suspended carrier biofilm reactor.* Desalination and Water Treatment, 2016. **57**(47): p. 22212-22222.
39. Suarez, C., et al., *Thickness determines microbial community structure and function in nitrifying biofilms via deterministic assembly.* Scientific Reports, 2019. **9**(1): p. 5110.
40. Satoh, H., et al., *Macroscale and microscale analyses of nitrification and denitrification in biofilms attached on membrane aerated biofilm reactors.* Water Research, 2004. **38**(6): p. 1633-1641.
41. Ansari, F., et al., *Factors Affecting Biofilm Formation in in vitro and in the Rhizosphere*. 2017. p. 275-290.
42. Chaudhary, D.S., et al., *Biofilter in water and wastewater treatment.* Korean Journal of Chemical Engineering, 2003. **20**(6): p. 1054.
43. Sagberg, P. and K.G. Berg, *Cost optimisation of nitrogen removal in a compact nitrogen and phosphorus WWTP.* Water science and technology, 2000. **41**(9): p. 147-154.

44. Bakken, L., A.K. Marsteng, and S. Nadeem, *Emissions of N2O, CH4, and CO2 from VEAS waste water treatment plant.* 2012. p. 20.
45. Molstad, L., P. Dörsch, and L. Bakken, *Improved robotized incubation system for gas kinetics in batch cultures*. 2016, Technical report.
46. Molstad, L., P. Dörsch, and L. Bakken, *Robotized incubation system for monitoring gases (O2, NO, N2O N2) in denitrifying cultures.* Journal of microbiological methods, 2008. **71**: p. 202-11.
47. Wang, Q., et al., *Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy.* Applied and environmental microbiology, 2007. **73**(16): p. 5261-5267.
48. Nierychlo, M., et al., *MiDAS 3: An ecosystem-specific reference database, taxonomy and knowledge platform for activated sludge and anaerobic digesters reveals species-level microbiome composition of activated sludge.* Water Research, 2020. **182**: p. 115955.
49. Hammer, Ø., D.A. Harper, and P.D. Ryan, *PAST: Paleontological statistics software package for education and data analysis.* Palaeontologia electronica, 2001. **4**(1): p. 9.
50. Tamura, K. and M. Nei, *Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees.* Molecular biology and evolution, 1993. **10**(3): p. 512-526.
51. Kumar, S., et al., *MEGA X: molecular evolutionary genetics analysis across computing platforms.* Molecular biology and evolution, 2018. **35**(6): p. 1547-1549.
52. Daims, H., *The Family Nitrospiraceae*, in *The Prokaryotes: Other Major Lineages of Bacteria and The Archaea*, E. Rosenberg, et al., Editors. 2014, Springer Berlin Heidelberg: Berlin, Heidelberg. p. 733-749.
53. Baz-Lomba, J.A., et al., *Assessing Alternative Population Size Proxies in a Wastewater Catchment Area Using Mobile Device Data.* Environmental Science & Technology, 2019. **53**(4): p. 1994-2001.
54. Ras, M., et al., *Extracellular polymeric substances diversity of biofilms grown under contrasted environmental conditions.* Water Research, 2011. **45**(4): p. 1529-1538.
55. Torresi, E., et al., *Biofilm Thickness Influences Biodiversity in Nitrifying MBBRs—Implications on Micropollutant Removal.* Environmental Science & Technology, 2016. **50**(17): p. 9279-9288.
56. Wegen, S., B. Nowka, and E. Spieck, *Low Temperature and Neutral pH Define "Candidatus Nitrotoga sp." as a Competitive Nitrite Oxidizer in Coculture with Nitrospira defluvii.* Applied and environmental microbiology, 2019. **85**(9): p. e02569-18.
57. Maixner, F., et al., *Nitrite concentration influences the population structure of Nitrospira-like bacteria.* Environmental Microbiology, 2006. **8**(8): p. 1487-1495.
58. Blackburne, R., et al., *Kinetic characterisation of an enriched Nitrospira culture with comparison to Nitrobacter.* Water research, 2007. **41**(14): p. 3033-3042.
59. Daims, H., et al., *Novel Nitrospira-like bacteria as dominant nitrite-oxidizers in biofilms from wastewater treatment plants: diversity and in situ physiology.* Water Science and Technology, 2000. **41**(4-5): p. 85-90.
60. Spasov, E., et al., *High functional diversity among Nitrospira populations that dominate rotating biological contactor microbial communities in a municipal wastewater treatment plant.* The ISME Journal, 2020. **14**(7): p. 1857-1872.
61. Belser, L. and E. Schmidt, *Growth and oxidation kinetics of three genera of ammonia oxidizing nitrifiers.* FEMS Microbiology Letters, 1980. **7**(3): p. 213-216.
62. Hagopian, D.S. and J.G. Riley, *A closer look at the bacteriology of nitrification.* Aquacultural Engineering, 1998. **18**(4): p. 223-244.
63. Winkler, M.K.H., et al., *Unravelling the reasons for disproportion in the ratio of AOB and NOB in aerobic granular sludge.* Applied Microbiology and Biotechnology, 2012. **94**(6): p. 1657-1666.
64. Winkler, M.-K.H., Q.H. Le, and E.I.P. Volcke, *Influence of Partial Denitrification and Mixotrophic Growth of NOB on Microbial Distribution in Aerobic Granular Sludge.* Environmental Science & Technology, 2015. **49**(18): p. 11003-11010.

65. Daims, H., et al., *In situ characterization of Nitrospira-like nitrite-oxidizing bacteria active in wastewater treatment plants.* Applied and environmental microbiology, 2001. **67**(11): p. 5273-5284.
66. Daims, H., et al., *Complete nitrification by Nitrospira bacteria.* Nature, 2015. **528**(7583): p. 504-509.
67. Haaijer, S.C.M., et al., *A novel marine nitrite-oxidizing Nitrospira species from Dutch coastal North Sea water.* Frontiers in microbiology, 2013. **4**: p. 60-60.
68. Watson, S.W., et al., *Nitrospira marina gen. nov. sp. nov.: a chemolithotrophic nitrite-oxidizing bacterium.* Archives of Microbiology, 1986. **144**(1): p. 1-7.
69. Stamper, M.A. and K.J. Semmen, *Chapter 23 - Basic Water Quality Evaluation for Zoo Veterinarians*, in *Fowler's Zoo and Wild Animal Medicine*, R.E. Miller and M. Fowler, Editors. 2012, W.B. Saunders: Saint Louis. p. 177-186.
70. Princic, A., et al., *Effects of pH and oxygen and ammonium concentrations on the community structure of nitrifying bacteria from wastewater.* Applied and environmental microbiology, 1998. **64**(10): p. 3584-3590.
71. Ruiz, G., D. Jeison, and R. Chamy, *Nitrification with high nitrite accumulation for the treatment of wastewater with high ammonia concentration.* Water Research, 2003. **37**(6): p. 1371-1377.
72. Amatya, I., et al., *Role of pH on biological Nitrification Process.* Journal of the Institute of Engineering, 2011. **8**.
73. Kirchman, D.L., *Degradation of organic material.* Processes in microbial ecology. Oxford University Press Inc., New York, NY, 2012: p. 79-98.
74. Kindaichi, T., T. Ito, and S. Okabe, *Ecophysiological interaction between nitrifying bacteria and heterotrophic bacteria in autotrophic nitrifying biofilms as determined by microautoradiography-fluorescence in situ hybridization.* Applied and Environmental Microbiology, 2004. **70**(3): p. 1641-1650.
75. Rittmann, B.E., J.M. Regan, and D.A. Stahl, *Nitrification as a source of soluble organic substrate in biological treatment.* Water Science and Technology, 1994. **30**(6): p. 1-8.
76. Wang, J., et al., *Factors affecting simultaneous nitrification and denitrification (SND) in a moving bed sequencing batch reactor (MBSBR) system as revealed by microbial community structures.* Bioprocess and Biosystems Engineering, 2020. **43**(10): p. 1833-1846.
77. Hink, L., et al., *The consequences of niche and physiological differentiation of archaeal and bacterial ammonia oxidisers for nitrous oxide emissions.* The ISME journal, 2018. **12**(4): p. 1084-1093.
78. Pester, M., et al., *amoA-based consensus phylogeny of ammonia-oxidizing archaea and deep sequencing of amoA genes from soils of four different geographic regions.* Environmental Microbiology, 2012. **14**(2): p. 525-539.
79. Poly, F., et al., *First exploration of Nitrobacter diversity in soils by a PCR cloning-sequencing approach targeting functional gene nxrA.* FEMS microbiology ecology, 2008. **63**(1): p. 132-140.
80. Daelman, M., et al., *Methane and nitrous oxide emissions from municipal wastewater treatment–results from a long-term study.* Water Science and technology, 2013. **67**(10): p. 2350-2355.

Appendix A: Media for batch experiment

The medium used for batch experiments with Leca biofilm at VEAS (Section 2.2.) was made of macronutrients (Table A.1.), micronutrients (Table A.2.) and 10 MΩ.cm water from PURELAB Chorus 2 (ELGA Purelab®). Per one litre medium it was added 10 mL of stock solution.

Table A. 3-1. ***Macronutrients for one litre medium.***

Macronutrients	**Gram (g)**
$NaHCO_3$	1 g
$(NH_4)2SO_4$	0.47 g
K_2HPO_4	0.40g

Table A. 3-2. Micronutrients in 10 mL stock solution.

Micronutrients	**Gram (g)**
$MgSO_4$-$7H_2O$	2.5
$CaCl_2$-$2H_2O$	1.5
$FeCl_2$-$4H_2O$	0.2
$MnCl_2$-$4H_2O$	0.55
$ZnCl_2$	0.068
CoCl-$6H_2O$	0.12
NiC_2-$6H_2O$	0.12
EDTA	2.8

Appendix B: Overview of all Leca biofilm samples from nitrifying filters at VEAS for Illumina sequencing of 16S rRNA analysis.

Monthly samples are sampled from PHA7 with the new washing procedure with three repetitions of step D2-D6 (Section 2.2.) and from PHA8 with the original washing procedure. There was also taken Leca biofilm samples from the batch experiment where Leca samples were exposed to one, three and five repetitions. Samples marked in grey were destroyed during transport, DNA extraction or sequencing.

Table B. 1. Overview of Leca biofilm sample for 16S rRNA analysis. Number of nitrifying biofilter, type of samples (Month, depths, samples from batch experiment), time for sampling, depth and name of samples. Samples marked in grey were destroyed during transport, DNA extraction or sequencing.

Filter	Type	Time	Depth	Name
81	Month	08:10	Top	M_NIT81_2309
82	Month	08:00	Top	M_NIT82_2309
83	Month	08:05	Top	M_NIT83_2309
84	Month	08:15	Top	M_NIT84_2309
71	Month	08:27	Top	M_NIT71_2309
72	Month	08:20	Top	M_NIT72_2309
73	Month	08:23	Top	M_NIT73_2309
74	Month	08:30	Top	M_NIT74_2309
71	Month	12:46	Top	M_NIT71_0309
72	Month	12:40	Top	M_NIT72_0309
73	Month	12:43	Top	M_NIT73_0309
74	Month	12:50	Top	M_NIT74_0309
81	Month	12:30	Top	M_NIT81_0309
82	Month	12:20	Top	M_NIT82_0309
83	Month	12:25	Top	M_NIT83_0309
84	Month	12:35	Top	M_NIT84_0309
71	Month	-	Top	M_NIT71_2008
72	Month	-	Top	M_NIT72_2008
73	Month	-	Top	M_NIT73_2008
74	Month	-	Top	M_NIT74_2008
81	Month	-	Top	M_NIT81_2008
82	Month	-	Top	M_NIT82_2008
83	Month	-	Top	M_NIT83_2008
84	Month	-	Top	M_NIT84_2008
71	Month	12:26	Top	M_NIT71_2306
72	Month	12:35	Top	M_NIT72_2306
73	Month	12:42	Top	M_NIT73_2306
74	Month	12:29	Top	M_NIT74_2306
81	Month	13:45	Top	M_NIT81_2306
82	Month	13:06	Top	M_NIT82_2306
83	Month	13:40	Top	M_NIT83_2306
84	Month	13:00	Top	M_NIT84_2306
71	Month	13:19	Top	M_NIT71_2805
72	Month	13:15	Top	M_NIT72_2805
73	Month	13:17	Top	M_NIT73_2805

Table B.1. Continued

74	Month	13:23	Top	M_NIT74_2805
81	Month	12:45	Top	M_NIT81_2805
82	Month	12:53	Top	M_NIT82_2805
83	Month	12:55	Top	M_NIT83_2805
84	Month	12:50	Top	M_NIT84_2805
71	Month	13:23	Top	M_NIT71_3004
72	Month	13:27	Top	M_NIT72_3004
73	Month	13:28	Top	M_NIT73_3004
74	Month	13:25	Top	M_NIT74_3004
81	Month	13:12	Top	M_NIT81_3004
84	Month	13:15	Top	M_NIT84_3004
82	Month	13:18	Top	M_NIT82_3004
83	Month	13:20	Top	M_NIT83_3004
71	Batch	08:43	Top	B1_NIT71_1906
71	Batch	12:23	Top	B1_NIT71_1906
71	Batch	08:42	Top	B1_NIT71_1806
71	Batch	12:18	Top	B1_NIT71_1806
71	Batch	08:35	Top	B1_NIT71_1706
71	Batch	12:25	Top	B1_NIT71_1706
71	Batch	09:05	Top	B1_NIT71_2406
71	Batch	12:38	Top	B1_NIT71_2406
72	Batch	09:30	Top	B3_NIT72_1206
72	Batch	13:13	Top	B3_NIT72_1206
71	Batch	08:35	Top	B3_NIT71_3006
71	Batch	12:57	Top	B3_NIT71_3006
74	Batch	08:25	Top	B5_NIT74_0307
74	Batch	12:06	Top	B5_NIT74_0307
74	Batch	08:20	Top	B5_NIT74_0207
74	Batch	11:45	Top	B5_NIT74_0207
71	Batch	08:15	Top	B5_NIT71_0107
71	Batch	11:50	Top	B5_NIT71_0107
72	Batch	08:30	Top	B3_NIT72_1206
72	Batch	12:35	Top	B3_NIT72_1206
83	Batch	09:05	Top	BO_NIT83_2906
83	Batch	12:45	Top	BO_NIT83_2906
81	Batch	08:25	Top	BO_NIT81_2606
81	Batch	11:50	Top	BO_NIT81_2606
82	Batch	09:30	Top	BO_NIT82_1006
82	Batch	13:13	Top	BO_NIT82_1006
73	Depth	10:25	Top	MH_NIT73_T_1609
73	Depth	10:50	Upper mid	MH_NIT73_UM_1609
73	Depth	11:00	Lower mid	MH_NIT73_LM_1609
73	Depth	11:10	Bottom	MH_NIT73_B_1609
74	Depth	10:10	Top	MH_NIT74_T_1609
74	Depth	10:00	Upper mid	MH_NIT74_UM_1609
74	Depth	09:50	Lower mid	MH_NIT74_LM_1609
74	Depth	09:30	Bottom	MH_NIT74_B_1609
74	Depth	08:20	Top	MH_NIT74_T_2608
74	Depth	08:25	Upper mid	MH_NIT74_UM_2608
74	Depth	08:33	Bottom	MH_NIT74_B_2608

Table B.1. Continued

74	Depth	08:37	Lower mid	MH_NIT74_LM_2608
73	Depth	08:49	Top	MH_NIT73_T_2608
73	Depth	08:50	Upper mid	MH_NIT73_UM_2608
73	Depth	08:52	Lower mid	MH_NIT73_LM_2608
73	Depth	08:54	Bottom	MH_NIT73_B_2608
81	Depth	10:07	Upper mid	MH_NIT81_UM_2306
81	Depth	10:00	Lower mid	MH_NIT81_LM_2306
81	Depth	09:50	Bottom	MH_NIT81_B_2306
81	Depth	13:45	Top	MH_NIT81_T_2306

Appendix C: Buffer solutions

Recipe over components in 50x TAE-buffer are listed in Table B.1. TAE-buffer was prepared by diluting 40 mL 50x TAE-buffer in 1960 mL MQ-water.

Table C. 1. Recipe for 50x TAE-buffer.

Component	Amounts
Tris base	242 g
Glacial acetic acid	57.1 mL
0.5M EDTA pH 8.0	100 ml
dH2O	Up to 1L

Table C. 2. Recipe for 1x Tris-EDTA buffer (TE-buffer)

Component	Amounts	Final concentration
2M Tris	2.5 ml	10.0 mM
0.5M EDTA pH 8.0	1.0 ml	1.0 mM
dH2O	496.5 ml	

Appendix D: DNA Extraction protocol - PowerSoil® DNA Isolation Kit (Mo Bio)

EXPERIENCED USER PROTOCOL

PowerSoil® DNA Isolation Kit

Catalog No. 12888-50 & 12888-100

Please wear gloves at all times

1. To the **PowerBead Tubes** provided, add 0.25 grams of soil sample.
2. Gently vortex to mix.
3. **Check Solution C1**. If **Solution C1** is precipitated, heat solution to 60°C until dissolved before use.
4. Add 60 µl of **Solution C1** and invert several times or vortex briefly.
5. Secure **PowerBead Tubes** horizontally using the MO BIO Vortex Adapter tube holder for the vortex (MO BIO Catalog# 13000-V1-24) or secure tubes horizontally on a flat-bed vortex pad with tape. Vortex at maximum speed for 10 minutes.

Note

If you are using the 24 place Vortex Adapter for more than 12 preps, increase the vortex time by 5-10 minutes.

6. Make sure the **PowerBead Tubes** rotate freely in your centrifuge without rubbing. Centrifuge tubes at 10,000 x g for 30 seconds at room temperature. **CAUTION**: Be sure not to exceed 10,000 x g or tubes may break.
7. Transfer the supernatant to a clean **2 ml Collection Tube** (provided).

Note

Expect between 400 to 500 µl of supernatant. Supernatant may still contain some soil particles.

8. Add 250 µl of **Solution C2** and vortex for 5 seconds. Incubate at 4°C for 5 minutes.
9. Centrifuge the tubes at room temperature for 1 minute at 10,000 x g.
10. Avoiding the pellet, transfer up to, but no more than, 600 µl of supernatant to a clean **2 ml Collection Tube** (provided).
11. Add 200 µl of **Solution C3** and vortex briefly. Incubate at 4°C for 5 minutes.
12. Centrifuge the tubes at room temperature for 1 minute at 10,000 x g.
13. Avoiding the pellet, transfer up to, but no more than, 750 µl of supernatant into a clean **2 ml Collection Tube** (provided).
14. Shake to mix **Solution C4** before use. Add 1200 µl of **Solution C4** to the supernatant and vortex for 5 seconds.

15. Load approximately 675 µl onto a **Spin Filter** and centrifuge at 10,000 x g for 1 minute at room temperature. Discard the flow through and add an additional 675 µl of supernatant to the **Spin Filter** and centrifuge at 10,000 x g for 1 minute at room temperature. Load the remaining supernatant onto the **Spin Filter** and centrifuge at 10,000 x g for 1 minute at room temperature.

Note

A total of three loads for each sample processed are required.

16. Add 500 µl of **Solution C5** and centrifuge at room temperature for 30 seconds at 10,000 x g.

17. Discard the flow through.

18. Centrifuge again at room temperature for 1 minute at 10,000 x g.

19. Carefully place spin filter in a clean **2 ml Collection Tube** (provided). Avoid splashing any **Solution C5** onto the **Spin Filter**.

20. Add 100 µl of Solution C6 to the center of the white filter membrane. Alternatively, sterile DNA-Free PCR Grade Water may be used for elution from the silica Spin Filter membrane at this step (MO BIO Catalog# 17000-10).

21. Centrifuge at room temperature for 30 seconds at 10,000 x g.

22. Discard the **Spin Filter**. The DNA in the tube is now ready for any downstream application. No further steps are required.

We recommend storing DNA frozen (-20° to -80°C). **Solution C6** contains no EDTA. To concentrate the DNA see the Hints & Troubleshooting Guide.

Thank you for choosing the PowerSoil® DNA Isolation Kit!

Appendix E: SequalPrep™ Normalization Plate (96) Kit (Invitrogen™)

invitrogen™

SequalPrep™ Normalization Plate (96) Kit

Catalog no: A10510-01 **Store at room temperature (15–30ºC)**

Contents and Storage

The components included with the SequalPrep™ Normalization Plate (96) Kit are listed in the table below. Sufficient reagents are included to perform 10 × 96 purification/normalization reactions. Upon receipt, **store all components at room temperature (15–30°C)**. Store plates for up to 6 months.

Components	Quantity
SequalPrep™ Normalization Plate (96)	2 bags of 5 plates each
SequalPrep™ Normalization Binding Buffer	40 ml
SequalPrep™ Normalization Wash Buffer	50 ml
SequalPrep™ Normalization Elution Buffer (10 mM Tris-HCl, pH 8.5)	40 ml

Description

The SequalPrep™ Normalization Plate Kit allows simple, one-step, high-throughput amplicon purification and normalization of PCR product concentration (2–3 fold range) via a limited binding capacity solid phase. Each well of the SequalPrep™ Normalization Plate can bind and elute ~25 ng of PCR amplicon. Eluted PCR amplicon can be subsequently pooled and subjected to a variety of massively parallel sequencing analyses. The SequalPrep™ Normalization Plate is compatible with any automated liquid handling workstations without the need for shakers, magnets, or vacuum. The SequalPrep™ Normalization Plate Kit when used with SequalPrep™ Long PCR Kit provides a complete PCR enrichment and amplicon normalization system that is designed to complement amplicon sequencing workflows such as next-generation sequencing.

The conventional next generation sequencing workflows require laborious sample prep methods consisting of amplicon purification, quantitation, and manual normalization to adjust amplicon concentration. The SequalPrep™ Normalization Plate Kit eliminates the tedious amplicon quantitation and manual normalization steps.

SequalPrep™ Normalization Plate Kits utilize ChargeSwitch® Technology that provides a switchable surface charge depending on the pH of the surrounding buffer to facilitate nucleic acid purification. Under low pH conditions, the positive surface charge of the ChargeSwitch® coating binds the negatively charged nucleic acid backbone. Proteins and other contaminants (such as short oligonucleotide primers) are not bound and are simply washed away.

System Overview

The SequalPrep™ Normalization Plate Kit is a solid phase, high-throughput amplicon purification and normalization system in a 96-well plate format. PCR products (5–25 µl) are added to a SequalPrep™ Normalization Plate well and mixed with the Binding Buffer. DNA binding to the plate is performed at room temperature for 1 hour. The wells are washed with Wash Buffer to efficiently remove contaminants. Purified PCR products are eluted using 20 µl Elution Buffer at normalized concentrations.

System Specifications

Starting Material:	At least 250 ng PCR product (amplicon) per well
DNA Fragment Size:	100 bp to 20 kb
Elution Volume:	20 µl
DNA Yield:	Up to 25 ng per well
Normalization Range:	2–3-fold
Plate Dimensions:	Standard SBS (Society for Biomolecular Screening) footprint, semi-skirted 96-well plate
Plate Capacity:	0.2 ml

Accessory Products

The following products may be used with the SequalPrep™ Normalization Plate Kit. For details, visit www.invitrogen.com.

Product	Quantity	Catalog no.
SequalPrep™ Normalization Wash Buffer	4 × 50 ml	A10510-03
SequalPrep™ Long PCR Kit with dNTPs	1,000 units	A10498
Platinum® PCR Supermix	100 reactions	11306-016
Platinum® PCR Supermix High Fidelity	100 reactions	12532-016
Quant-iT™ PicoGreen® dsDNA Assay Kit	1 kit	P7589
PureLink™ Foil Tape	50 tapes	12261-012
E-Gel® *96* gels 1% (or 2%)	8 gels	G7008-01 (G7008-02)

Part no: 100003531 Rev. date: 5 May 2008

General Guidelines

- Wear a laboratory coat, disposable gloves, and eye protection when handling reagents and plate.
- Always use proper aseptic techniques when working with DNA and use only sterile, DNase-free tips to prevent DNase contamination.
- If you are using only part of the plate for DNA purification, cover unused wells with the Plate Seal and leave them attached while purifying DNA in the other wells. The plates can be stored at room temperature for up to 6 months.
- The SequalPrep™ Normalization Plates are compatible for use with automated liquid handling workstation; the workstation must be capable of handling and manipulating 96-well plates.
- If you are using automated liquid handling workstations for purification, you may need additional Wash Buffer depending on your type of workstation. See previous page for Wash Buffer ordering information.

Generating PCR Amplicon

You can generate the PCR amplicon using a method of choice. General recommendations for generating PCR amplicons are listed below:

- To obtain the best results, we recommend using the SequalPrep™ Long PCR Kit with dNTPs (page 1) which provides a robust system for long-range, high-fidelity PCR for use in next-generation sequencing applications.
- Other commercially available PCR supermixes and enzymes such as Platinum® PCR Supermix (page 1), Platinum® PCR Supermix High Fidelity (page 1), or equivalent are suitable for use.
- Perform PCR in a separate plate. **Do not** use the SequalPrep™ Normalization Plate to perform PCR.
- You need at least 250 ng amplicon per well to use with the SequalPrep™ Normalization Plate (see below).

Sample Amount

To achieve robust normalization, we recommend adding at least 250 ng/well of amplicon. This input amount is easily achieved using only a fraction of most PCR amplification reactions. An average efficiency PCR (20 µl reaction volume) produces product in the range of 25–100 ng/µl, allowing you to purify 5–10 µl using the SequalPrep™ system.

Elution Options

Depending on the nature of the downstream application and target nucleic acid concentrations desired, the SequalPrep™ kit offers the flexibility to elute purified DNA in a variety of options.

The **standard elution** method described in the protocol below is designed to elute purified DNA from each well using 20 µl elution volume to obtain each amplicon at a concentration of 1–2 ng/µl.

The **optional sequential elution** method is designed to sequentially elute multiple rows or columns using the same 20 µl of elution buffer to obtain higher amplicon concentrations. The amplicon concentrations will be additive as sequential wells are eluted. For example, dispense 20 µl of elution buffer into the first column (A1–H1), mix well, and incubate for 5 minutes at room temperature. Then, simply move this column of elution buffer to the next column (A2–H2), and again incubate for 5 minutes. Continue this step to obtain your specific elution needs for the downstream application of choice.

Materials Needed

- PCR reactions containing amplicons of the desired length (see **Generating PCR Amplicon**, above)
- DNase-free, aerosol barrier pipette tips
- *Optional:* automated liquid handling workstation capable of handling and manipulating 96-well plates
- *Optional:* PureLink™ Foil Tape (see previous page)

Binding Step

1. Transfer the desired volume of PCR product (5–25 µl PCR reaction mix, at least 250 ng amplicon/well) from the PCR plate into the wells of the SequalPrep™ Normalization plate.
2. Add an equivalent volume of SequalPrep™ Normalization Binding Buffer.

   For example: To purify 10 µl of PCR product, add 10 µl SequalPrep™ Normalization Binding Buffer.
3. Mix completely by pipetting up and down, or seal the plate with PureLink™ Foil Tape (page 1), vortex to mix, and briefly centrifuge the plate.
4. Incubate the plate for 1 hour at room temperature to allow binding of DNA to the plate surface. Mixing is not necessary at this stage.

   Note: Incubations longer than 60 minutes do not improve results. However, depending on your workflow you may perform overnight incubation at room temperature for the binding step.
5. **Optional:** If >25 ng DNA/well yield is desired, transfer the amplicon/Binding Buffer mixture from Step 4 to another, fresh well/plate to sequentially bind more DNA. Perform DNA binding at room temperature for 1 hour.

   Note: After binding is complete, you can remove the amplicon/Binding Buffer mixture from the well and store at –20ºC for up to 30 days to perform additional purifications at a later time.
6. Proceed to **Washing Step**, next page.

Washing Step

1. Aspirate the liquid from wells. Be sure not to scrape the well sides during aspiration.

   Note: If you wish to store the amplicon/Binding Buffer mixture for additional purifications at a later time, aspirate the liquid from wells into another plate and store at –20ºC for up to 30 days.

2. Add 50 µl SequalPrep™ Normalization Wash Buffer to the wells. Mix by pipetting up and down twice to improve removal of contaminants.

3. Completely aspirate the buffer from wells and discard.

   To ensure complete removal of wash buffer and maximize elution efficiency, you may need to invert and tap the plate on paper towels depending on the pipetting technique or instrument used. A small amount of residual Wash Buffer (1–3 µl) is typical and does not affect the subsequent elution or downstream applications.

4. Proceed to **Elution Step**, below.

Elution Step

Review **Elution Options** (previous page).

1. Add 20 µl SequalPrep™ Normalization Elution Buffer to each well of the plate.

   Note: Do not use water for elution. If you need to elute in any other buffer, be sure to use a buffer of pH 8.5–9.0. If the pH of the buffer is <8.5, the DNA will not elute efficiently.

2. Mix by pipetting up and down 5 times or seal the plate with PureLink™ Foil Tape (page 1), vortex to mix, and briefly centrifuge the plate. Ensure that the buffer contacts the entire plate coating (up to 20 µl level).

3. Incubate at room temperature for 5 minutes.

4. Transfer and pool the purified DNA as desired or store the eluted DNA at 4°C (short-term storage) or –20°C (long-term storage) until further use.

Expected Yield and Concentration

The expected DNA concentration is 1–2 ng/µl when using 20 µl elution volume. The expected DNA yield is ~25 ng/well normalized.

Optional: DNA Quantitation

The SequalPrep™ Normalization Plate Kit is designed to eliminate the quantitation and manual dilution steps typically performed for normalization in next-generation sequencing workflows. You can pool the eluted amplicon and use the pooled amplicons directly for your downstream applications without DNA quantitation.

However, if your downstream application requires DNA quantitation, you may determine the yield of the eluted amplicon using Quant-iT™ PicoGreen® dsDNA Assay Kit (page 1). We **do not** recommend using UV spectrophotometric measurements (A_{260}/A_{280} nm), as this method is inaccurate for low DNA concentrations.

Downstream Applications

The SequalPrep™ Normalization Plate Kit is designed to produce purified PCR products with normalized concentrations and substantially free of salts and contaminating primers. PCR amplicons purified from this system can be used individually or pooled in any downstream application for which normalization is an important sample preparation criterion such as next generation sequencing applications.

Pooled amplicons purified using the SequalPrep™ Normalization Plate Kit have produced successful data from massively parallel sequencing-by-synthesis on the Illumina/Solexa Genome Analyzer indicating that the amplicon purity is suitable for other next-generation sequencing platforms (Roche/454 FLX, Applied Biosystems SOLiD™ system). For detailed sample preparation guidelines, refer to the instrument manufacturer's recommendations.

Continued on next page

Appendix F: Up-concentration of sample by Amicon® Ultra-0.5 Centrifugal Filter Devices (Merck Millipore)

User Guide

Amicon® Ultra-0.5 Centrifugal Filter Devices

for volumes up to 500 µL

For research use only;
not for use in diagnostic procedures

Introduction

Amicon® Ultra-0.5 centrifugal filter devices provide fast ultrafiltration, with the capability for high concentration factors and easy concentrate recovery from dilute and complex sample matrices. The vertical design and available membrane surface area provide fast sample processing, high sample recovery (typically greater than 90% of dilute starting solution), and the capability for 30-fold concentration. Typical processing time is 10 to 30 minutes depending on Nominal Molecular Weight Limit (NMWL). Solute polarization and subsequent fouling of the membrane are minimized by the vertical design, and a physical deadstop in the filter device prevents spinning to dryness and potential sample loss. Efficient recovery of the concentrated sample (retained species) is achieved by a convenient reverse spin step after collecting the filtrate. Amicon® Ultra-0.5 devices are supplied non-sterile and are for single use only.

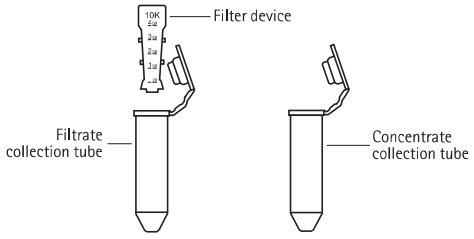
The Amicon® Ultra-0.5 product line includes 5 different cutoffs (Nominal Molecular Weight Limit, NMWL). These devices are for research use only and not for use in diagnostic procedures.

- Amicon® Ultra 3K device — 3,000 NMWL
- Amicon® Ultra 10K device — 10,000 NMWL
- Amicon® Ultra 30K device — 30,000 NMWL
- Amicon® Ultra 50K device — 50,000 NMWL
- Amicon® Ultra 100K device — 100,000 NMWL

Applications

- Concentration of biological samples containing antigens, antibodies, enzymes, nucleic acids (DNA/RNA samples, either single- or double-stranded), microorganisms, column eluates, and purified samples
- Purification of macromolecular components found in tissue culture extracts and cell lysates, removal of primer, linkers, or molecular labels from a reaction mix, and protein removal prior to HPLC
- Desalting, buffer exchange, or diafiltration

Materials Supplied



The Amicon® Ultra-0.5 device is supplied with two microcentrifuge tubes. During operation, one tube is used to collect filtrate; the other to recover the concentrated sample.

Required Equipment

Centrifuge with fixed angle rotor that can accommodate 1.5 mL microcentrifuge tubes

CAUTION: To avoid damage to the device during centrifugation, check clearance before spinning.

Suitability

Preliminary recovery and retention studies are suggested to ensure suitability for intended use. See the "How to Quantify Recoveries" section.

Device Storage

Store at room temperature.

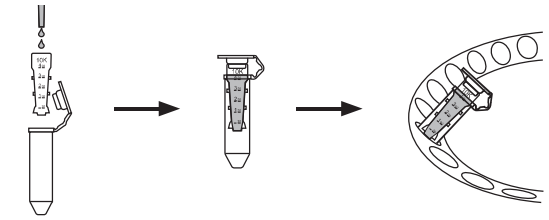
Prerinsing

The ultrafiltration membranes in Amicon® Ultra-0.5 devices contain trace amounts of glycerine. If this material interferes with analysis, pre-rinse the device with buffer or Milli-Q® water. If interference continues, rinse with 0.1 N NaOH followed by a second spin of buffer or Milli-Q® water.

CAUTION: Do not allow the membrane in Amicon® Ultra filter devices to dry out once wet. If you are not using the device immediately after pre-rinsing, leave fluid on the membrane until the device is used.

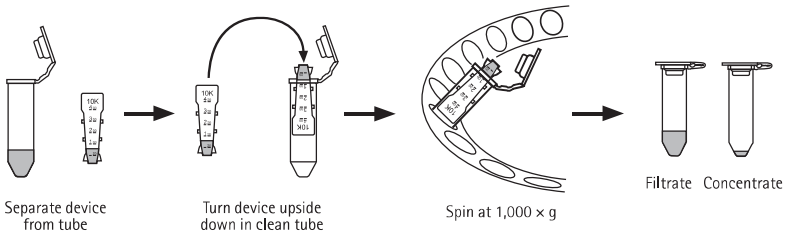
How to Use Amicon® Ultra-0.5 Centrifugal Filter Devices

1. Insert the Amicon® Ultra-0.5 device into one of the provided microcentrifuge tubes.
2. Add up to 500 µL of sample to the Amicon® Ultra filter device and cap it.
3. Place capped filter device into the centrifuge rotor, aligning the cap strap toward the center of the rotor; counterbalance with a similar device.
4. Spin the device at 14,000 × g for approximately 10–30 minutes depending on the NMWL of the device used. Refer to Figure 1 and Table 2 for typical spin times.



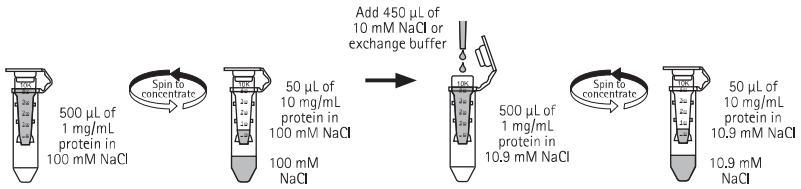
5. Remove the assembled device from the centrifuge and separate the Amicon® Ultra filter device from the microcentrifuge tube.
6. To recover the concentrated solute, place the Amicon® Ultra filter device upside down in a clean microcentrifuge tube. Place in centrifuge, aligning open cap towards the center of the rotor; counterbalance with a similar device. Spin for 2 minutes at 1,000 × g to transfer the concentrated sample from the device to the tube. The ultrafiltrate can be stored in the centrifuge tube.

   NOTE: For optimal recovery, perform the reverse spin immediately.



Desalting or Diafiltration

Desalting, buffer exchange, or diafiltration are important methods for removing salts or solvents in solutions containing biomolecules. The removal of salts or the exchange of buffers can be accomplished in the Amicon® Ultra-0.5 device by concentrating the sample, discarding the filtrate, then reconstituting the concentrate to the original sample volume with any desired solvent. The process of "washing out" can be repeated until the concentration of the contaminating microsolute has been sufficiently reduced. See example below.



Performance – DNA Concentration

The Amicon® Ultra-0.5 30K device provides the best balance between recovery and spin time for double-stranded DNA for base pairs ranging from 137 to 1159. To achieve maximum PCR product recovery and primer removal with primers greater than 20 bases, one or two additional spins with Tris-EDTA (TE) buffer are recommended.

Table 1. Typical recovery of nucleotides from Amicon® Ultra-0.5 devices

PCR Product (base pairs)	PCR Primer (bases)	PCR Recovery (%)	PCR Primer Removal (%)	TE Washes (number)
137	10	≥ 95	≥ 90	0
	20	≥ 90	≥ 85	1
	48	≥ 90	≥ 75	2
301	10	≥ 90	≥ 90	0
	20	≥ 85	≥ 90	1
	48	≥ 90	≥ 80	2
657	10	≥ 95	≥ 90	0
	20	≥ 90	≥ 90	1
	48	≥ 95	≥ 90	2
1159	10	≥ 90	≥ 90	0
	20	≥ 90	≥ 95	1
	48	≥ 95	≥ 95	2

Spin conditions: 40° fixed angle rotor, 14,000 × g, room temperature, 100 µL PCR and 400 µL TE buffer for a starting volume of 500 µL, 20–30 µL final volume, 10 minute spin, n=12.

Appendix G: Average abundance of bacterial community

Average abundance of bacterial community classified on class level for the new and original washing procedure are presented in Figure F.1. Average Bray Curtis similarity from samples taken directly prior and after the washing procedure in June and start of July showed in Figure F.2. Se detailed information of samples used in Section 2.2.1.

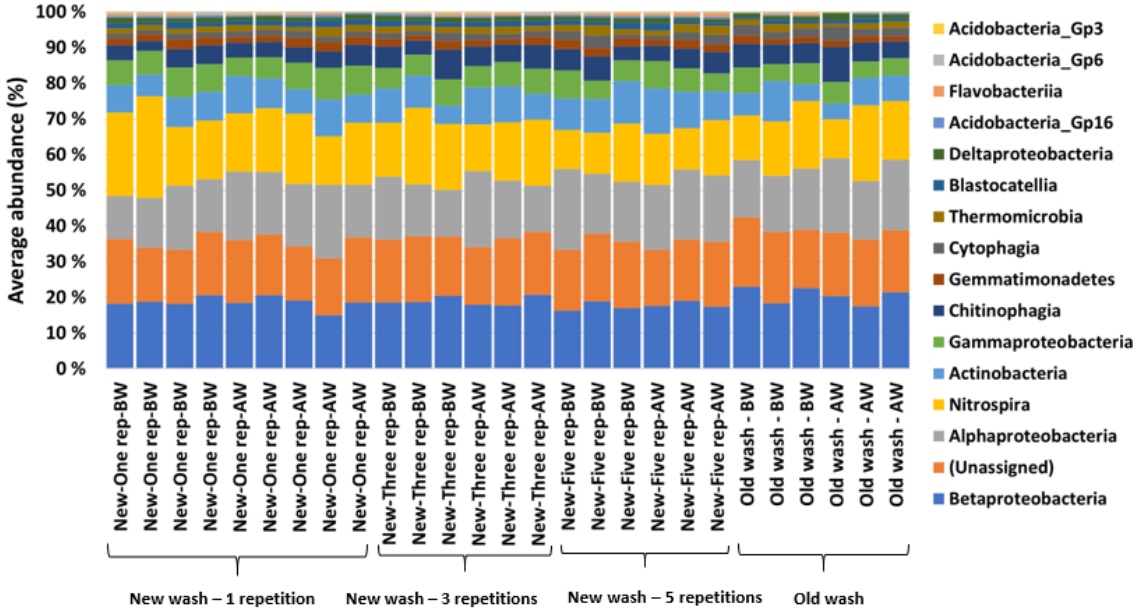


Figure G. 1. Average abundance (%) of bacterial community classified on class level for washing procedures before (BW) and after wash (AW). New washing procedure with one (New-One rep), three (New-Three rep) and five repetitions (New-Five rep) and original washing routine (Original wash).

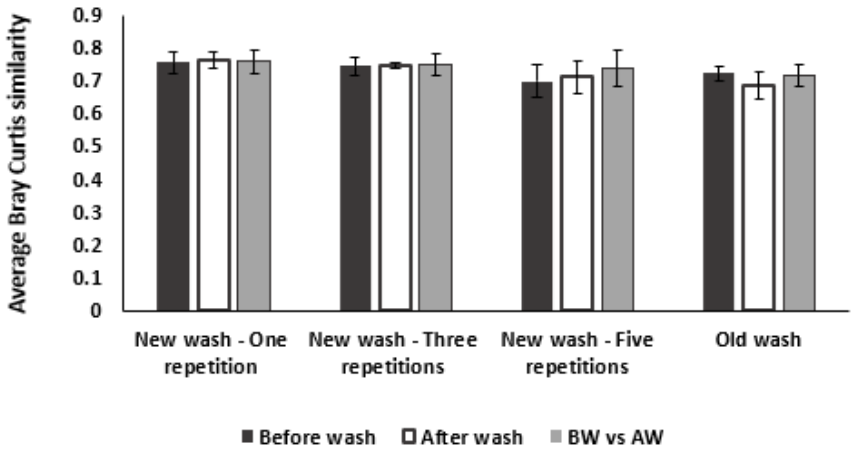


Figure G. 2. Average Bray Curtis similarities for Leca biofilm from nitrifying filter before (BW) and after washing (AW) at VEAS. New washing procedure was conducted with One, Three and Five repetitions and Original wash followed standard procedure (See Materials and method for description of washing procedure). Average is calculated within samples Before wash and After wash, for three replicates for all calculations except New washing procedure - One repetition AW which had four replicates and New wash - Three repetitions AW which had two replicates. Average is calculated between samples before and after wash (BW vs AW). Error bars represent standard deviation.

Appendix H: Leca sampled for robotized incubation experiment

Detailed information on sampling of Leca biofilm sampled for the robotized incubation experiment conducted at NMBU.

Table H. 1. Detailed information for Leca biofilm samples used in robotized incubation experiment at NMBU. Overview of filters where Leca biofilm was sampled before or after the process of washing. Date and time since the last washing procedure was conducted in the nitrifying filters were the samples were collected. All samples were collected 02.11.2020 at approximately 09:00 AM.

	New wash		**Original wash**	
Before or After	Before wash	After wash	Before wash	After wash
Filter:	NIT74	NIT73	NIT83	NIT84
Date	01.11.2020	02.11.2020	02.11.2020	02.11.202
Time	21:45	06:30	02:30	08:15

Appendix I: Water quality factors during small scale batch-experiments

Ammonium and turbidity in wastewater was plotted together with nitrate production in Figure 3.13.. It was assumed that there was no large variation in wastewater entering PHA7 and PHA8 (See 2.2. Figure 2.1.2.). There was only monitored ammonia concentrations going into PHA8 and not in PHA7 at VEAS, and since it was assumed that incoming wastewater was similar for both PHA7 and PHA8, ammonia concentrations in wastewater entering PHA8 were used for the calculations.

The assumption of similarity of incoming wastewater in PHA7 and PHA8 was substantiated by the fact that Ammonia-N determined in the incoming wastewater (inlet) in PHA8 was positively correlated with turbidity in the incoming wastewater for nitrifying filters in PHA7 (Pearson correlation = 0.811, p-value = 9.79E-09). Ammonium-N measured in the inlet for PHA8 and Ammonium-N out (outlet) of PHA7 was also positively correlated (Pearson correlation = 0.702, p-value = 5.28E-06). Ammonia-N out of PHA7 followed the concentration of ammonia -N inlet PHA8.

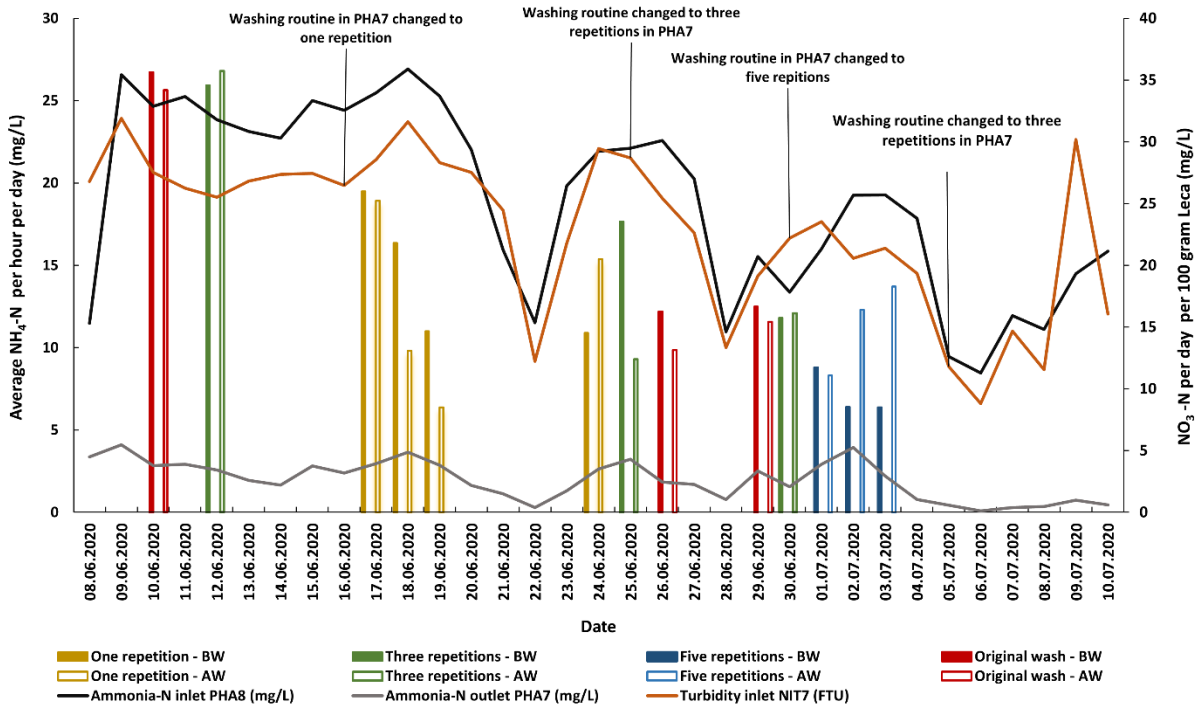


Figure I. 1. Ammonia concentration and turbidity in inlet and ammonia concentration in outlet water to nitrifying filters and the nitrification capacity for Leca biofilm samples taken immediately before and after the washing procedure. Bars representing nitrate production per day per 100-gram Leca in mg/L for Leca biofilm samples taken immediately before and after the washing procedures. Black and grey line visualising average ammonium concentration per day in mg/L in inlet in process hall eight (PHA8) and ammonia-N out (outlet) of nitrification filters in process hall seven (PHA7). Brown line visualising turbidity in inlet for nitrifying filters in PHA7 (NIT7). Average values are calculated from measurements done every hour for 24 hours (total of 24 measurements per day) from 08.06.2020 to 01.07.2020.

Appendix J: Ammonium concentrations in wastewater from March to September in 2020 at VEAS

The ammonium-N concentrations are automatically measured out of sedimentation tank 8 to the inlet of process hall eight every hour for 24 hours during the year. Figure J.1. shows the measured values from March to September in 2020.

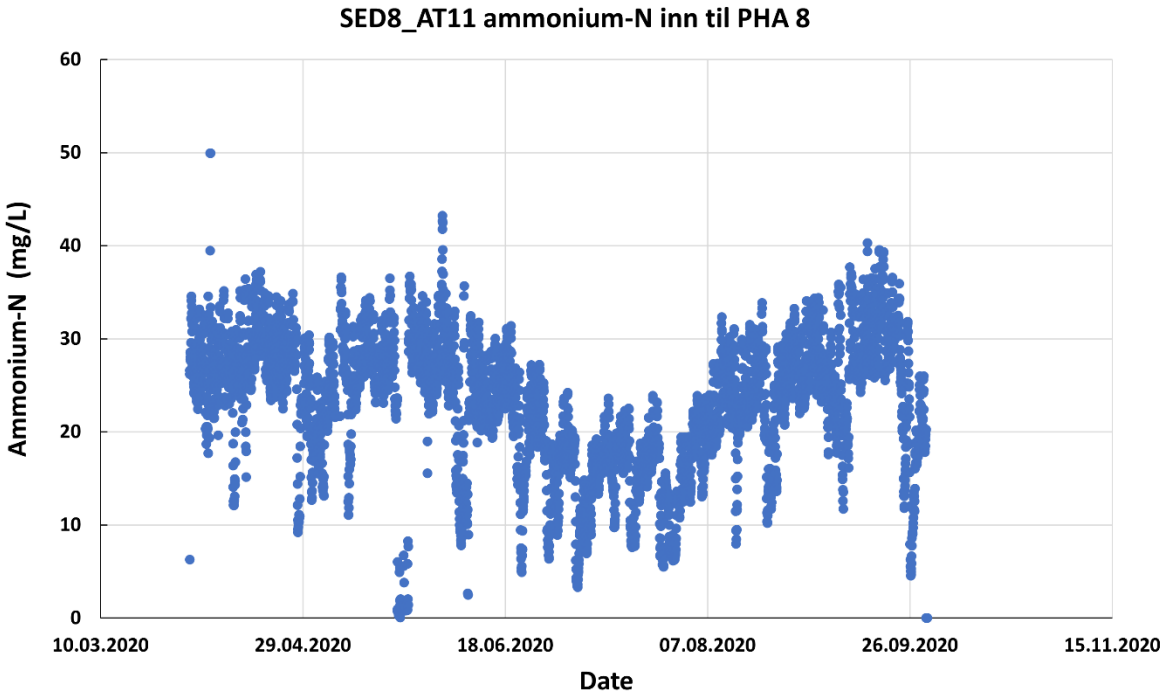


Figure J. 1. Ammonium-N concentrations from sedimentation tank 8 (SED8) to process hall eight (PHA8) from March to September in 2020. Measurements are done every hour.

NTNU
Norwegian University of
Science and Technology