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IGOR ZAYTSEV

Bioaugmentation in LWA-filled horizontal subsurface flow filters for wastewater treatment: Impact of flow regime, temperature and donor system

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ABBREVIATIONS

AWP	activated sludge wastewater treatment plants
BOD_7	biological oxygen demand (7 days)
CW	constructed wetland
HLR	hydraulic loading rate
HSSF	horizontal subsurface flow
KoBio MCs	MCs with the addition of microbial suspension from Kodijärve CW
LWA	light weight aggregates
LECA	light expanded clay aggregates
MSc	mesocosms
NH_4-N	ammonia nitrogen
NO_2-N	nitrite nitrogen
NO_2-N	nitrite nitrogen
N_{tot}	total nitrogen
P	phosphorus
PaBio MCs	MCs with the addition of microbial suspension from Paistu CW
PE	purification efficiency
SD	standard deviation
TOC	total organic carbon
VSSF	vertical subsurface flow

ABSTRACT

The possibilities to optimize pollutant removal in hybrid compact CWs were studied. The measurements in these systems showed that for the successful functioning of hybrid CWs in a cold climate, the optimal hydraulic load should be ≤20 mm d^{-1}, with a recirculation rate of 100–300%. The usage of bioaugmentation to enhance the denitrification of a newly established LECA-based horizontal subsurface flow (HSSF) soil filter receiving pre-treated wastewater from a vertical flow filter was studied in two subsequent trials. The first trial was a half-year-long pilot-scale trial and the second was a one-year-long trial based on the data of the first trial.

In the first trial, bioaugmentation was performed in the cold season and low hydraulic loading was applied. The pilot-scale experiment offers evidence regarding the survival and reproduction of introduced microbes taken from an LECA-based HSSF constructed wetland with similar internal conditions, after bioaugmentation into newly established LECA-based HSSF CW mesocosms. Bioaugmentation resulted in a trend towards higher and more stable denitrification in the supplemented mesocosms during the nearly half-year study period.

In the second trial, bioaugmentation was performed in the warm season, and high hydraulic loading was applied. Two HSSF filters with the same LWA substrate but different wastewater flow regimes were used as donor systems for the bioaugmentation. NO_3-N concentrations in the outflows of all variants of studied MCs were significantly influenced by the time factor. Post-hoc comparison indicated that MCs bioaugmented with the sediment suspension from a similar HSSF had significantly lower NO_3-N concentrations than the control MCs, whereas MCs bioaugmented with the sediment suspension from a HSSF where recirculation of the treated wastewater was used did not show significant differences compared to the control MCs. This finding emphasizes the importance of similarity of flow regime and water parameters in choosing a donor system for bioaugmentation.

1. INTRODUCTION

In accordance with the European Union Water Directive, in the near future all wastewater, even that from small-scale sources, must be treated before being emitted into the environment. Constructed wetlands are thus often the only alternative in rural areas (Vymazal, 2007).

According to data from the Ministry of the Environment, nearly 30 constructed wetlands have been established in Estonia at the present time, of which 20 are soil filters, but the total number of constructed wetlands in the world is in the tens of thousands (Kadlec and Knight, 1996).

Hybrid constructed wetlands (CWs) which combine the aerobic and anaerobic properties of vertical and horizontal subsurface flow parts to improve the removal of organics and especially nitrogen (Vymazal, 2005), are the most practical ecotechnological wastewater treatment method in the Estonian conditions (Noorvee et al., 2005). In Estonia, the use of soil filters is preferred, because water flow in soil reduces the cooling of water, as well as the energy loss that results from evaporation and convection, permitting temperature-sensitive processes to take place even in cold climate conditions. In the trials presented in this thesis we studied a horizontal subsurface flow constructed wetland based on the LWA substrate. We were interested in the possibility of enhancing the denitrification of a newly established LWA-based HSSF soil filter receiving pretreated wastewater from a vertical flow filter.

There is extensive evidence that CWs need an adaptation period in order to achieve the water purification efficiency that is typical of the system (Kadlec and Knight, 1996). Development of the treatment capacity for nitrogen and carbon transformation takes time. One possibility for the shortening of the adaptation period of a subsurface flow CW is to accelerate the development of the necessary characteristics of the local microbial community through bioaugmentation.

Our study was based on the assumption that newly established LWA-based HSSFs are not suitable for the rapid development of a denitrifying microbial community based on inflowing denitrifiers. Therefore we presumed that HSSF CWs should be supplemented with microbes taken from a wetland that has similar internal conditions, such as a similar substrate and inflowing wastewater parameters, and has already achieved the necessary purification results.

1.1. Objectives

We seek to find possibilities to optimize pollutant removal in hybrid compact CWs. This aim includes the determination of optimal loading and operational regimes for LWA-based two-stage hybrid constructed wetland systems in cold climate conditions for the treatment of municipal and agro-industrial wastewater using the recirculation of the treated wastewater. The main objective of this PhD dissertation is to enhance denitrification in HSSF flow filter mesocosms

using the bioaugmentation method. The sub-objectives for achieving this main goal are:

I a) to ascertain whether the transfer of the microbial community from a working CW having the same substrate as the original CW would make it possible to shorten the initial community formation period during the adaptation period of the newly-established CW and more rapidly raise the denitrification efficiency in the CW to an acceptable level during the pilot-scale trial.

b) to determine suitable amounts of sediment suspension for bioaugmentation for subsequent trials.

II a) to prove that the use of bioaugmentation on the new substrate of the LWA-based HSSF filter of a hybrid constructed wetland may have a significant positive effect on the denitrification efficiency of the filters.

b) to determine how the addition of sediment suspension, which has a low content of organics and nutrients and has been taken from an HSSF filter that has a similar substrate, influences denitrification during the one-year period after filling the horizontal filter with new LWA. We sought to discover how bioaugmentation works, 1) if the donor system has a similar substrate, flow regime and wastewater pre-treatment level to the study system, 2) when only the substrate is similar and the changes in the working regime of the donor system may have hindered denitrification of the microbial community.

2. LITERATURE REVIEW

2.1. Constructed wetlands

Wastewater treatment with wetland technology has gathered increasing popularity worldwide. The main advantages of CWs are: 1) lower operational costs and 2) less energy demand compared with other wastewater treatment technologies. The energy expenditures required for the exploitation and establishment of wetland wastewater purification systems are much lower than those for conventional systems (Kadlec and Knight, 1996; Vymazal, 2001). On the other hand, the lifetime and wastewater purification efficiency of constructed wetlands vary greatly (Bastian and Hammer, 1993; Crites and Tchobanoglous, 1998).

Kadlec and Knight (1996) say that the continuation of effectively functioning CWs for wastewater treatment is limited. Vymazal (2001) divides CW systems into free water surface (FWS) and sub-surface flow (SSF) systems on the basis of the type of water flow. SSF systems can by further divided into vertical sub-surface flow (VSSF) and horizontal sub-surface flow (HSSF) systems.

The different treatment wetlands that are in operation in many countries have in most cases been used for the purification of domestic and rainfall water, and also to a lesser extent for agricultural wastewater, industrial wastewater and landfill leachate (Vymazal et al., 2001). One of the most effective ways to treat the wastewater of small settlements and rural areas is by using subsurface flow CWs.

Subsurface flow constructed wetlands are known to be efficient in the removal of both biological oxygen demand (BOD_7) and total suspended solids (TSS) from wastewater. Nevertheless, nitrogen (N) and phosphorus (P) removal is known to be somewhat problematic (Brix et al., 2001, Vymazal et al., 1998).

In addition to their satisfactory performance, multifunctionality in terms of biodiversity and landscape services, and cost-effectiveness, CWs have one significant disadvantage: their high area requirement (Vymazal et al., 2001). On the other hand, the large area (volume) of wetland systems makes CWs better adapted to changing hydraulic and nutrient loadings, and therefore usable in under-populated areas (objects with variable wastewater flow rates and pollution loads). The idea of compact systems is to sustain systems with lower area requirements and lower building costs. In cold climates, CWs are often designed with a reserve in order to compensate for lower temperatures during winter (Jenssen et al., 1993; Wallace et al., 2001; Werker et al., 2002). Providing measures that can help achieve proper results without over-dimensioning would make CWs a much more attractive wastewater treatment technology.

Hydraulic loading and hydrologic detention time are key factors of purification efficiency in all kinds of constructed wetlands (Kadlec and Knight, 1996). Excessively high hydraulic load has been reported as a main limiting

factor of purification processes in both subsurface flow wetland systems (De Sousa et al., 2001).

Temperature is another key factor controlling purification processes in CWs (Kadlec and Knight, 1996). Nevertheless, the influence of temperature in full-scale treatment wetlands and pilot systems demonstrates temperature effects that differ from the results acquired at the laboratory scale or by modelling. Various subsurface flow CWs show a much more diverse pattern of temperature effects on purification processes. The LWA- or Filtralite-P-based hybrid CWs combined from VSSF and HSSF filters in series and treating domestic wastewater show equally high long-term efficiency in both summer and winter (Jenssen et al., 1993; Maehlum et al., 1995; Maehlum et al., 1999; Jenssen et al., 2005; Öövel et al., 2007).

In contrast, some studies report lower NH_4-N and P removal in subsurface flow wetlands in winter (Sikora et al., 1995; Steer et al., 2002), whereas Kushk et al. (2003) report a significantly lower N removal in an experimental HSSF in the winter season.

2.2. Hybrid constructed wetlands

Hybrid constructed wetlands combine the aerobic and anaerobic properties of vertical and horizontal subsurface flow constructed wetlands to improve the removal of organics, especially nitrogen.

Hybrid systems for combined systems are comprised most frequently of subsequential vertical flow (VF) and horizontal flow systems (HF) (Vymazal et al., 2005). HF systems cannot provide nitrification because of their limited oxygen transfer capacity, whereas VF systems, on the other hand, do provide good conditions for nitrification, but no denitrification occurs in these systems (Vymazal et al., 2005). Therefore in a combined system the advantages of the VF and HF systems are combined to complement each other (Vymazal et al., 2005). It is possible to produce an effluent low in BOD, which is fully nitrified and partly denitrified and hence has a much lower total-N outflow concentration (Cooper et al., 1999).

2.2.1. Recirculation systems

One possible operational method to achieve proper results without over-dimensioning subsurface flow (SSF) CWs and free surface water (FSW) CWs is the recirculation of wastewater (Sun et al., 2003). The recirculation of wastewater as a procedure to enhance aeration and purification processes has been used in various pilot- and full scale SSF and FSW CWs for the treatment of different types of wastewater (Sun et al., 2003; Green et al., 2002; Brix et al., 2002; Tchobanoglous and Schroeder, 1987). Re-circulation provides additional oxygen transfer for aerobic microbial activities into the wastewater, and also

enhances contact between pollutants and microorganisms due to the mixing of inflowing and outflowing wastewater (Connoly et al., 2003). In addition, as the suspended solids are predominantly removed by filtration, recirculation of the effluent increases the chances for the suspended solids to be trapped in the system (Sun et al., 2003). Also, recirculation of the wastewater is one of the possible operational methods that can be used to compensate small area and short retention time.

2.3. LWA-based horizontal subsurface flow filters

In our study we used filter material that is generally referred to as LWA. In some cases the synonym LECA is also used (see Brix et al., 2001). LWA-based systems have good water conductivity, which lowers the risk of clogging, low heat conductivity and, in the case of high Ca and Mg content, also a high phosphorus adsorption capacity, and are therefore successfully applied in Norway, which has similar climatic conditions to those in Estonia (Zhu et al., 1997; Harris and Maehlum, 2003; Jenssen and Krogstad, 2003).

2.3.1. The role of denitrification in nitrogen removal

Vymazal et al., (2001) describe mechanisms that ultimately remove nitrogen from wastewater: ammonia volatilization, denitrification, plant uptake (with biomass harvesting), ammonia adsorption, ANAMMOX and organic N burial. Other processes (e.g., ammonification or nitrification) "only" convert N among various N forms but do not actually remove nitrogen from the wastewater. Denitrification is considered to be a major removal mechanism for nitrogen in most types of constructed wetlands. Denitrification is one part of the nitrogen removal process and reduces nitrate (NO_3^-) to dinitrogen (N_2). Different requirements for the presence of oxygen for nitrification and denitrification are the major obstacle to achieving higher nitrogen removal in many treatment wetlands.

Environmental factors known to influence denitrification rates include the absence of O_2, redox potential, soil moisture, temperature, pH value, the presence of denitrifiers, soil type, organic matter, nitrate concentration and the presence of overlying water (Vymazal, 1995).

2.3.2. Bioaugmentation

The development of the treatment capacity for nitrogen and carbon transformation takes time. A suitable microbial community for wastewater treatment in constructed wetlands usually develops spontaneously from the microbes that were previously present in the substrate and the microbes that arrive with the

inflowing wastewater (Decamp and Warren, 2001; Silyn-Roberts and Lewis, 2001; Stevik et al., 2004). One means to shorten the adaptation period of a subsurface flow CW is to accelerate the development of the necessary characteristics of the local microbial community through bioaugmentation. The main advantage of bioaugmentation is on its ability to significantly accelerate the removal rate of pollutants over a relatively short period.

Bioaugmentation is the supplementing of microbes that have certain necessary metabolic traits into polluted soil or water, with the aim of accelerating the biodegradation of pollutants (Scow and Hicks, 2005). Bioaugmentation is usually performed using microbial strains or mixes of strains that: (1) are isolated from the same polluted site and grown in the selective media containing the pollutant; (2) have specific metabolic pathways that are not present in the environment; (3) have been genetically modified (Scow and Hicks, 2005). At present there is limited information about experiments using bioaugmentation in constructed wetlands for wastewater treatment. Bioaugmentation has been reported as a suitable measure to enhance microbial activities in polluted soils (Andreoni et al., 1998) and wetlands (Simon et al., 1999). Bioaugmentation is also used to enhance the biodegradation of pollutants (gasoline, petrol etc.) (Van Veen, 1997). Few studies have been performed on the efficiency of bioaugmentation in treatment wetlands in connection with the degradation of pesticides (Runes et al., 2001), organic chemicals (Simon et al., 2004), PAHs (Yu et al., 2005) and the removal of heavy metals (Park et al., 2008; Lampis et al., 2009).

The microbial communities of different substrates have different structures, and therefore the best results in bioaugmentation have been achieved with microbial strains or microbial communities that have been isolated from the same polluted environment, because microbial communities that are isolated from the same environment are adapted to it, whereas introduced microbes will be competed out (Simon et al., 2004; Bento et al., 2005).

Heinaru et al., (2005) report that microbial populations together degrade pollutants more efficiently than a single strain, due to the presence of partners which use the various intermediates of the degradation pathway more efficiently.

Thompson et al. (2005) state that regardless of the approach chosen, the isolation and characterization of the appropriate microorganisms as well as their survival and catabolic activity in the contaminated environment are the key factors for successful bioaugmentation.

Key factors that determine the efficiency of the bioaugmentation process: (1) the ability of bioaugmented microbes to degrade the target contaminant; (2) the competence of introduced microbes to compete with the indigenous population for the necessary nutrients; (3) the simplicity of use or distribution of the bioaugmetation method; (4) the high stability during storage of the microbial material, and; (5) the low costs associated with the production, storage, and transportation of microbial material (Romich et al. 1995).

Bouchez et al., (2000) and Genry et al. (2004) mention several reasons for the possible failure of bioaugmentation, which include abiotic factors such as extremes in temperature, water content, pH, nutrient availability, low availabi-

lity or potentially toxic level of pollutants and biotic factors such as the production of antibiotics or antagonistic interactions.

2.3.3. Competition and survival of introduced microorganisms

The main factor that causes the failure of bioaugmentation is the rapid decrease in the population size of the introduced microbes (Ramos et al., 1991; Thiem et al., 1994). The fate of introduced microorganisms in various environments is controlled by: 1) physicochemical parameters Evans et al., (1993); 2) nutrient availability (Fujita et al., 1994; Goldstein et al. 1985; Barcina et al., 1997); 3) and the existence of microniches (Posma et al., 1990). The competition of the bioaugmented and autochtonous microbes can be controlled by adding specific nutrients that are degradable only by bioaugmented microbes (Ogunseitan et al. 1991, Van Veen et al. 1997) or by changing the operation parameters of the system in which bioaugmentation is applied (Blumenroth and Wagner-Döbber, 1998; Fijuta et al. 1994). In addition, it has been reported that the survival of bacteria added to soil was improved by the pre-adaptation of the strains on a minimal medium with soil extract (Timmis 1997).

Also, Cho et al. (1997) and Wolter et al. (1997) state that inoculum carrier is one of the primary factors that affects bioaugmentation, and an ideal carrier material for microbial cells in the bioaugmentation process transfers the microorganisms without affecting their population or capacity to degrade chemicals.

2.3.4. Bioaugmentation for the enhancement of denitrification in CWs

There are quite a few studies about the use of bioaugmentation to boost N removal in CWs.

Rustige and Nolde (2006) have performed laboratory experiments with specially adapted biomass that has been added to columns imitating constructed wetlands; Paredes et al. (2006) have inoculated a subsurface flow constructed wetland with anammox bacteria, which have an extremely low growth rate; Zou et al. (2009) used bioaugmentation with amended soil and report that bioaugmentation caused enhanced nitrogen removal in a laboratory-scale subsurface wastewater infiltration system; Andersen et al. (2006) state that bioaugmentation with denitrifying bacteria *Comamonas denitrificans* ATCC 700936T yielded positive results. Experiences with the application of bioaugmentation for the remediation of polluted soils or sediments in natural wetlands (Park et al., 2008), meanwhile, are not applicable to subsurface flow CWs for wastewater treatment, because the CWs have a constant inflow of pollutants, whereas polluted wetland sites usually do not.

3. MATERIALS AND METHODS

3.1. Bioaugmentation trials

The research done for this PhD analyses two sequential trials with LECA-based horizontal flow soil filter filled with new substrate. These trials started in different seasons: the first one on 28th January 2008 and the second one on 5th June 2009.

The first trial (Publication 3) was a pilot-scale approach planned to determine proper amounts of donor material for bioaugmentation using a donor system with similar substrate, and to obtain data for further study.

The second bioaugmentation trial (Publication 4) was begun in the summer period in order to allow the faster growth of microbes due to the higher water temperatures than in the previous trial, and its set-up was based on data obtained from the above-mentioned pilot-scale experiment. We assumed that the lowering of the wastewater retention time inside the HSSF MCs of the study system should make the differences in NO_3-N concentrations in parallel outflows, when control MCs are compared to bioaugmented MCs, more noticeable. Wastewater retention time in the HSSF MCs was lowered to as low as 1.2 days and kept stable during the entire trial period.

3.2. Site description

The study system is located on the territory of the active sludge wastewater treatment plant (AWP) of Nõo village in Southern Estonia. The wastewater (domestic wastewater combined with effluents from the dairy and meat industries) is pumped into the CW before it reaches the grid of the AWP.

The exact water volume is controlled by a timer-operated pump. A certain amount of wastewater is first pumped into a septic tank (2 m^3). After the septic tank, wastewater flows by gravity through the interim well to the VSSF pre-treatment filters. During the first trial VSSF pre-treatment filters, total area was 3 m^2 (Publication 3). After the end of the first trial, the area of the vertical pre-treatment filter was increased twice, to 6 m^2, to avoid overloading it (Publication 4). This change was introduced to ensure that wastewater flowing into the HSSF part of the hybrid CW has a similar TOC/BOD_7 ratio and NO_3-N concentrations to the beginning of the pilot-scale experiment described in Publication 3.

Pre-treated wastewater flows into the distribution box, where wastewater is divided equally between 21 parallel MC cells (for each MC cell: length – 1.5 m, width – 0.2 m, depth – 0.6 m) (Fig. 1).

The HSSF MCs were filled with LWA with particle size of 2–4 mm.

In both trials we had a total of 9 MCs used for the bioaugmentation experiment with LWA. During the first trial, from the building of the system to 10th

July 2009, the studied filters were covered with 5-cm-thick insulation slabs to stabilize water temperature during the study period (Publication 2).

At the time of the second trial we used the same insulation slabs only, to avoid the freezing of wastewater due to low temperatures in the middle of the study period, from 29th October 2009 until 5th April 2010 (Publication 4).

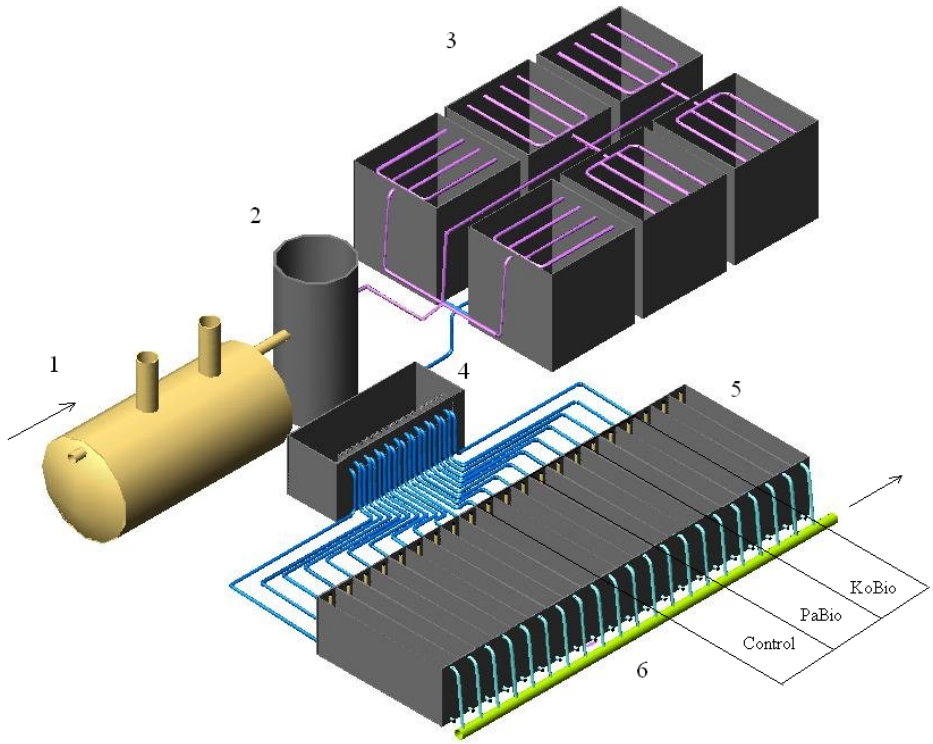

Figure 1. The design of the Nõo study system as it was at the time of the second trial. 1 – Septic tank, 2 – interim well, 3 – vertical flow pre-treatment filter, 4 – wastewater distribution box for the inflows of the HSSF mesocosms, 5 – HSSF mesocosms, 6 – outflow pipes and collector pipe. MCs used for the trial: **Control** – control MCs, **PaBio** – MCs with microbial suspension from Paistu CW, **KoBio** – MCs with microbial suspension from Kodijärve CW. MCs not surrounded by a rectangle were not used in the bioaugmentation trial.

3.3. Description of experiments

Both trials were performed with LECA-based HSSF soil filter mesocosms filled with fresh, germ-free LECA and inoculated with a microbial community suspension (hereinafter 'sediment suspension'). Sediment suspension was produced by the robust mechanical treatment of the substrate collected from the inflow parts of the LECA-based HSSF soil filters of the hybrid CWs. During the first trial (trial 1) we used sediment suspension collected from the HSSF part of the hybrid CW that treats the wastewater of Paistu Basic School in Viljandi County, Estonia (donor system 1). This donor system has been in operation since 2002 (Publication 3). In the second trial (trial 2) we added the other donor system (donor system 2). This system treated the wastewater from a home for the elderly in Kodijärve, Tartu County, Estonia. This donor system was renovated and has been operating as a new system since 2005 (Publication 4).

The main reason for the investigation of the donor material from the Kodijärve horizontal filter was to see whether and how much the difference in the working regime of a donor system with the same substrate influences the results of bioaugmentation.

The main difference between the donor systems regarding our experiment in trial 2 was the age and stability of the conditions for the development of a denitrifying microbial community.

From December 2006 to December 2008, wastewater flowing into the Kodijärve hybrid CW has been re-circulated using different re-circulation regimes, whereas recirculation and the vertical filter of the Kodijärve hybrid CW were not in use during the 6-month period before the collection of the substrate for bioaugmentation (from December 2008 to June 2009) (Table 1).

The HSSF filter bed of Paistu hybrid CW received wastewater pre-treated in a vertical subsurface flow (VSSF) filter. In this respect, the Paistu HSSF filter bed had a relatively stable environment compared to the Kodijärve HSSF filter bed.

Table 1. Information about working regimes and the values of BOD_7 and NO_3-N (mean ± s.d.; N) in CWs whose HSSF filter beds were used for the collection of sediment suspensions for bioaugmentation. The values of the Paistu CW were adapted from Öövel et al. (2007) (Publication 4)

Donor system	Period of data collection	Working regime		Average BOD_7 (mg/l)			Average NO_3-N (mg/l)	
		Pretreat- ment in VF	Recircu- lation	VF inflow	HF inflow	HF outflow	HF inflow	HF outflow
Kodi- järve (1)	December 2008 to June 2009	No	No	–	90.7±42.4 (N=3)	10.1±15,5 (N=3)	0.030±0.02 (N=3)	0.10±0.12 (N=3)
	December 2006 to December 2008	Yes	Yes	80.3±26.8 (N=9)	34±33 (N=9)	3.9±6.1 (N=9)	22±16 (N=9)	12±10 (N=9)
Paistu (2)	30 October 2003 to 15 October 2005	Yes	No	98.1±46.9 (N=18)	18.8±10.2 (N=18)	5.5±5.9 (N=18)	calculated value: 32.9	calculated value: 16.0

The water quality parameters presented in Table 1 indicate that both donor systems worked well for the mineralization of organic material and denitrification. Very low NO_3 values in Kodijärve during the period from December 2008 to June 2009 indicate that in the VSSF the nitrification process did not function well (Publication 4).

Both donor systems contained LWA with a particle size of 2–4 mm. The substrate for the extraction of sediment suspension containing the expected microbial community was collected at a depth of 30–40 cm from the water-saturated substrate layer of the HSSF filter bed of the donor hybrid CW. The water inside the substrate samples was replaced with the water collected from the outflow well of the HSSF filter bed in order to lower the coliform content in the sediment suspension resulting from the treatment. The water level inside the vessel, which contained 8 litres of LWA, was 1 cm below the surface of the LWA. The sediment suspension (stock suspension) from donor substrates was produced by treating 8 litres of the substrate mixture and added water in a concrete vibrator for 4 minutes. After the treatment, the sediment suspension (stock suspension) was separated from the LWA. Bioaugmentation was performed by mixing the sediment suspension (stock suspension) with wastewater that had been left in the MCs for one week in the first trial and for three days in the second trial, then drained and used for re-filling with the purpose of inoculation. The MCs were inoculated 1–3 hours after production of the suspension. Three replicas of control MCs and three replicas of MCs that were bioaugmented with different concentrations of the sediments from the same donor system (Publication 3) or sediments from two different donor systems (Publication 4) (9 MCs in total in both case) were used in our experiment.

The water in the MCs was kept stagnant for 4 days in the first trial and for 3 days in the second trial, in order to let the introduced microbial community to stabilize, attach and start the formation of biofilms before the regular working regimes was commenced on 28 January 2008 in the first trial and on 8 June 2009 in the second trial (Publication 3 and 4).

In the first trial (Publication 3) we had two different microbial concentrations for the inoculation of mesocosms. Through repeated analysis, we determined the correct numbers of final abundance of the introduced cells in the microbial suspension and wastewater mix: 1118±602 introduced cell/ml drained wastewater and five times higher concentration, 5177±3009 introduced cell/ml drained wastewater (Table 2) using the method for counting bacteria in stock solution (Publication 3).

At present there are more progressive methods for determination total number of bacteria and species composition microbes in the donor material, but we did not need these methods for our experiments. The method presented does not enable calculation of the exact numbers of introduced cells, but only visual determination and comparison of the relative amount of microbes in the microbial suspension of different donor materials. Therefore in the second trial (Publication 4) only the content of C and N were measured in introduced sediments.

3.3.1. Method for counting bacteria in stock solution

In order to determine the microbial count of the stock solution, water samples were vacuum filtered through black polycarbonate nuclepore filters (with a pore size of 0.2 μm). Filters were placed on microscope slides and were then stained with 6 μl DNA-specific fluorescent stain SybrGreen I together with mounting medium Moviol in one step (Lunau et.al., 2005). Microbes were counted using epifluorescence microscope Olympus BX51 with an oil immersion objective.

For this purpose, preparations were captured digitally with a built-in Olympus DP71 camera using the cell^{B} program. Nearly 20 pictures (at least 200 cells) were taken of each preparation, and microbes were enumerated from the pictures as a total count (Publication 3).

3.3.2. Amounts of introduced sediment

During the first trial we introduced 1.03 l and 5.15 l of stock suspension per 150 l wastewater-filled LWA of each individual mesocosm cell for the bioaugmentation experiments with the Paistu donor material, and at the time of the second trial we introduced 0.77 l and 0.68 l with the Paistu and Kodijärve donor material respectively (Table 2). The amount of the mix of drained substrate water and stock suspension was 50 l per each individual mesocosm.

3.3.3. Substrate sampling and analysis

During the first trial, soil samples were collected from each mesocosm prior to the second water sampling event on 27 May 2008, and the second trial soil samples were collected from each MC after the first water sampling, on 6 July 2009. Substrate samples in both trials were collected using a similar method: one soil sample consisted of 5 subsamples that were collected at even distances along the longitudinal axis of the individual mesocosm from a depth of 25–35 cm and were mixed (Publication 3 and 4).

3.3.4. Biolog Ecoplate analyses

In the first trial, Biolog Ecoplates (Biolog Inc., USA) were used to differentiate the microbial consortia from the composite soil samples of three replicas of the control mesocosms and bioaugmented mesocosms. Each plate contains 31 individual carbon test substrates and tetrazolium dye for the indication of bacterial growth. Soil samples were analyzed on the day they were collected from the wetland. Nearly 1.5 g of each individual composite soil sample was crushed in the brayer and suspended on a Vortex in 30 ml sterile tap water. After the settling of the heavier particles, 1 ml of the suspension was dissolved in 30 ml of sterile tap water and suspended on a Vortex.

Table 2. Average amounts of introduced sediments. Stock suspension per mesocosm (litre) and content of added dry inorganic and organic matter (mg per kg substrate DM), estimated count of microbial cells in stock suspension (cells/ml) and estimated final abundance of introduced cells in stock suspension and wastewater mix (cells/ml) in the first and second trial. Paistu A – MCs with the addition of microbial suspension (concentration 1) from Paistu CW in the first trial; Paistu B – MCs with the addition of microbial suspension (concentration 2) – five-fold higher concentration from Paistu CW in the first trial; Paistu – MCs with the addition of microbial suspension from Paistu CW in the second trial; Kodi – MCs with the addition of microbial suspension from Kodijärve CW in the second trial

Trials	Donor material	Amount of introduced stock suspension per mesocosm (litre)	Added dry matter (mg per kg substrate DM)	Added inorganic matter (mg per kg substrate DM)	Added organic matter (mg per kg substrate DM)	Estimated count of microbial cells in stock suspension (cells/ml)	Estimated final abundance of introduced cells in stock suspension and wastewater mix (cells/ml)
First trial	Paistu A	1.03 l	631	517	114	55396	1600±850 (1118±602)
	Paistu B	5.15 l	3155	2583	572	55396	8060±4250 (5177±3009)
Second trial	Paistu	0.77 l	536	516	20	589114	9033±3574
	Kodi	0.68 l	402	392	10	147407	2015±819

The suspension of bacteria obtained from each soil sample was used for the inoculation of Biolog Ecoplates. Well colour development was measured with a spectrophotometer at 24, 48, 72, 96, 120 and 146 h after inoculation. Data obtained from the 96 h incubation were used for further analysis (Publication 3).

3.4. Detection of substrate carbon and substrate nitrogen

During both experiments the content of C and N was detected in the composite soil samples (Figure 2). Substrate samples were analysed using "The Determination of Nitrogen in Soil by the Kjeldahl Method", and organic matter was analysed using the "Loss on Ignition" method in the Laboratory of Biochemistry at the Estonian University of Life Sciences. For the calculation of carbon numbers from the numbers of organic matter, we used the conversion factor of 0.49.

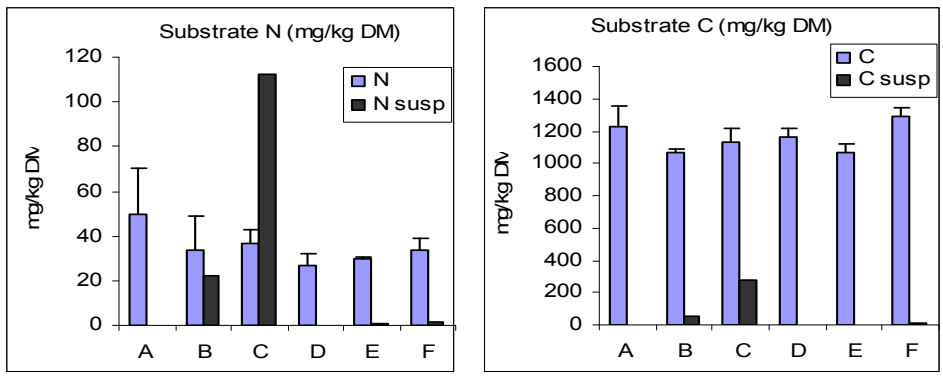

Figure 2. The average and standard deviation values of soil C and soil N (mg/kg DM) in the substrate samples collected from the studied mesocosms (MCs). The amounts of C and N (mg/kg DM) that were added with sediment suspension are shown as C suspension and N suspension for C and N respectively. A – control MCs during the first trial; B – MCs with the addition of microbial suspension (concentration 1) from Paistu CW in the first trial; C – MCs with the addition of microbial suspension (concentration 2 – five-fold higher concentration) from Paistu CW in the first trial; D – control MCs during the second trial; E – MCs with the addition of microbial suspension from Paistu CW in the second trial; F – MCs with the addition of microbial suspension from Kodijärve CW in the second trial.

The content of dry matter and the concentrations of C and N of the introduced stock suspension were also detected (see: Table 2). The amounts of C and N and dry matter added with sediment suspension during the bioaugmentation were calculated, considering that the sediment suspension diluted in drained wastewater and used for the refilling of the MCs was evenly distributed throughout the substrate.

3.4.1. Water sampling and analysis

Water samples were collected during the study period at the time of the first trial, from 24 January to 10 July 2009 (3 water sampling events) and during a second trial from 01 June 2009 to 07 July 2010 (9 water sampling events). During the both trials, water samples were taken from the inlets and the outlets of HSSF mesocosms.

Water samples were analyzed for BOD_7, N_{tot}, NH_4–N, NO_2–N, NO_3–N and TOC (during the first trial TOC was only analyzed in selected samples during the second and the third water sampling events, at the time of the second trial TOC was analyzed in every individual sampling time) (Table 3). We used Standard Methods for the Examination of Water and Wastewater (APHA, 1989) in the laboratory of Tartu Environmental Research Ltd (Publication 3).

During the second trial, water quality parameters N_{tot}, NH_4-N, NO_2-N, NO_3-N and TOC were analyzed using Dr. Lange cuvette tests (Hach-Lange, Germany). Water samples for BOD_7 were analyzed using Standard Methods for the Examination of Water and Wastewater (APHA, 1998) in the laboratory of Tartu Environmental Research Ltd (Table 3). Water temperatures inside the mesocosms and wastewater retention times are shown in Table 3.

Table 3. Sampling dates and experimental parameters in hybrid constructed wetland in Nõo pilot system during both bioaugmentation trials.

Trials	Sampling time	Numbers of days from launch	Numbers of days between sequential selected events	Water quality parameters	Hydraulic retention time (days)	Water temperature ºC (average)
First trial of bioaugmentation (24 January – 10 July 2009)	25 February	32	32	BOD_7, N_{tot}, NH_4–N, NO_2–N, NO_3–N	4.2	4
	28 May	124	92		3.3	11
	10 July	167	43		1.7	14
Second trial of bioaugmentation (08 June 2009- 10 June 2010)	1 July	23	23	BOD_7, N_{tot}, NH_4–N, NO_2–N, NO_3–N, and TOC	1.2	18,6
	20 July	42	19			17,2
	11 August	64	22			18,2±0.4
	7 September	91	27			14.4±0.1
	5 October	119	28			7.0±0,3
	2 November	147	28			3.0±0.2
	12 January	218	71			1.6±0.6
	19 April	315	97			5.2±1.0
	6 July	393	78			21.8±0.2

3.4.2. Data analysis

During both trials, the preliminary calculation of data was performed using *Microsoft Excel 2003* (Richmond, WA, USA), and *Statistica 8.0* (Statsoft Inc., Tulsa, OK, USA) was used for the statistical analysis.

In the first trial, we also used the Biolog microplate method, in which optical density values divided by average well colour development (AWCD) were analyzed using principal component analysis (PCA) and were used for the calculation of the Shannon index of diversity:

$$H = -\sum p_i \ln(p_i)$$

where: p_i is the relative abundance of each species, calculated as the proportion of individuals of a given species to the total number of individuals in the community: n_i/N. Species evenness was calculated according to the following formula:

$$J' = H' / H'_{\max}$$

where:

$$H'_{\max} = \ln S$$

and S is the number of species (species richness).

Selected water quality indicators were checked for normality, and Pearson or Spearman Rank Order correlation coefficients between these indictors were detected (Publication 3).

In both the first and second trials, Kolmogorov-Smirnov, Lillefors, and Shapiro-Wilk's tests were applied to test the variables for a normal distribution. For normally distributed data we used the t-test and paired t-test, whereas the non-parametric Mann-Whitney U test and Wilcoxon Matched Pairs test was used in cases where data were not normally distributed. We used repeated ANOVA measurements to check the possible effect of bioaugmentation, as the same microcosms were measured repeatedly. The repeated measurements factor (time) had 6 levels. The categorical factor had 3 levels: Control, PaBio and KoBio. When the values in time levels did not follow the normal distribution, log-transformation was applied to normalize them. The Fisher LSD test was used for multiple post-hoc comparison of treatment methods. To measure the relation between two variables, Pearson correlation coefficient (r) was calculated. A significance level of α=0.05 was set in all cases (Publication 4).

3.5. The recirculation systems

In publication 1 and publication 2 we studied compact hybrid CW experimental systems situated in Nõo and Rämsi. These LWA-based systems were established in summer 2005. Pollutant removal in these hybrid compact CWs was optimized by regulating hydraulic loading rates and operational regimes. In these trials the recirculation of the treated wastewater was changed during six subsequential sampling periods. Recirculation rates during different operational regimes were from 0 to 600%. We consider the HSSF CW in Nõo (Publication 2) to be an analogue to the Kodijärve system, which was used as a donor system for bioaugmentation. Since the summer of 2005, wastewater was re-circulated from the outflow well to the inflow well (to enhance nitrification-denitrification) and filter material LWA was used (Figure 3) in the Kodijärve HSSF CW.

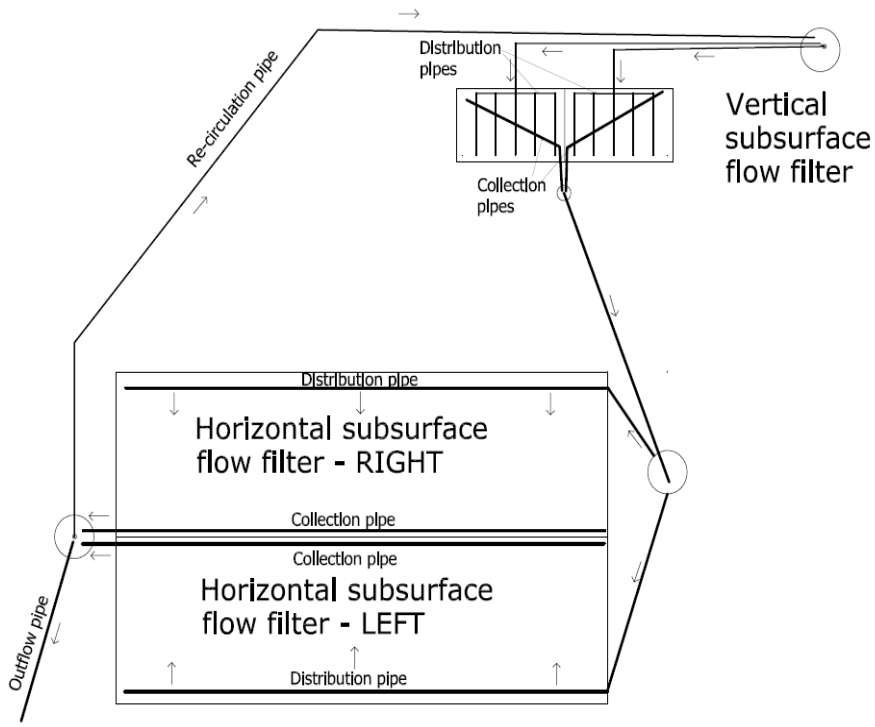

Figure 3. Schematic layout of the hybrid constructed wetland in Kodijärve, Estonia (after reconstruction in 2005).

The main reason to use donor material from the recirculation system in our bio-augmentation trial was that the substrate of the donor system was similar to HF mesocosms, whereas the wastewater flowing into the HF was a mix of re-circulated and non-recirculated wastewater and was supposed to support a different microbial community.

4. RESULT AND DISCUSSION

4.1. Recirculation experiments

Recirculation experiments (Publication 1 and Publication 2) show that with a decrease in wastewater and pollutant load and the greater re-circulation of the wastewater, purification efficiency increased in terms of most water quality indicators. Therefore, higher recirculation is a good solution to improve purification performance in overloaded systems. As expected, purification efficiency in both pilot systems analyzed in Publication 2 had a significant negative correlation with hydraulic loading entering the CW system. The main reason for the unsatisfactory results during the first periods (operational regimes) were the overloaded systems (in terms of both hydraulic, nutrient and organic matter load). Likewise, it is reasonable to assume that the communities of microorganisms were insufficiently developed in the filter material. We generally assume that the main effect of the recirculation is to increase aeration in vertical pre-treatment filters. Our results suggest that optimal hydraulic load for the hybrid CWs in cold climates should be up to 20 mm d^{-1}, with a recirculation rate of 150–300% (Publication 1). This hydraulic load is low, and thus other methods for the optimization of purification processes in hybrid CWs should also be considered.

4.2. Impact of bioaugmentation on microbial activity

This analysis is concerned with the impact of different factors on the formation of the denitrification capacity of an HSSF filter after bioaugmentation. During the first and second trials, the conditions in the Paistu HSSF CWs donor system differed only in terms of seasonal temperature regimes.

The first trial comparison of the NO_2–N and NO_3–N concentrations (mg l^{-1}) in the outflows of the studied HSSF CW mesocosms showed that the mesocosms that were bioaugmented with concentration 2 (microbial suspension with five-fold higher concentration of microbial cells in the first trial) tended to have better purification results over all three sampling events (Fig. 2 in Publication 3). The mesocosms that were bioaugmented with concentration 1 (microbial suspension from Paistu CW in the first trial) had a weaker trend of improved purification results in the first and second sampling events compared with concentration 2, because these data were not used in the third water sampling event. Also, the abundance of introduced cells of concentration 1 was too low to have an effect at the time of the first water sampling event, which occurred 4 weeks after bioaugmentation (Publication 3). In addition to the trend towards better purification results, bioaugmentation seems to have stabilized NO_2–N and NO_3–N concentrations in the outflows of the mesocosms, lowering the variability of outflow concentrations. The latter effect is observable as the lower

standard deviations of average NO_2–N and NO_3–N outflow concentrations and also the lower variation coefficients of these average outflow concentrations in the outflows of bioaugmented mesocosms compared to the outflows of control mesocosms during subsequent water sampling events (Fig. 2 and Table 1 in Publication 3). NO_3-N concentrations in the inflows and outflows of the studied MC during both trials are shown in Figure 4.

The second trial, which was launched in summer, gave positive results. In order to verify the effect of bioaugmentation on NO_3-N concentrations in the outflows of all variants of the studied MCs, repeated ANOVA measurement (Fig. 4) was used. Data analysis shows that concentrations of NO_3-N in the outflows of all variants of the studied MCs were significantly influenced by the time factor ($p<0.001$, ANOVA), and that the interaction of time and treatment was also a significant factor ($p<0.05$, ANOVA). The time factor in this study is directly related to water temperature. Water temperature is one of the key factors that determine denitrification in constructed wetlands (Vymazal, 1995). Post-hoc comparison indicated that bioaugmented MCs with the sediment suspension from the Paistu HSSF had significantly lower NO_3-N concentrations than the control MCs ($p<0.05$, Fisher LSD test). This finding is consistent with the fact that the flow regime and essential parameters of the wastewater flowing into the HSSF part of our study system resembled much more closely that of the Paistu HSSF filter bed than the Kodijärve HSSF filter bed. More successful bioaugmentation with the donor material from the similar system is consistent with statements by Simon et al. (2004) and Bento et al. (2005) about the improved survival of introduced microbes that are isolated from the same environment.

Since the 2nd, 3rd and 7th sampling times were not used for the analyses, the discussion of the influence of the time factor indicates only the 1st, 4th, 5th, 6th, 8th and 9th sampling times.

The values of NO_3-N concentrations in the outflow of the bioaugmented MCs compared to the outflows of control MCs were somewhat but not significantly lower on the first sampling occasion (Fig. 4). At the 9th sampling occasion the effect of bioaugmentation already seems to have been over-shadowed by the effect of the development of an autochtonous denitrifying microbial community (Johanson et al., 1998). During the 9th sampling occasion NO_3-N removal was equally high in all three MCs, and was 86.9% in MCs with the addition of microbial suspension from Paistu CW.

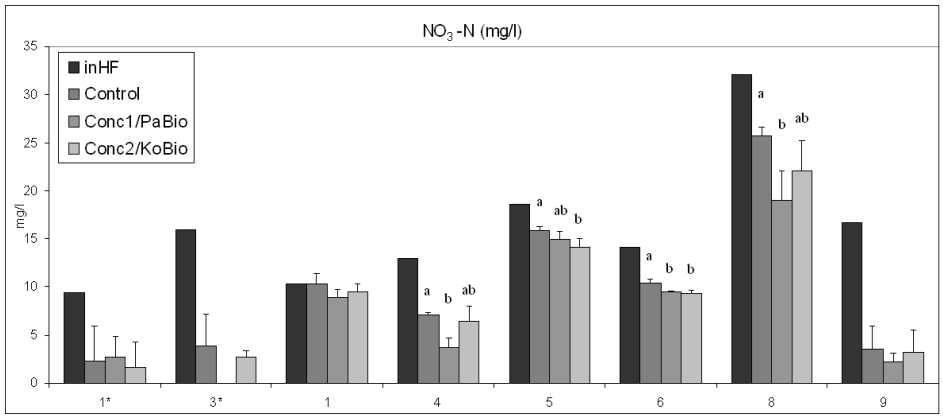

Figure 4. NO_3-N concentrations (mg/l) in the inflow and the average and standard deviation values of NO_3-N concentrations (mg/l) in the outflows (mean ± s.d.; N=3) of studied mesocosms at subsequent sampling times during the first trial and during the second trial (1 – 1st sampling occasion, 4 – 4th sampling occasion, etc.). Sampling times of the first trial are marked with an asterisk (*). The letters above the outlet columns indicate significant differences (t-test, $p<0.05$ or $p<0.01$) within a sampling occasion. In HF – inflow, Control – control MCs, Conc1/PaBio – MCs with the addition of microbial suspension with lower concentration in the first trial or microbial suspension from Paistu CW in the second trial, Conc2/KoBio – MCs with the addition of microbial suspension with higher concentration in the first trial or microbial suspension from Kodijärve CW in the second trial.

On the 4th sampling occasion, the NO_3-N concentration in the outflows of the MCs with the addition of microbial suspension from Paistu CW was 1.93 times lower than in the outflow of the control MCs ($p<0.01$, t-test) (Fig. 4). NO_3-N removal in the MCs with the addition of microbial suspension from Paistu CW was 1.57 times higher than that of the control MCs (45.8%) during the 4th sampling occasion. Similarly, at the 8th sampling time there was a remarkably lower NO_3-N concentration in the outflows of the MCs with the addition of microbial suspension from Paistu CW than in the outflows of control MCs ($p<0.05$, t-test) (see: Fig 4.).

During the 5th and 6th water sampling occasions, the effect of bioaugmentation that resulted in significant differences between the MCs ($p<0.05$, t-test) was relatively weak due to relatively low temperatures, and was also present in MCs bioaugmented with sediment suspension from the Kodijärve HSSF filter. The pattern of the 8th sampling time resembles to that of the 4th. The main reason for this may be the higher temperature compared to the sampling events at 5th and 6th water sampling times. Generally, the above-mentioned results of the comparison of MCs at individual sampling times were variable, and the detected differences in outflow concentrations of nitrate between the MCs were in most cases relatively small (Publication 4).

It is reasonable to assume that at low temperatures, the role of biofilms is relatively limited, and denitrification depends on the microbes of inflowing wastewater (because it is relatively warmer than the substrate of the HSSF).

The impact of water temperature is one of the most important factors controlling wastewater purification in CWs (Kadlec and Knight, 1996). It may be important for the survival of introduced cells. The development of biofilms in mesocosms at lower water temperatures is limited (Madden et al., 2010). Experiments with bioaugmentation were performed in different temperature conditions. In Publication 4, the word time factor is used to indicate different sampling times. Water temperature data in the all studied mesocosms was shown in Table 3. During the first and second trials, water temperatures in the studied MCs in winter were practically identical. At the time of the second trial, water temperatures remained stable during the first three water sampling times, and began to drop from the 4^{th} water sampling time.

In the first trial the final abundance of introduced cells per ml drained wastewater was lesser than in the second trial (Table 2). This situation can be explained by the impact of higher temperature at the time of bioaugmentation. We assume that in winter, a higher abundance of introduced cells may be necessary to overcome the slow development of the microbial community in the bioaugmented system (Murphy et al., 1997; Reynold et al., 1989; Jenneman et al. 1985).

The growth and development of bioaugmented microbial communities in the first sampling periods have a similar pattern during both trials, whereas average purification efficiency differed due to different retention times (Table 3). For example, in Publication 4, the significantly lower average NO_3-N purification efficiency of all studied LWA-filled MCs (N=9) on the first sampling occasion compared to the second and third sampling occasions ($p<0.01$, Wilcoxon Matched Pairs test), when water temperatures were similar, may indicate that the growth of denitrifying microbial communities had already reached a plateau at the time of the second sampling, since Truu et al. (2009) state that the formation of the biofilms in the new system usually already occurs within 100 days after launching of the system.

At the times of both trials, purification efficiencies (%) (in the second trial we used the word "removal") of the studied water quality indicators were calculated for all water sampling events with two exceptions (Fig. 2 in Publication 3 and Table 2 in Publication 4).

During the second trial, the average NO_3-N removal of all of the studied LWA-filled MCs was relatively low due to the very high load (Table 2, Publication 4). In the first trial the average purification efficiency of NO_3–N remained at nearly 80% during the first and third water sampling events. At the time of the second trial the average purification efficiency of NO_3–N was 39.6% ± 22.5% during the entire study period.

At the time of the first trial, hydraulic retention time during the sampling events decreased gradually to find the optimal regime for the detection of differences in denitrification (see Table 3). Hydraulic retention time during the second trial was a stable 1.2 days during the entire study period. The average mass removal rates during the first and second trials are shown in Table 4.

Table 4. Mass removal rate (mg m^2 day^{-1}) during both trials.

MCs and sampling times in Trial 1			MCs and sampling times in Trial 2						
Sampling time	25.02.08	10.07.08	Sampling time	01.07.09	07.09.09	05.10.09	02.11.09	19.04.10	06.07.10
MCs Control	284±146	1214±326	MCs Control	0±159	829±42	380±63	514±64	886±126	1818±327
MCs Conc. 1	268±90	–	MCs PaBio	200±133	1301±144	509±116	640±9	1819±427	2015±138
MCs Conc. 2	309±106	1334±74	MCs KoBio	105±103	910±212	616±113	663±46	1397±443	1866±309

The results presented in Table 4 show that the relationship between the changing of the retention time and the resulting changes in NO_3-N mass removal is not clear, whereas there was, as expected, a clear increasing trend in NO_3-N mass removal over time.

4.3. Biolog-ecoplates data

During the first trial, microbial consortia of the studied mesocosms were compared using Biolog-ecoplates data. The ordination plot in Figure 5 shows that the composite soil sample of the mesocosms that received the highest quantity of the introduced microbial community had the most distant (separate) location from the composite soil samples of the studied mesocosms and composite samples of the mesocosms that were augmented with a humic substance-rich fraction of different soils as the main difference from studied mesocosms (two composite samples of the mesocosms that were augmented with a humic substance-rich fraction of different soils were included in the PCA as reference, for purposes of comparison).

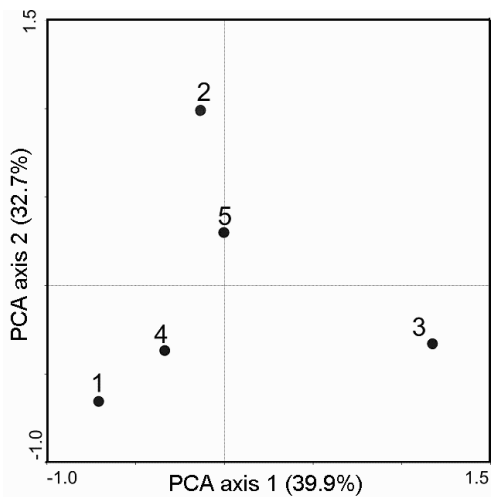

Figure 5. Results of the PCA of the Biolog-ecoplates data. 1 – composite soil sample of the control mesocosms (n=3), 2 – composite soil samples of the mesocosms with microbial suspension addition (1600±850 cell ml^{-1} water) (n=3), 3 – composite soil samples of the mesocosms with the addition of a microbial suspension with five-fold higher concentrations (8060±4250 cells ml^{-1} water) (n=3), 4 and 5 – reference composite soil samples (n=3) of the mesocosms with added humic substance-rich fraction of soil (included in PCA for comparison) (Publication 3).

The data on humic substance-amended mesocosms were not included in this study and are therefore not discussed in detail. At the same time, the use of these data could help to achieve a better understanding of the ordination of samples, as these environments are comparable to that of our study. The high distances between the location of the studied samples compared to the outsourced samples and the most separated location of the sample collected from the mesocosm that was bioaugmented with the highest concentration of microbial suspension shows that the structure of the microbial communities was closely related to the presence and amount of the introduced microbial suspension 4 months ago.

4.4. Substrate carbon and nitrogen

The amounts of C and N and dry matter that were added with sediment suspension during the bioaugmentation were calculated, considering that the sediment suspension diluted in drained wastewater and used for the refilling of the MCs was evenly distributed throughout the substrate. During the first trial, bioaugmentation resulted in the addition of 56.1 mg C and 22.5 mg N per kg of substrate dry matter inside MCs that were bioaugmented with the microbial suspension with concentration 1 from Paistu HSSF (B in Fig. 2).

During the second trial the bioaugmentation resulted in the addition of 5.1 mg C and 0.8 mg N per kg of substrate dry matter inside MCs that were bioaugmented with the microbial suspension from Paistu HSSF (E in Fig. 2) and 9.9 mg C and 1.6 mg N per kg of substrate dry matter inside MCs that were bioaugmented with the microbial suspension from Kodijärve HSSF (F in Fig. 2).

We assume that in winter, temperature and microbial activity were low, and more C and N accumulated in the substrate of the donor systems' filter beds. This assumption is logical, since it has been found that lower water temperatures support the accumulation of organic sediments in CWs (Mustafa et al., 2008). C and N concentrations in donor materials collected from Paistu HF during the first trial were respectively 11 times and 28 times higher than during the second trial. During the second trial the amount of C added with the sediment suspension comprised only 0.48% and 0.77% of total substrate C in the MCs that were bioaugmented with the donor material from Paistu HSSF and Kodijärve HSSF respectively. Therefore it is possible to assume that the amount of introduced C and N in the substrate did not play an important role in the acceleration of development of the denitrifying microbial community. There was also a significantly lower concentration of soil C inside the MCs that were bioaugmented with the lower concentration of sediment suspension from Paistu HSSF (C) than inside the control MCs ($p<0.05$, Mann-Whitney U-test) (Fig. 2). In the second trial we can observe a similar trend, but no significant difference was demonstrated there. We assume that at the time of substrate sampling, the microbial community from Paistu HF had already consumed soil organics in the MCs that were bioaugmented with the lower concentration of sediments, but not

in the MCs that were bioaugmented with higher concentrations of sediments. In the second trial, substrate C inside the MCs that were bioaugmented with the sediment suspension from Paistu HSSF had significantly lower concentrations than those inside the MCs that were bioaugmented with the sediment suspension from Kodijärve HSSF ($p<0.01$, Mann-Whitney U-test). Previous findings support the assumption that the microbes introduced from Paistu HSSF may be better able to degrade recalcitrant organic matter, whose ratio to easily degradable organics is presumed to be high in the Paistu HF and Nõo HF, but not as high in the Kodijärve HF, where recirculation was needed in addition to pre-treatment in the vertical filter.

4.5. TOC concentrations, BOD_7 concentrations and TOC/BOD_7 ratio

In Publication 3, the TOC/BOD_7 ratio had a positive correlation with a serial number of the purification stage, with $r = 0.63$ ($p<0.05$, Spearman correlation). We assume that this correlation represents the growth of the ratio of the recalcitrant fraction of TOC, which is obviously a widespread phenomenon in the degradation gradients of the CWs, as the study by Nguyen (2000) reports that over 90% of the organic matter accumulated in the studied CW was present as stable organic matter fractions. In the same case (Publication 3), the average BOD_7 value of the pre-treated wastewater flowing into the HSSF filter of the donor system (see: Material and methods) was, according to Öövel et al. (2007), 18.8 ± 10.2 mg l^{-1} during the period from November 2003 to November 2005, e. g. wastewater was sufficiently purified to result in a high TOC/BOD_7 ratio.

In Publication 4, TOC concentrations, BOD_7 concentrations and the TOC/BOD_7 ratio in the outflows of all variants of the studied MCs were significantly influenced by the time factor ($p<0.001$, repeated ANOVA measurements), which is connected with seasonal changes in temperature. A post-hoc comparison indicated that MCs bioaugmented with the sediment suspension from the Paistu HSSF had significantly lower TOC/BOD_7 ratios and significantly higher BOD_7 concentrations than the control MCs ($p<0.05$, Fisher LSD test), whereas contrary to expectations, a similar comparison regarding TOC did not reveal any significant differences. Therefore the comparison of the TOC, BOD_7 and TOC/BOD_7 ratio values in the inflow into the MCs with the values of the same indicators in the outflow from the control MC and the outflows of either bioaugmented MCs during the 1^{st}, 4^{th}, 5^{th}, 6^{th}, 8^{th} and 9^{th} sampling occasions (the average outflow concentrations of three replica of studied treatment variants were used as independent observations) was performed using a paired t-test or Wilcoxon Matched Pairs test.

The analysis presented in the Results and Discussion of publication 3 leads to the assumption that the microbial community introduced from the Paistu HSSF filter bed had, in addition to a somewhat better capacity to degrade recalcitrant organics, a relatively low ability to degrade aerobically degradable

organics. Both findings are consistent with the statements of Simon et al. (2004) and Bento et al. (2005) that the microbial community for bioaugmentation must be taken from a similar environment. The following are possible explanations for these findings: 1) some of the TOC may need anaerobic conditions for degradation, despite the fact that most of the TOC can be removed by aerobic processes; 2) the microbial community bioaugmented from Paistu HSSF may have contained a much lower percentage of aerotolerant microbes than the microbial community bioaugmented from the Kodijärve HSSF or the microbial community flowing in from the VSSF, for the following possible reasons: a) wastewater from the Kodijärve HSSF filter bed, recirculated through the VSSF filter, may have contained facultative anaerobes capable of surviving in the VSSF filter; b) and/or an autochtonous microbial community of control mesocosms and an autochtonous part of the microbial community of the MCs bioaugmented with sediment suspension from the Kodijärve HSSF was probably in the development stage, which may have caused a relatively low demand of oxygen for the degradation of recalcitrant organic matter, which in consequence may have allowed aerobic microbes that were inflowing from the VSSF community to have better availability of some oxygen left in the wastewater of the inflow part of HSSF mesocosms. Of all of the analyzed average pollutant removal values of the studied MCs at individual water sampling sessions (N=9, for every individual water sampling session), only the average TOC removal of the studied MCs increased during the intense study period (from June 2009 to November 2009, N=4), as it had a strong positive correlation with the sequential number of the sampling session ($r=0.98$, $p<0.05$). The average BOD_7 removal of all of the studied MCs (Fig. 4 in Publication 4) was high throughout all of the analyzed sampling sessions (sampling Nos. 1, 4, 5, 6). Therefore it is reasonable to assume that the slow increase in TOC removal over time was related to the increase in the number of microbes capable of degrading relatively recalcitrant organic matter left in the wastewater after pre-treatment in the VSSF filter, as Fontaine et al. (2003) state that these microbes (K-strategists) have lower growth rates. These findings show that bioaugmentation with the sediment suspension from a similar environment for the acceleration of the formation of denitrification capacity inside new LWA substrates of HSSFs in hybrid CWs can be considered in HSSFs where the ratio of recalcitrant organics to easily degradable organics in inflowing wastewater is high enough to cause a low growth rate among microbes.

5. CONCLUSIONS

Studies of the optimization of pollutant removal in compact hybrid CW systems have shown that for the successful functioning of hybrid CWs in a cold climate, optimal hydraulic load should be ≤20 mm d^{-1}, with a recirculation rate of 100–300%. With a decrease in wastewater and pollutant load and the larger recirculation of the wastewater, purification efficiency increased in terms of most water quality indicators.

Bioaugmentation resulted in a change in the structure of the microbial community and in a trend towards higher and more stable denitrification of the supplemented mesocosms during the nearly half-year study period.

We presume that inflowing free-living denitrifiers are less adapted than bioaugmented ones. The reasons for this effect may be the following: 1) stressful environmental conditions in the LECA filter for inflowing microbes caused by the chemical properties of the LECA; 2) the absence of easily degradable organic matter due to the effect of pre-treatment systems, as inflowing microbes have a poorer ability to degrade recalcitrant organic matter.

The development of denitrification capacity in the LWA substrate of HSSF CW mesocosms with high hydraulic loading rate can be significantly enhanced by the addition of sediment suspension taken from an LWA-based HSSF that is part of a hybrid constructed wetland and has a similar water quality, similar pre-treatment level of inflowing wastewater and similar flow regime. In full-scale CWs where longer retention time is used to overcome possible low nitrate removal during peak-flows, the effect of bioaugmentation may not be detectable most of the time, except during peak flow events. The relatively variable effect of bioaugmentation over time shows that it probably has less importance for full scale operation compared with other factors. Nevertheless, experiments with bioaugmentation in larger scale systems applying high hydraulic load and better temperature isolation in the winter period are needed to achieve the maximal possible positive effect of bioaugmentation for the acceleration of the development of denitrification capacity in newly built or restored HSSF components of CWs.

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SUMMARY IN ESTONIAN

Bioaugmentatsioon LWA substraadiga horisontaalfiltris reovee puhastuseks: voolurežiimi, temperatuuri ja doonorsüsteemi mõju

Käesoleva doktoritöö eesmärgiks oli LWA-põhise kompaktse hübriidsüsteemi lämmastikuärastuse võime tõstmise uurimine. Seetõttu on töö 1. ja 2. publikatsioonis käsitletud tagasipumpamise kasutamisel põhinevaid katsesüsteeme, aga 3. ja 4. publikatsiooniga seotud töö eksperimentaalne osa ja töö põhisisu keskendub küsimusele, kas kompaktse hübriidse tehismärgala horisontaalvoolulise filtri lämmastikuärastuse võime väljakujunemist saab kiirendada, kasutades selleks bioaugmentatsiooni.

Põhjapoolse parasvöötme tingimustes on hübriidseid tehismärgalasid otstarbekas ehitada kompaktsena. Tehismärgala kompaktsena ehitamine võimaldab märgala mahu ja pindala suhte tõstmise teel soojuskadusid vähendada ja talveperioodil puhastusprotsessi toimumiseks vajalikku temperatuuri säilitada.

Puhastussüsteemi rajamise ja käigushoidmise kulude optimeerimisel on süsteemi mahu vähendamiseks soovitav kasutada puhastatava reovee tagasipumpamist. Tagasipumpamise kasutamine hübriides süsteemis põhineb peale hübriidsüsteemis kombineeritud reoveepuhastuse etappide kordamise ka sisse- ja väljavoolava reovee segamisel, tõstes toitainete ja mikroorganismide kontakti. Käesoleva töö 1. ja 2. publikatsioonis käsitletud Nõos ja Rämsis paiknenud kompaktsetes hübriidsetes süsteemides tehtud katsetes on selgunud, et tagasipumbatava reovee hulga tõstmisega on võimalik ka suure hüdraulilise koormuse korral saavutada nõuetele vastavat lämmastiku ja orgaanilise aine ärastust. Selleks on optimaalne kui kogu süsteemi hüdrauliline koormus on ≤20 mm päevas ja tagasipumpamine100–300%.

Samas lisanduvad tagasipumpamise kasutamisega kulutused elektrienergiale ja sageli ka kulutused elektriliini toomiseks kuni puhastussüsteemile sobiva asukohani.

Juhul kui eelpool toodud põhjustel tagasipumpamise kasutamisest loobuda, tuleb hübriidsüsteemi mõõtmetelt kompaktsena hoidmiseks optimeerida vertikaalfiltris toimuvat aeroobset ja horisontaalfiltris toimuvat anaeroobset puhastusetappi (protsessi) eraldi. Käesoleva töö publikatsioonides 3 ja 4 on kirjeldatud kahte järjestikust katset, milles uuriti kergkruusa substraadil põhineva vertikaalvoolulisest eelpuhastusfiltrist saabuvat reovett puhastava horisontaalse pinnasfiltri lämmastikuärastuse tõstmist töötava hübriidse tehismärgala sarnase substraadiga horisontaalfiltrist võetud mikroobikooslusega bioaugmentatsiooni teel.

Esimene, pilootkatse, kestis pool aastat ja teine katse, mis põhines esimeses katses kogutud andmetel, kestis aasta. Esimeses katses viidi bioaugmentatsioon läbi talvel ja katseperioodi jooksul rakendati madalat hüdraulilist koormust. Selle katse tulemusena selgus, et vastrajatud horisontaalvoolulistesse mesokos-

midesse viidud mikroobikooslus jäi elama ja paljunes ning bioaugmentatsiooni tulemusel võis töödeldud mesokosmides katseperioodi jooksul täheldada kõrgema ja stabiilsema denitrifikatsiooni trendi.

Teises katses viidi bioaugmentatsioon läbi soojal aastaajal ja suure hüdraulilise koormuse juures kahest erinevast doonorsüsteemist võetud mikroobikooslusega. Nende hübriidsete tehismärgalade horisontaalfiltrites oli substraadiks kergkruus, aga süsteemide voolurežiimid olid erinevad. Katsete tulemusel leidsime, et nitraatlämmastiku kontsentratsioonid kõigi uuritud mesokosmide väljavooludes olid oluliselt mõjutatud ajategurist ($p<0.001$, korduvmõõtmistega dispersioonanalüüs), mis on peamiselt seotud sessoonse veetemperatuuri kõikumisega. Post-hoc võrdlus Fisheri testiga näitas, et sarnase voolurežiimiga doonorsüsteemist pärineva mikroobikooslusega bioaugmenteeritud mesokosmide väljavoolude nitraadikontsentratsioon oli oluliselt madalam kontrollmesokosmide omast ($p<0.05$), samas aga tagasipumpamise kasutamisega süsteemist võetud kooslusega bioaugmentatsioonil olulist mõju ei olnud. Seega saab hübriidse tehismärgala LWA substraadiga horisontaalvoolulise pinnasfiltri mesokosmide denitrifitseerimisvõime arenemist oluliselt kiirendada lisades mikroobikooslust, mis on võetud töötava hübriidse tehismärgala sama substraadiga horisontaalfiltrist, millesse sissevoolaval reoveel on sarnane vee kvaliteet, sarnane eelpuhastuse tase ja sarnane voolurežiim.

Ajas varieeruv ja katsetingimustest sõltuv bioaugmentatsiooni mõju tehtud katsete käigus mõõdetud lämmastikuärastusele näitab, et peale sobiva koosluse olemasolu mõjutavad sisseviidava koosluse denitrifikatsoonivõimet nii bioaugmenteeritava süsteemi veetemperatuuri sessonne kõikumine kui hüdrauliline koormus. Optimaalsete tingimuste korral võib bioaugmentatsioon märkimisväärselt tõsta horisontaalvoolulise filtri lämmastikuärastuse võimet. Selle kinnituseks on teise katse 4-ndal proovivõtu korral mõõdetud 1,93 korda madalam NO_3-N kontsentratsioon Paistu hübriidse tehismärgala horisontaalfiltrist võetud mikroobikooslusega bioaugmenteeritud mesokosmides võrreldes kontrollmesokosmidega ($p<0.01$, t-test) ja 1,57 korda kõrgem NO_3-N puhastusefektiivsus kui kontrollmesokosmides (vastavalt 71,9% bioaugmenteeritud ja 45,8% kontrollmesokosmides). Täismõõdus tehismärgalades, kus piikvooludega kaasneva madala lämmastikuärastuse kompenseerimiseks kasutatakse pikemat viibeaega, võib bioaugmentatsiooni mõju olla jälgitav ainult piikvoolu ajal. Samas võib bioaugmentatsioon suurema- või täismahulises kompaktse hübriidsüsteemi horisontaalfiltris lühikese viibeaja rakendamise korral anda parema tulemuse kui väikeses katsesüsteemis, kuna suurem süsteem võimaldab paremat talvist soojusisolatsiooni kui väike katsesüsteem.

Kokkuvõtteks võib öelda, et sarnasest keskkonnast pärineva kooslusega bioaugmentatsioon on kasutatav hübriidse tehismärgala denitrifikatsioonivõime tõstmiseks uue või vahetatud substraadiga horisontaalfiltri adaptatsiooniperioodi jooksul. Kuigi bioaugmentatsioon on puhastusprotsessi parandamiseks vaid üks võimalikest moodustest, millega manipuleerides saab lämmastiku ärastust optimeerida, võib bioaugmentatsiooni kasutamine aidata reovee puhastamise protsessi võrreldes varasemaga palju ökonoomsemaks muuta.

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PUBLICATIONS

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