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RELATIONSHIPS BETWEEN MICROBIAL CHARACTERISTICS AND ENVIRONMENTAL CONDITIONS IN A HORIZONTAL SUBSURFACE FLOW CONSTRUCTED WETLAND FOR WASTEWATER TREATMENT

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ABSTRACT

Nurk, K. 2005. Relationships between microbial characteristics and environmental conditions in a horizontal subsurface flow constructed wetland for wastewater treatment.

We studied several microbial characteristics (immobilized N, soil respiration, microbial biomass C, potential nitrification), microbial community structure (assessment of potential C source utilization patterns of the microbial community using Biolog-EcoPlates in 2001 and community-level PCR in 2002) and environmental parameters (the carbon, nitrogen and phosphorus content of the soil samples, the water quality and physicochemical indicators in water sampling wells) in the horizontal subsurface-flow (HSSF) constructed wetland (CW) for wastewater treatment in Kodijärve, Estonia in 2001 and 2002. The Kodijärve HSSF CW was constructed in October 1996 to purify the wastewater from a hospital for about 40 persons. The system consists of two beds, each 25*6.25*1m, which are filled with coarse sand. Before entering the system, wastewater flows through a two-chamber septic tank. The two beds show significant differences in hydraulic conditions and correspondingly in performance. In the dry bed, covered predominantly by *Scirpus sylvaticus*, the water table is 20–50 cm lower than in the neighbouring wet bed (*Phragmites australis*). In the summer of 2002 the vertical subsurface flow (VSSF) part, which functions as the pretreatment system for the aeration of the wastewater, was added to the HSSF system.

The higher microbial diversity and metabolic activity found in the wet bed is probably attributable to the enhanced availability of nutrients. Significant differences were found between the beds in the spatial distribution of the structure of the microbial community. The dry bed exhibited greater structural differences between the microbial community of the soil samples and lower metabolic activity in the lower layer. We assume that these differences were related to the availability and the quality of the sources of nutrients and to the variability of the aeration conditions in the different soil sites, whereas higher metabolic activity in the upper layer was related to the presence of organics of the decomposable plant material.

We found significant changes in microbial characteristics in both wet and dry HSSF beds between 2001 and 2002. The average value of the respiration of the soil samples enhanced 1.4 times, and the average value of the measured biomass C (SIR) increased 1.6 times. At the same time, immobilized N decreased 5.8 times, and potential nitrification 7.2 times. No significant differences in microbial characteristics were found between the wet and dry bed in either 2001 or 2002. The lower BOD_7 value after the building of the VSSF pretreatment filter in August 2002, and enhanced aeration in the autumn of 2002 resulted in a change in the structure of the microbial community. We assumed

that the proportion of the r-strategists within the microbial community increased, which allowed the rapid degradation of the easily degradable carbon source. The fact that the intensity of soil respiration and also the values of measured biomass-C are strongly related to the structure of the microbial communities is positive evidence that enhanced biomass-C (SIR) in 2002 was related to the dominance of r-strategist microbes in the microbial community.

The reduced BOD_7 value and enhanced aeration after the establishment of the VSSF mainly changed the distribution of the values of microbial characteristics in the vertical gradient. The sandy filter material with low humus content made the buffering system quite poor, and destabilized the selected characteristics of the microbial community, whereas the deposits of accumulated carbon, nitrogen, and phosphorus offered protection against changes in the microbial community: in sites with higher C, N, and P concentrations, the structure of microbial communities had changed to a lesser extent.

The effect of increased aeration by the VSSF pretreatment filter on the microbial community was limited, because the oxygen was already consumed in the inflow sides of both beds.

The microbial community of the lower layers was more vulnerable than that of the upper layers. This resulted in a weaker re-immobilization of N in the lower layers.

In 2001, the proportion of microbial immobilized N was 29.2% (8.7 kg a^{-1}) of the total amount of N retained in the soil filter. The average annual N removal from the system was 13.2 g m^{-2} a^{-1}. This relatively low value was significantly increased due to the establishment of a pretreatment VSSF filter in 2002.

ORIGINAL PUBLICATIONS

These theses are based on the following papers, which are included as appendices at the end of the thesis:

Publication I
Nurk, K., Truu, J., Truu, M., Mander, Ü. 2005. Microbial characteristics and nitrogen transformation in planted soil filter for domestic wastewater treatment. *Journal of Environmental Science and Health, Part A – Toxic/Haz. Subst. & Environmental Eng*. A40, 6–7, 1201–1214.

Publication II
Truu, J., **Nurk, K**., Juhanson, J., Mander, Ü. 2005. Variation of microbiological parameters within planted soil filter for domestic wastewater treatment. *Journal of Environmental Science and Health, Part A – Toxic/Haz. Subst. & Environmental Eng*. A40, 6–7, 1191–1200.

Publication III
Nurk, K., Truu, J., Mander, Ü. 200X. Impact of enhanced aeration and changed wastewater quality on the microbial characteristics of a horizontal subsurface flow planted soil filter for wastewater treatment. *The Science of the Total Environment.* (Submitted).

Publication IV
Mander, Ü., Teiter, S., Kuusemets, V., Lõhmus, K., Öövel, M., **Nurk, K**., Augustin, J. 2003. Nitrogen and phosphorus budgets in a subsurface flow wastewater treatment wetland. In: Brebbia, C.A. (Ed.) *Water Resources Management II,* WIT Press, Southampton, Boston, pp. 135–148.

Author's contribution

Publication I: The author is fully responsible for the fieldwork, data collection and analysis, most of the statistical analysis and is fully responsible for writing the manuscript.

Publication II: The author is fully responsible for the fieldwork and partly responsible for data collection and analysis, and for writing the manuscript.

Publication III: The author is fully responsible for the fieldwork, data collection and analysis, and is fully responsible for writing the manuscript.

Publication IV: The author is partly responsible for the fieldwork, data collection and analysis, and for writing the manuscript.

1. INTRODUCTION

Water purification efficiency in constructed wetlands (CW) is determined largely by the constructional parameters and optimal flow regime for the better conditioning of microbiological and geochemical nutrient transformation and retention processes.

The majority of the data about different CW-s describe wetland performance in terms of nutrient flows, accumulation and transformations for entire individual systems. The data concerning wastewater purification efficiency and the durability of CW-s vary greatly (Bastian and Hammer, 1993; Crites and Tchobanoglous, 1998). The high variability of the performance of constructed wetlands indicates that it depends not only on hydraulic load and pollutant concentrations, but also on the inner environmental conditions that vary between the different wetland types and individual wetlands. A deeper understanding of the limitations and possibilities of nutrient transformation in different wetland media would contribute to finding optimal constructional parameters and operational regimes.

The fact that the carbon and nitrogen retention and transformation in CW-s are mediated mainly by microbes is widely accepted. Microbial metabolism is responsible for the turnover of C and the transformation of organic and inorganic N forms and causes the nutrient transfer between the pools of wetland ecosystem and losses through gaseous emissions (Kadlec and Knight, 1996; Reddy and D'Angelo, 1997). Microbial C and N pools are interdependent and in dynamic balance with the other C and N pools of the wetland. These balances are influenced by the environmental factors that modify nutrient cycling inside the wetland ecosystem and losses from the wetland ecosystem.

The relationships between the microbial characteristics and environmental conditions in horizontal subsurface flow (HSSF) constructed wetlands (CW) have not at present been sufficiently studied.

The first studies of the relationships between the microbial characteristics and substrate of HSSF CW systems have until now focused on the organic matter accumulation and clogging of these systems. Tanner et al. (1998) and Nguyen (2000, 2001) focused on the clogging and maturation of gravel-bed HSSF CW-s and relationships between different organic matter fractions and microbial respiration, microbial biomass C and biomass N. The influence of the plants on the microbial mechanisms of carbon removal and microbial community consistence with molecular methods was assessed in the HSSF CW by Baptista et al. (2003). The other studies of microbial characteristics in subsurface flow systems have focused on vertical flow filters, for example the study by Felde and Kunst (1997), or Bahgat et al. (1999).

This thesis focuses on the HSSF CW in Kodijärve, Estonia. The different water tables and resulting different aeration of the wastewater cause signi-

ficantly different purification efficiencies in the parallel beds of Kodijärve HSSF (Noorvee et al., 2005).

1.1. Constructed wetlands

Constructed treatment wetlands are defined as engineered wetlands that utilize natural processes involving wetland vegetation, soil and their associated microbial assemblages to assist, at least partially, in treating an effluent or other water source, and can be classified as natural treatment systems. While conventional treatment relies largely on naturally occurring, biological pollutant transformations, it is energy intensive because it uses the input of the nonrenewable, fossil-fuel energies that predominate in the treatment processes. At the same time, natural treatment systems rely (to a greater or lesser extent) on renewable, naturally occurring energies of solar radiation, wind, gravitation, the chemical-free energy of rainwater, surface water, and groundwater; and storage of potential energy in biomass and soils. All natural treatment technologies are relatively land intensive; however, they have widely varying requirements for supplemental, fossil-fuel energy inputs; specific treatment capabilities; and different strengths and weaknesses for individual applications (Kadlec and Knight, 1996).

Vymazal (2001) divides constructed wetland systems on the basis of the type of water flow into the free water surface (FWS) and sub-surface flow (SSF) systems. SSF systems can be further divided into vertical sub-surface flow (VSSF) and horizontal subsurface flow (HSSF) systems.

Constructed wetlands are used for the treatment of different types of wastewater, which includes the treatment of primary settled and secondary treated sewage, tertiary effluent polishing; for disinfection, urban and rural run-off management, toxicant management, land-fill and mining leachate treatment, enhancement of in-stream nutrient assimilation, nutrient removal via biomass production and export, and groundwater recharge (Bavor et al., 1994). Different wetland types can be combined to exploit the advantages of specific systems to cover the needs of the different stages of nutrient transformation (Cooper et al., 1999; Vymazal, 2001).

1.1.1. Horizontal subsurface-flow constructed wetlands

HSSF CWs use a bed of soil, coarse sand or gravel as a substrate for the growth of rooted wetland plants. Wastewater flows by gravity, horizontally through the bed substrate, where it contacts microbes living in association with the substrate and plant roots. The bottom of the bed is sloped to minimize water flow overland (Kadlec and Knight, 1996). According to Kadlec and Knight (1996),

the oxygen diffusion in flooded soils is nearly 10,000 times slower than in aerobic soils, and a low dissolved oxygen level results in the accumulation of organic matter in wetland soils, because of a reduced level of microbial activity and organic decomposition. A common problem in HSSF constructed wetlands is clogging, which lowers hydraulic conductivity (Tanner et al., 1998). At the same time, the transition zone from aerobic to anaerobic conditions in the upper layers of HSSF systems offers extensive contact between soil air and soil water, as Heeb and Züst (1991) and also Noorvee et al. (2005) have found better BOD purification efficiencies when the transition zone between aerobic and anaerobic conditions have been wider. According to Wu et al. (2000), higher amounts of oxygen are diffused into the water in subsurface flow systems than in free-water systems. The HSSF CW systems are beneficial in cold climates because the water is not exposed to ambient air during the process, thus ensuring minimal energy losses through evaporation and convection (Wittgren and Maehlum 1997; Maehlum et al., 1999; Wallace et al., 2001; Werker et al., 2001)

1.2. The key processes responsible for C and N removal from wastewater

1.2.1. Carbon removal

Wetlands, which are typically characterized by the accretion of organic matter, are major sinks for C. The net accumulation of organic C is a result of the balance between primary production and heterotrophic respiration. The organic matter produced is deposited seasonally on the soil surface and may eventually be converted to a new soil layer, providing long-term storage of C and nutrients. In addition to internal production of C, effluents containing particulate and dissolved C are added to constructed wetlands, usually measured as BOD (bio-chemical oxygen demand). The BOD added to wetlands may be removed by (1) the settling of particulate BOD, and (2) the breakdown of soluble BOD during microbial respiration (Reddy and D'Angelo, 1997).

Decomposition of plant litter and particulate C is the main pathway for C removal and involves the gradual conversion of complex organic molecules to simple organic and inorganic constituents through: (1) abiotic leaching and fragmentation, (2) extracellular enzyme hydrolysis, and (3) the aerobic and anaerobic catabolic activity of heterotrophic microorganisms. Step (1) is a physical process, while steps (2) and (3) are microbially mediated reactions that are affected by substrate quality (e.g. cellulose, lignin, nutrient content), electron acceptors and environmental factors such as pH, temperature and nutrient availability (Reddy and D'Angelo, 1997).

1.2.2. Nitrogen removal

Nitrogen entering constructed wetlands is present in particulate and dissolved organic and inorganic forms (NH_4^+ and NO_3^-) forms. Particulate forms are removed through settling and burial, while the removal of dissolved forms is regulated by various biogeochemical reactions functioning in soil and the water column. Nitrogen reactions in wetlands effectively process inorganic N through nitrification and denitrification, ammonia volatilization, and plant and microbial uptake. Ammonification of dissolved organic N derived from detrital plant tissue or soil organic matter may be a source of inorganic N to the water column (Reddy and D'Angelo, 1997).

Organic N mineralization can be described as a function of C/N ratio, extracellular enzyme concentrations (such as protease), microbial biomass and soil redox conditions. Floodwater NH_4-N may be lost through ammonia volatilization, regulated by temperature, vegetation density, air movement above the water surface, mixing in the water column, NH_4^+ concentration, algal activity and associated pH fluctuations. In constructed wetlands, this process is most significant when the influent water contains high levels of NH_4-N, and pH exceeds 8.0. Nitrification occurs in the aerobic zones of the water column, soil water interface and root zone. The relative importance of these zones in overall nitrification depends on O_2 availability and NH_4-N concentration (Reddy and D'Angelo, 1997).

NO_3^- diffuses into anaerobic soil, where it is reduced to gaseous end products (N_2O and N_2) or NH_4-N. Denitrification rates are usually limited by NO_3^- concentration and diffusion of NO_3^- from aerobic zones to anaerobic sites. In a system with active denitrification, NO_3^- levels are usually low, and thus measurement of NO_3^- in soil and the water column does not provide a good indication of this process. Since denitrification is mediated by heterotrophic microorganisms, its rate may be indicated by available organic C (Reddy and D'Angelo, 1997).

1.3. Microbial C and N pools and the transfer of nutrients between the pools of the ecosystem

Nutrient transformation in the ecosystems can be described in terms of the changes of the nutrient amounts in the different nutrient pools. Differences between wetlands are largely due to differences in physical, chemical, and biological conditions that affect transformations and transport processes and treatment efficiency in the soil-water-plant system (Reddy and D'Angelo, 1997).

The pools of microbial biomass C and biomass N are formed by the processes of C assimilation and N immobilization and are interdependent because the balance of microbial C and N are genetically determined. The fate of NH_4^+

depends on the main factors that create specific conditions in the soil; these factors can support the stabilization of N by microbial immobilization, which is the microbial conversion of inorganic N (both ammonium and nitrate) into organic forms (Patrick, 1990; Jarvis et al., 1996). If these conditions do not support immobilization, N losses from soil microbial community or even from the ecosystem can occur through biological or non-biological processes. The processes of mineralization and immobilization are interdependent. The process of continuous transfer of mineralized N into organic materials in soil microbial biomass and the release of immobilized N back into soil inorganic pool is known as "Mineralization – Immobilization Turnover" or MIT (Patrick, 1990; Jarvis et al., 1996). The balance between soil C and soil N with microbial C and N depends on the intensity of soil C utilization through microbial C assimilation and respiration and soil N mineralization and immobilization and the processes that drain soil N, such as subsequent nitrification and denitrification or ammonia volatilization (Reddy and D'Angelo, 1997; Hart et al., 1994). The amount of N bound into microbial biomass in the soil depends both on N and C availability for the simultaneous turnover and assimilation of C together with coupled N immobilization in the soil (Barrett and Burke, 2000).

The intensity of the microbial processes of C mineralization, assimilation and coupled N immobilization are related to the amount of active microbial biomass and are controlled by the availability of O_2 or alternative electron acceptors – D´Angelo and Reddy (1999) found that C mineralization rates under aerobic conditions were 3 times faster than under anaerobic conditions with alternative electron acceptors such as NO_3^-, SO_4^{2-}; CO_2^-. C mineralization, assimilation and coupled N immobilization are also controlled by the temperature and quality of soil organic matter – such as the ratio of easily degradable soil C to recalcitrant soil C and by the availability of soil N (Reddy and D'Angelo, 1997; Magill and Aber, 2000; Whalen et al., 2000). Different microbial functional groups, which are specialized to different stages of organic matter decomposition, have different needs for N availability for C mineralization (Henriksen and Breland, 1999)

1.4. The relationships between aeration and redox gradients and zonal differences in nutrient transformation processes in CW systems

The environmental gradients of the wetlands include physical, hydrological, chemical, biological gradients, which change in space as well as in time (Mulamoottil *et al.*, 1996).

One of the main distinguishing features of wetlands is the presence of aerobic and anaerobic interfaces that create steep redox gradients in the range of +700 to –300 mV. These gradients are affected by at least three different

conditions: hydrological fluctuations, the presence of electron acceptors (such as O_2, NO_3^-, and SO_4^{2-}), and transport of O_2 by plants into the root zone. Under drained conditions, anaerobic microsites within the soil aggregates can result in steep redox gradients (Reddy and D'Angelo, 1997). Under flooded conditions, the diffusion of O_2 through floodwater maintains aerobic conditions at the soil-floodwater interface ranging in thickness from a few millimetres to about 2 cm. Redox gradients may also be set up as a result of buffering by alternate electron acceptors used during anaerobic respiration. For example, denitrification, FeOOH, and SO_4^{2-} reduction and methanogenesis are distinguished by distinctly different redox potentials. Thus redox gradients may be used as an indicator of potential nitrification-denitrification reactions, iron-oxide regulated precipitation of P and oxidation of CH_4, and sulfides, and the breakdown of toxic organic compounds (Reddy and D'Angelo, 1997).

The aeration and redox potentials are the most important factors influencing nutrient transformation processes in constructed wetlands. The differentiation of the microbial processes, such as nitrification and denitrification, along the O_2 and redox gradient is related to both the vertical zonality and microzonal growth of the microbes (Reddy and D'Angelo, 1997). Microzonal growth in biofilms and detrital aggregates allows improved exchange of essential metabolites between different functional groups of the microbes (Paerl and Pinckney, 1996). This allows anaerobic activities to occur in aerobic habitats (Storey et al., 1999). Biofilms, flocs and microbial mats are responsible for most microbial conversions in natural environments (Beer and Schramm, 1999).

Microzonal growth is space-effective because of the short distances for the diffusion of the metabolites and can also be space-effective in terms of wastewater purification. Microzonal growth needs a substrate for the attachment of microbes and is thus supported in CW systems, which have large specific contact areas between water and substrate, such as in subsurface flow systems. The higher specific contact area between the water and substrate in these systems results in generally higher water purification efficiency compared to free-water systems (Kadlec and Knight, 1996).

1.5. The structure and functional parameters of microbial communities

The studies of microbial communities in conventional wastewater treatment plants has shown that cultivation-dependent approaches do not allow a proper description of the composition and dynamics of the communities actually present (Wagner and Loy, 2002).

As the functional parameters of microbial communities in HSSF CW-s have not been sufficiently studied, there are also few studies of the community structure of these systems. Ibekwe et al. (2003) characterized microbial composition

in two CWs treating dairy washwater using denaturing gel electrophoresis (DGGE). Baptista et al. (2003) applied the DGGE approach and fluorescent *in situ* hybridization for the assessment of the abundance of selected bacterial groups in an HSSF CW. There are also some papers that characterize the microbial community composition in CWs on the basis of DNA extraction and subsequent cloning from HSSF wetland leachate and free-water CW sediments (Walsh et al., 2002; Lloyda et al., 2004). The cultivation- based methods such as the single carbon source utilization patterns with BIOLOG-plates allow one to gain information concerning the structural differences of microbial communities, but these results are biased due to the selectivity of these methods. Thus the labour-effective approach of the community level DNA fingerprints PCR and subsequent DGGE analysis is appropriate for the studies to achieve first basic knowledge of the microbial community composition and structural differences of the microbial community in individual CW systems. These approaches can be used to provide the studies of the functional parameters of microbial characteristics with supplementary information.

1.6. Main objectives

The main objectives of this thesis are: 1) to study the relationships between zonal differences in environmental conditions and microbial characteristics such as microbial biomass C, microbial biomass N, soil respiration, potential nitrification and single carbon source utilization patterns in two parallel beds of the Kodijärve HSSF CW, 2) to find out how the microbial community reacted to the aeration of the inflowing wastewater, to the higher evapotranspiration from the wetland and to the lowered BOD_7 value of the wastewater, 3) to determine functional differences in the microbial community in the different sites of the CW, 4) to find the relationships between the studied functional microbial parameters and between the genotypic fingerprint of the microbial community and 5) to evaluate the fraction of microbial biomass N in the wetland's N balance.

2. MATERIALS AND METHODS

2.1. Study site

The Kodijärve planted sand filter was established in October 1996 by the Centre for Ecological Engineering Tartu (CEET), and it treats effluent from a hospital (about 40 PE). The constructed wetland is located 120 m downhill from the house on the shore of a small hypertrophic lake named Väike Kodijärv (Viinakoja Lake; 3.5 ha). Before entering the system, wastewater flows through a two-chamber septic tank. In the summer of 2002, the VSSF part, as a pretreatment system for better aeration of the wastewater, was added to the HSSF system (Fig. 1).

The HSSF filter of the Kodijärve hybrid CW system consists of two parallel beds, each 25*6.25*1m, filled with coarse sand. The wastewater enters the system through an intervaller well that divides the water over two Thomson weirs to the beds, where it seeps through the inlet pipes and into the sand. The wastewater is shared between the beds in equal flow rates. The beds have different hydraulic conductivity and are called a dry (right) bed and a wet (left) bed due to the different water levels therein. The outlet pipes installed in the centre collect the purified water to the outlet well where the stand pipe allows the regulation of the water table in the beds. The bottom and sides of the chambers are isolated with PVC foil. From the outlet well the water flows through a channel to the natural reed stands on the lake shore (Mander *et al.*, 2001). Eighteen water sampling wells, 9 in each bed, are distributed evenly throughout the sand filter (Fig. 1).

The dry bed was dominated mainly by *Scirpus sylvaticus* (wood club-rush) with some *Urtica dioica* (nettle), *Epilobium hirsutum* (hairy willow-herb) and some single individuals of other species. In the wet bed, about 60% of the bed was dominated by wood club-rush; in the rest of the bed, patches of reed (*Phragmites australis*) and some single individuals of other species grew (Mander *et al.,* submitted).

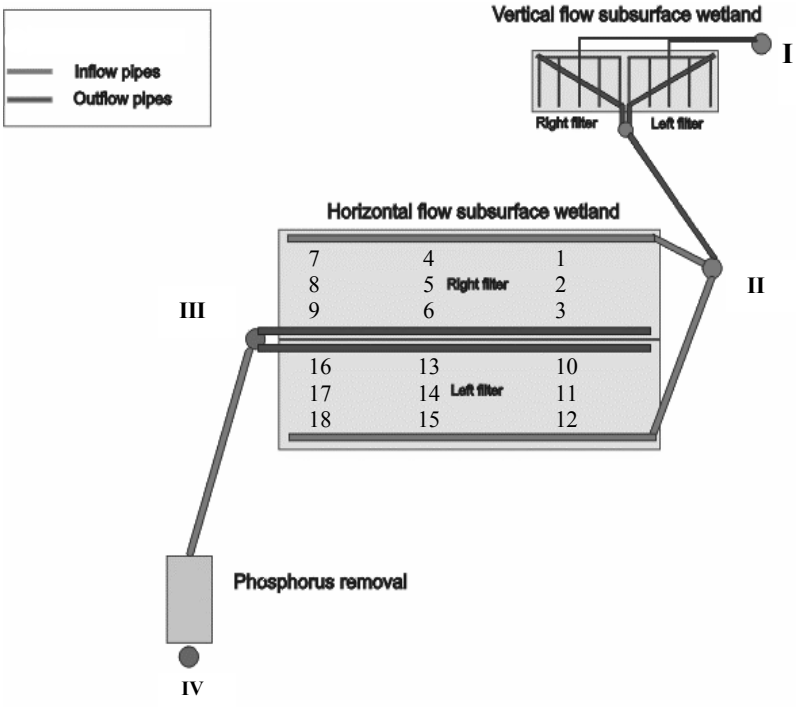

Figure 1. Diagram of the hybrid constructed wetland in Kodijärve, Estonia. Water sampling sites: I – inflow to the VSSF filter, II – inflow to the HSSF filter, III – outflow from the HSSF filter, IV – outflow from the phosphorus removal bed. 1–18: water sampling wells within HSSF filter beds.

2.2. Soil and water sampling

Soil samples for microbiological and parallel soil samples for chemical analyses and water samples for standard water quality analysis (APHA; 1989) from inflow; outflow and 18 wells were collected at the beginning of October 2001 (4 October) and at the end of October 2002. Soil samples for the measurement of potential nitrification were stored in small plastic bags at 4ºC until analyses were performed. Soil samples for other analyses were stored in 500 ml plastic boxes at room temperature. Soil samples were randomly collected with a soil core drill around individual water wells, to ensure the even and statistically relevant distribution of different soil sites with different humus content, clay particles etc. in soil particles. Thus a balanced distribution of soil samples throughout the top layer of the wetland is represented. Soil samples were collected randomly in a 1 m radius around the water sampling wells near the inflow (samples around wells No. 1, 4, 7 in the dry bed and 12, 15, 18 in the wet bed) and outflow (samples around wells No 3, 6, 9 in the dry bed and 10, 13, 16

in the wet bed) in both dry and wet beds at a depth of 0–10 cm ("upper layer") and 20–30 cm ("lower layer") (Fig. 1).

2.3. Meteorological data

Air and soil temperature, wind velocity, solar radiation, precipitation and evapotranspiration were measured using a DAVIS Groweather automatic weather station installed close to the CW. The summer of 2002 was significantly warmer and dryer than that of 2001 (Mander et al., 2005).

2.4. Microbiological methods

All microbial characteristics related to the detection of the microbial C and N pools and the potential capacities of the processes that modify those pools were measured in 2001 and 2002. These characteristics were measured and calculated according to Schinner et al. (1996). Here is a brief description of these methods (the detailed description is in the following sections). 1) For the measurement of soil respiration (g CO_2 kg^{-1} (dm) d^{-1}), respired CO_2 was trapped in an NaOH solution and determined by titration with HCl after the addition of BaCl. 2) Biomass C (g C kg^{-1} (dm)) was measured using the SIR method, which is based on the measurement of initial respiratory response (CO_2 outcome as for the detection of soil respiration), after the addition of glucose into the soil sample, and is thus an indirect method for microbial biomass C assessment. 3) Immobilized N (g NH_2-N kg^{-1} (dm)) was measured using the fumigation-extraction technique coupled with ninhydrin-reactive nitrogen (NH_2-N of the proteins and nucleic acids) detection. 4) For the measurement of potential nitrification (mg N kg^{-1} (dm) d^{-1}), the soil samples were incubated for 5 h at 25 ˚C in 20 ml of 1 mM ammonium sulfate solution in a shaking water bath so as to saturate the solution with oxygen. Further nitrite oxidation to nitrate was inhibited by adding 0.1 ml 1.5 M sodium chlorate, and the nitrite was detected colorimetrically.

In 2001, the analysis of the potential C source utilization pattern of soil microbial community by Biolog-plates was used to differentiate the microbial community structures based on the functional differences between communities. This method is also described herein.

The molecular methods of the analysis of microbial community structure that are important for the understanding of some considerations presented in this thesis based on Truu et al. (2005) (Publication III) include the community-level PCR of the extracted DNA from the soil samples. These PCR analyses included the amplification of the DNA that should be specific for 1) eubacteria; 2) archaea; 3) ammonia-oxidizing bacteria, and also included 4) ammonium

monooxygenase specific primers. The PCR products resulting from the DNA amplification with these primers were separated in denaturing gradient gel electrophoresis (DGGE) to obtain banding patterns for the further community level genotypic analyses.

2.4.1. Potential C source utilization pattern of microbial community using Biolog-plates

The analysis of multiple substrate metabolism using assemblages of bacterial strains may be used to differentiate inocula from environmental samples. Biolog-plates, 96 well microtiter plates containing nutrients, a single carbon test substrate in each well and a tetrazolium redox dye to monitor substrate oxidation can be used for this purpose (Garland and Mills, 1991).

The soil samples collected from the wetland were used for Biolog-plate analyses after 4 to 5 days. 1 g of individual soil was suspended with a mixer in 100 ml sterile tap water. After the settling of the heavier parts of the soil, 1 ml of the suspension, which presumably consisted of bacteria and some lighter material, was dissolved in 100ml of sterile tap water. The suspension of bacteria obtained from each soil sample was used for further analysis on Biolog plates.

The Biolog EcoPlates had 96 wells, with 3 replicas of 32 wells: 31 wells for each substrate and one for control without substrate. Portions of samples were inoculated into plates with a multipipettor (0.15 ml into each well). The substrate use pattern of the microbial community was measured as tetrazolium dye colour development from 31 substrate wells against control cell colour development. Individual microbial communities have specific reproducible substrate use patterns, which can be analysed in order to measure and characterize the relationships between different microbial communities. The tetrazolium dye development was measured using a Lab Systems Multiscan scanning spectrophotometer 24, 48 and 72 h after inoculation of the plates with a suspension of the bacteria. The data obtained were used to calculate average well color development (AWCD) for the measurements of the different times for each BIOLOG-plate. To calculate AWCD, the measured well colour developments (colour response) of each of the response wells were subtracted from the colour response of respective control wells (response well values lower than control well values were accounted to be 0). The transformed well colour development values were obtained in this way. The average value for every substrate was calculated for three replicas of 31 substrate wells. The average well colour development was calculated as the average value of all transformed well colour development values for substrate wells. AWCD for 24, 48 and 72 h were compared. The data of 72 h incubation were found to be the most appropriate for further analysis.

2.4.2. Determination of soil respiration and microbial biomass C

There are different methods that may be used to record the respiratory response of soil microbiota. To evaluate soil respiration and soil microbial biomass, we used methods based on the NaOH solution-trapped CO_2 measurements by titration.

2.4.2.1. Soil respiration by titration

Microbial respiration or basal respiration, an index of microbial activity, was determined as the amount of carbon dioxide (CO_2) evolved over the 24 hour aerobic incubation of 20 g moist field samples at 25 ºC in carefully sealed 250 ml glass bottles. Respired CO_2 was trapped in 20 ml of 0.05 M NaOH solution and determined by titration with 0.1 M HCl, after 0.5 M $BaCl_2$ addition to precipitate the absorbed CO_2 as barium carbonate, using a phenolphtalein indicator. The amount of absorbed CO_2 was calculated as the difference between the mean volume of HCl consumed by four samples and the mean volume of four controls (bottles without respiring soil samples), according to the following formula (Schinner et al., 1996):

$$((C–S)*2.2*100)/(SW*\%dm) = mg\ CO_2/(g\ dm\ 24\ h), \quad (1)$$

where:

C	–	mean volume of HCl consumed by controls (ml)
S	–	mean volume of HCl consumed by samples (ml)
2.2	–	conversion factor (1 ml of 0.1 M HCl corresponds to 2.2 mg CO_2)
SW	–	initial soil weight (g)
100/%dm	–	factor for soil dry matter.

2.4.2.2. Substrate-Induced Respiration

To evaluate soil microbial biomass, we used a method called Substrate-Induced Respiration (SIR). The principle of SIR is to record maximum initial respiratory response, which is considered to be proportional to the amount of microbial carbon present in the soil sample (also proportional to microbial biomass), after glucose (substrate) amendment. Applying a conversion factor derived from the calibration of substrate-induced respiration to the chloroform-fumigation incubation technique, values can be converted to mg biomass-carbon. The SIR method detects only metabolically active aerobic microorganisms. Dead or dormant biomass is not detected.

The method used to record respiration is principally the same as above (soil respiration by titration). SIR coupled with CO_2 measurements by titration is called the Isermeyer Technique.

2.4.2.2.1. Isermeyer Technique

For the detection of SIR, 20g of field-moist soil mixed with an optimised amount of glucose were incubated for 4 h at 22ºC. Each measurement for individual soil consisted of four samples against four blanks (bottles without soil samples). Conventionally, 500 ml bottles and 0.1M NaOH are used for CO_2 trapping. We used 250 ml laboratory bottles and 20ml 0.05 M NaOH to trap CO_2 evolved from the glycose-amended soil sample, considering the O_2 amount for respiration and NaOH amount for CO_2 trapping will not be limiting for the soils to be analysed (these considerations are based on earlier experiences in the same lab).

The pattern of respiratory response has to be recorded immediately for at least 8 h after glucose amendment. The Isermeyer Technique is only suitable with soils that show no increase in their respiration rates during the first 6 h after amendment (no proliferation of microorganisms under these conditions). A failure to detect a stable respiration rate for at least 3 h after amendment indicates that the soil population tested is not in a resting state. Given this, no microbial biomass determinations using the SIR method are possible. Growing microbial biomass cannot be detected. Thus preconditioning is needed to achieve the balance between soil microbial population and substrates. The soil samples were stored for 2 months at room temperature before biomass detection to achieve stabilised conditions. It is recommended that the concentration of glucose that leads to the maximum release of CO_2 under the experimental conditions be determined.

For the determination of the amount of glucose that leads to the maximum CO_2 release, we tested different amounts of glucose. The tested amounts were 1000, 2000, 3000, 4000, 5000, 6000, 7000 and 8000 micrograms per 1g of soil. The CO_2 release was measured after 4 h of incubation.

To test whether the soil microbiota is in a resting state or not, different incubation times from 45 minutes to 8 hours were used. CO_2 emission rates appeared to show a decrease after 3 h incubation. We assumed that maximum initial respiration response occurred during the first 2 or 3 hours, and rapid CO_2 emission slowed down somewhere between 2 or 3 to 4 hours. After initial maximum respiration response, microbes began to adapt their metabolic pathways for growth under renewed circumstances, or microbial metabolic processes could not be detected due to changing microbial respiratory quotient (RQ – emitted CO_2 (mol) / respired O_2 (mol)). During maximum initial respiration response RQ is found to be 1 (Dilly *et al.,* 2000). Thus maximum initial

respiration response indicates the overall metabolic activity of the aerobic microbial population of the soil sample.

The incubation time chosen to be applied to SIR was 4 h. Microbial biomass C was calculated according to the following formula (Schinner et al., 1996)):

$$((B–S)*2.2*100*100)/(X*SW*\%dm) = mg\ CO_2/(100\ g\ dm*h), \quad (2)$$

where

SW	–	initial soil weight (g)
B	–	mean volume of HCl consumed by blanks (ml)
S	–	mean volume of HCl consumed by samples (ml)
2.2	–	conversion factor (1 ml 0.1 M HCl corresponds to 2.2 mg CO_2)
100	–	conversion factor (100 g dm)
X	–	incubation time (h)
100/% dm	–	factor for soil dry matter

Assuming a respiratory quotient of 1, 1 mg CO_2 100 g^{-1} dm h^{-1} corresponds to 20.6 mg biomass-C/ 100 g dm. This factor for SIR was estimated from a correlation to the fumigation – incubation technique using agricultural mineral soils in Central Europe.

2.4.3. Determination of potential nitrification

The ability of the soil's microbial population to nitrify under optimal conditions for nitrification (O_2 saturated medium, non-limiting and non-inhibiting NH_4^+ supply, appropriate temperature) is the soil's ability for potential nitrification. For the determination of Potential Nitrification, soil samples were incubated for 5 h at 25ºC in an ammonium sulfate solution. Nitrite released during incubation was extracted with potassium chloride and determined colorimetrically at 520 nm. Saturation of O_2 was achieved by shaking the solution. Further nitrite oxidation to nitrate was inhibited using sodium chlorate.

In order to detect the potential nitrification of individual soil, measurements of 2 samples and 1 control were performed. 20 ml of 1mM $(NH_4)_2\ SO_4$ solution and 0.1 ml of 1.5M $NaClO_3$ (different concentrations of sodium chlorate were previously tested to specify a sufficient amount for total inhibition) were added to 5 g of field-moist soil and placed in 50 ml Erlenmeyer flasks, mixed briefly and closed with caps. Two tubes (samples) were incubated for 5 h on a shaker, and the third tube (control) was stored for 5 h at –20ºC.

After incubation, the control was thawed at room temperature. 5 ml of 2 M KCl was added to the samples and control, mixed briefly, and after that samples and control were filtered immediately.

For colorimetric analysis, 5 ml of filtrate, 3ml of NH_4Cl buffer (0.19 M, pH 8.5) and 2 ml of colour reagent were mixed and allowed to stand for 15 minutes at room temperature, then measured at 520 nm against the reagent blank. To prepare the calibration curve, 5 ml calibration standards were treated like the soil filtrates. Calibration standards contained 0, 0.2, 0.4, 0.8 and 1 microgram NO_2–N ml^{-1}.

Potential nitrification is expressed as the amount of NO_2–N released from 1 g of soil over 5h and calculated according to the following formula (Schinner et al., 1996):

$$((S–C)*25.1*1000*100)/(5.5*\%dm) = ng\ N/(g\ dm\ 5\ h), \quad (3)$$

where:

S	–	mean value of samples (mg N)
C	–	control (mg N)
25.1	–	volume of extract (ml)
1000	–	conversion factor (1 mg N = 1000 ng N)
5	–	aliquot of filtrate (ml)
5	–	initial soil weight (g)
100/% dm	–	factor for soil dry matter

2.4.4. Detection of biomass-N

There are different methods for determining the amount of N immobilised into the soil biomass. These methods are based on the killing of the bacteria to make N available for extraction and detection. In order to detect biomass-N, the fumigation-extraction technique coupled with ninhydrin–reactive N detection was used.

2.4.4.1 Ninhydrin–reactive N using fumigation-extraction technique

Biomass–N is bound into amino acids. To measure the amino acid nitrogen content of soil biomass, 10 g of soil was measured into the 2 Erlenmeyer flasks (samples), and 3 ml of ethanol-free chloroform was added. The flasks were closed with caps and fumigated for 24 h at 25ºC in the dark. Fumigation with chloroform is needed for the breakdown of the cells. After fumigation the chloroform was removed using repeated evacuation. 2 samples and 2 controls (same amount of non-fumigated soil) were extracted with 50 ml 2 M KCl solution, shaken for 1 h, then filtered.

For the analyses, 2 ml of the filtrate was mixed with 0.5 ml 0.4 M sodium citrate solution in the test tubes. Subsequently, 2 ml of ninhydrin reagent mixture was added. Test tubes were held in a boiling water bath for 30 minutes and then cooled under running water. After cooling, 5 ml of ethanol was added, and the tubes were shaken. The solutions were measured photometrically at 570 nm against the reagent blank within 1 h. Calibration standards containing 0, 0.35, 1.4 and 2.8 microgram leucine-N/ ml were applied. For the calculation of the results, the following formula was used (Schinner et al., 1996):

$$(S*V*100)/(SW*ml*\%dm) = \mu g \text{ ninhydrine-reactive N/ g dm}, \quad (4)$$

where:

S	–	mean value of samples (µg N)
V	–	extraction volume (ml)
SW	–	initial soil weight (g)
ml	–	aliquot of filtrate (ml)
100/% dm	–	factor for soil dry matter

The conversion factor 3.1 was applied for the final calculation of the results.

2.5. Statistical analysis

2.5.1. Analysis of the data from 2001

The data were transformed using Microsoft Excel, and statistical analyses were performed with Statistica 6 (StatSoft Inc.) software. All parameters were controlled for normality, and if necessary transformed (normalized) prior to analysis (log, ln, atan and square root functions were used for this purpose). The microbiological characteristics of the two beds and of the respective layers (the upper layer of the dry bed versus the upper layer of the wet bed and the lower layer of the dry bed versus the lower layer of the wet bed) were compared using a t-test.

The respective microbiological data from two layers (upper layer versus lower layer) and two rows (or sides) (inflow versus outflow) were analyzed using a paired t-test, and data between three transects were analyzed using ANOVA and Duncan tests in both beds separately. Transect 1 in the dry bed included samples from both layers around water sampling wells No 1 and 3, transect 2 – No 4 and 6, transect 3 – No 7 and 9; transect 1 in the wet bed included samples around wells No 10 and 12; transect 2 – No 13 and 15, and transect 3 – No 16 and 18 (Fig.1). We determined Pearson and Spearman Rank Order correlation coefficients between the microbiological characteristics, soil chemistry, water quality indicators and gaseous emissions from the wetland. We

used the average values of microbiological and soil chemistry data from the upper and lower layers and also both layers separately for correlation analyses. Biolog microplate data were also analysed, using principal component analysis (PCA) and the Shannon index of diversity. The structures of the microbial consortia in both beds were compared using a multivariate randomization test.

2.5.2. Microbial data analysis after the building of the pretreatment VSSF filter in 2002

The analysis of the microbial data in the next year, i.e. 2002, included the comparison of microbial characteristics measured in 2002 with the microbial characteristics obtained in 2001.

We compared the dry and wet beds of the wetland with a t-test or Mann-Whitney U-test, and the upper and lower layers in both beds with a paired t-test or Wilcoxon Matched Pairs test.

The respective parts of the wetland in 2001 vs. 2002 were compared using a paired t-test or Wilcoxon Matched Pairs test. To provide the correct basis for the comparison of the patterns of respective microbiological characteristics, the compared pairs (upper vs. lower layer and dry bed vs. wet bed) of the respective characteristics in the same year were checked simultaneously in 2001 and 2002 for nonparametric tests, and some of the data that had a normal distribution were also analyzed using a Wilcoxon Matched Pairs test (for the comparison of the layers) or a Mann-Whitney U-test (for the comparison of the beds).

We also determined Pearson and Spearman Rank Order correlation coefficients between the microbiological characteristics, soil chemistry, and water quality indicators.

Additional analysis of the data from 2002, which was not added to Publication III, included the comparison of the transects and inflow and outflow sides in 2001 with the respective parts in 2002, using paired t-tests or Wilcoxon Matched Pairs tests. The inflow vs. outflow sides were compared with paired t-tests or Wilcoxon Matched Pairs tests, and transects were compared with ANOVA or Kruskal-Wallis ANOVA in 2002. The compared pairs of the microbial characteristics were, where necessary, also replaced by a nonparametric test using the Wilcoxon Matched Pairs tests for the comparison of the inflow vs. outflow sides, and the Kruskal-Wallis ANOVA for the comparison of the transects.

3. RESULTS AND DISCUSSION

3.1. Spatial patterns of microbial indicators in parallel beds of Kodijärve HSSF CW in 2001

The higher diversity (Fig. 2, b)) and higher metabolic activity (t-test, $p<0.001$) (Fig. 2, a)) in the wet bed than in the dry bed is probably related to the greater availability of nutrients from the wastewater in the wet bed. This conclusion is supported by the fact that the average nitrogen immobilization of the upper and lower layers and BOD_7 concentration was positively correlated ($r = 0.85$, $p<0.05$) in the wet bed, but not in the dry bed. The lower metabolic activity (paired t-test, $p<0.05$) (Fig. 2, a) in the lower layer of the dry bed is probably caused by the lower stability of the aeration and feeding conditions, which is influenced by the fluctuation of the water level due to weather conditions and also by the deep water table below the lower layer, whereas the upper layer of the dry bed had better availability of organics of dead plant material (Nurk et al., 2005; Publication I).

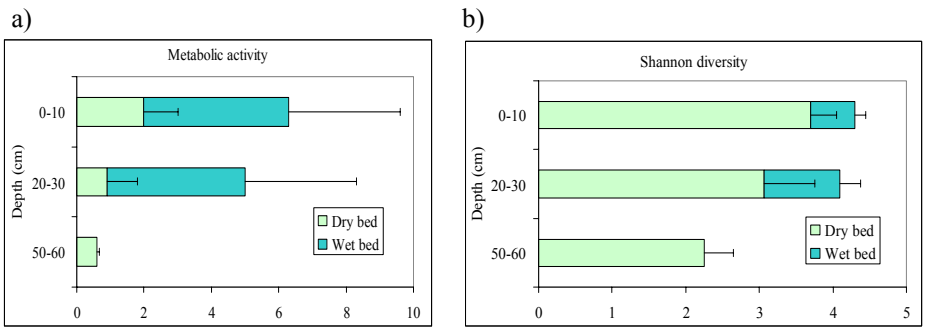

Figure 2. The average and standard deviation values of metabolic activity (a) and Shannon diversity (b) of the microbial consortia in the Kodijärve HSSF filter. In the dry bed, some additional samples were analyzed from the 50–60 cm layer of the filter material (Nurk et al., 2005; Publication I).

The structure of the microbial community was found to differ between the two beds ($p<0.01$, multivariate randomization test). The greater spatial differences in the microbial community structure of the dry bed (PCA, Fig. 3) are probably attributable to the greater differences in aeration between the layers due to the lower water table. The latter conclusion is supported by the greater number of detected differences between the microbial characteristics of the upper and lower layers of the dry bed (Nurk et al., 2005; Publication I).

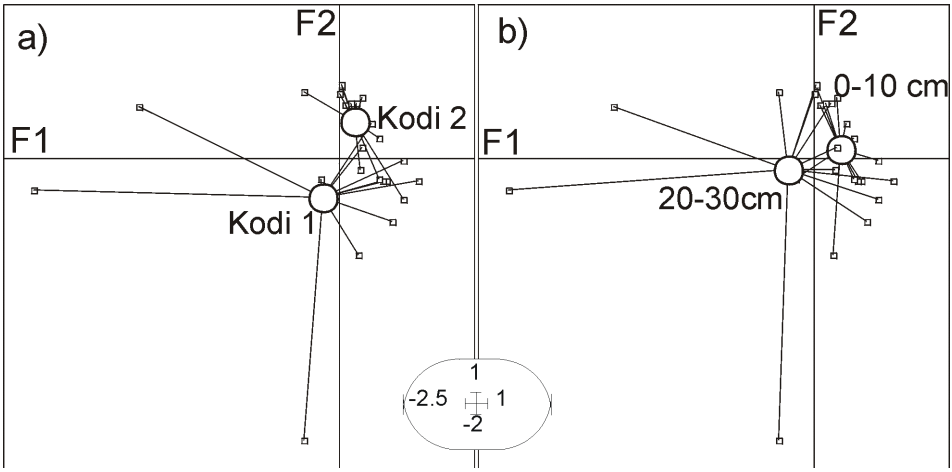

Figure 3: Results of the principal component analysis of the Biolog-plates data of the Kodijärve HSSF CW. a) centroids of beds, Kodi 1 – dry bed, Kodi 2 – wet bed; b) centroids of the soil layers of both beds, 0–10 cm – upper layer, 20–30 cm – lower layer. Axes F1 and F2 describe 38.7% and 23.92% of the variability, respectively.

We assume, based on the results of the correlation analysis, that in the lower layer of the dry bed, the low water table and the changing water level have caused the relative depletion of easily degradable soil C compared to the other layers of the wetland. The lower layer of the dry bed was probably dominated by the facultatively anaerobic bacteria (Nurk et al., 2005; Publication I).

3.2. The analysis of the impact of environmental conditions on the changes in microbiological characteristics in 2001 vs. 2002

The values of all of the studied microbial characteristics differed significantly in 2001 and 2002 – the average value of soil respiration increased 1.4 times, and the average value of the measured biomass C (SIR) increased 1.6 times ($p<<0.01$, paired t-tests). At the same time, immobilized N decreased 5.8 times, and potential nitrification 7.2 times ($p<<0.01$, Wilcoxon Matched Pairs test). That resulted in an extremely high biomass C:N ratio in 2002, when the average biomass C:N ratio was 10.4-fold higher than in 2001 ($p<<0.01$, Wilcoxon Matched Pairs test), and the ratio of average biomass C to average immobilized N was 9.4 times higher than in 2001. These changes were related to the lowering of BOD_7, the enhanced aeration of the inflowing wastewater after construction of the VSSF pretreatment filter in August 2002 (Noorvee et al., 2005) and

the extraordinarily dry summer of 2002 with higher temperatures and evapotranspiration (Mander et al, 200X; submitted), which can cause: (1) the reduction of consumable organics, (2) enhanced oxygen availability for respiration. These environmental factors modified the inner conditions in the soil filter and were considered to be the main factors responsible for the changes in microbial characteristics.

Additional comparison of the biomass N:P ratios of the entire soil filter in 2001 vs. 2002 revealed much lower variation between the two years: the average immobilized N versus average biomass P ratio was 2.6 in 2001 and 3.0 in 2002.

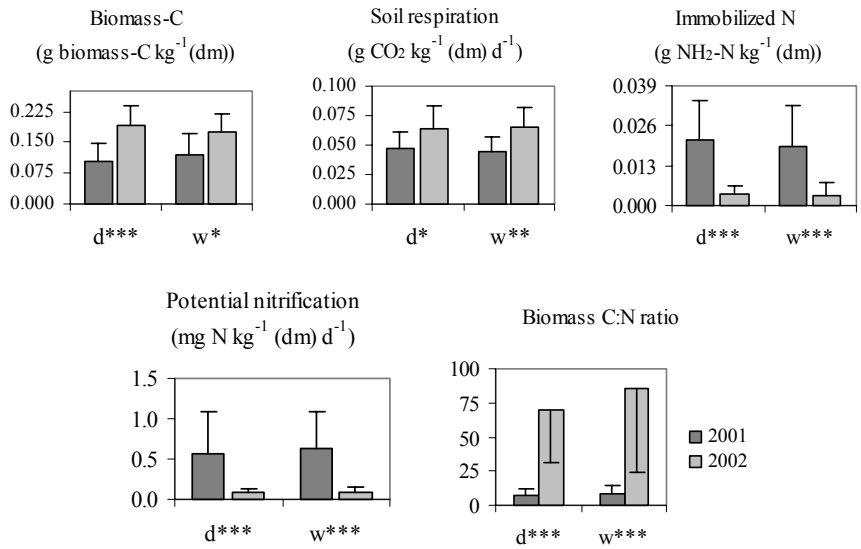

Figure 4. The average and standard deviation values of microbial characteristics in the dry bed (d) and wet bed (w) of the Kodijärve HSSF CW in 2001 and 2002. The detected significant differences in the respective values in these years are indicated as * for $p<0.05$, ** for $p<0.01$ and *** for $p<<0.01$. For soil respiration and biomass-C, the paired t-test was used, and for the other microbial characteristics the Wilcoxon Matched Pairs Test was used (Nurk et al., submitted; Publication III)

We assumed that the lowered organics of the wastewater caused the decrease in the previous microbial community that was proportional to immobilized N, whereas higher biomass C, which was measured using the SIR method, was related to the growth in the proportion of r-strategic heterotrophic microbes (Lebuhn et al., 1994; Sharma et al., 1998), which are capable of the rapid turnover of easily degradable organics. We used correlation analysis to collect additional information about the relationships between microbial characteristics, soil chemical parameters in the upper and lower layer and water quality indi-

cators from the water sampling wells in the dry and wet beds (Nurk et al., submitted; Publication III).

The comparison of the dry and wet beds offered stronger evidence of the growth of biomass-C and weaker evidence of the growth of respiration in the dry bed than in the wet bed (Fig. 4).

Due to the lower water table, the studied layers of the dry bed were more aerobic and in 2001 also already had, due to the poorer availability of wastewater, less soil organics for respiration, which caused a weaker aeration effect and lower organics of the wastewater in 2002. Less evidence of the growth of biomass C in the wet bed was probably caused by the slower degradation of organics by the heterotrophic microbes that were adapted to anaerobic conditions and were less capable of the rapid degradation of easily degradable carbon than the aerobes.

We found the differences in the number of significant correlations between the layers and the beds in 2001 and 2002 (Table 1).

Table 1. The number of significant correlations between microbial characteristics and environmental parameters studied in both beds of the Kodijärve HSSF filter.

	Dry bed		Wet bed	
	2001	2002	2001	2002
Layers together	14	26	24	6
Upper layer	6	21	17	3
Lower layer	8	5	7	3

Heterotrophic processes are nearly 3 times faster in aerated conditions (D'Angelo, Reddy; 1999), and the stability of the conditions supports the establishment of multiple relationships between the different indicators (Golovlev, 2001). We also consider that the changes in environmental conditions had a different impact in the dry and wet beds. We assume that:

1) the changes in the conditions, which favoured the growth of aeration, were more fundamental in the wet bed than in the dry bed, and that caused the delayed formation of new balances in the wet bed.
2) the lower number of correlations in the wet bed was also caused by the slow processes due to low aeration, because the increase in aeration was not sufficient to make the wet bed as aerobic as the dry bed.
3) the higher number of significant correlations in the wet bed than in the dry bed in 2001 is mainly due to the high number of correlations in the upper layer and indicates that earlier, the microbial characteristics of the wet bed were well balanced with the environmental conditions, and in the upper layer depended on a multitude of factors that were of equal importance. These extra factors in the upper layer compared to the lower layer of the wet bed were probably caused by: a) the presence of the aeration gradient that

modified the conditions, b) the thin humus layer in the topsoil. At the same time, the altered aeration conditions of the dry bed (Nurk et al., 2005; Publication I) did not allow for the establishment of balances between the multiple factors.

4) the increase in the number of significant correlations in the dry bed in 2002 can be explained by the relative stability and rapid establishment of the new balances due to the high aeration in the upper layer, where most of the significant correlations were found. The changes in the environmental conditions in the dry bed in 2002 had, in contrast to the wet bed, the same direction as the processes before the change in the conditions, and allowed for the establishment of new balances within 2 months. We assume that the upper layer differed from the lower layer of the dry bed mainly due to the higher aeration and the presence of the humus layer.

The results of the correlation analyses were used to provide background information for the analysis of the differences in the microbial characteristics of the layers in 2002 and the analysis of the changes in the microbial characteristics in 2001 vs. 2002 (Nurk et al., submitted; Publication III).

The changes in microbial characteristics in the inflow and outflow side of both beds and in the transects across the longitude axis of the wetland were analysed in addition to the data from the added publications in section 3.2.3.

3.2.1. The changes in the pattern of microbial characteristics in 2001–2002

The comparison of respective layers (Nurk et al., 2005; Publication III), inflow and outflow sides and transects along the main water flow in both beds revealed significant differences for many of the characteristics of the compared parts. The comparison of the resulting spatial patterns of the compared values of microbial characteristics in the upper vs. lower layer, inflow vs. outflow side and between the transects in 2002 with the respective patterns in 2001 show that the change in the conditions had changed the patterns of the studied characteristics.

The change in the consistence and aeration of wastewater and enhanced evapotranspiration mainly influenced the zone of the gradient of the aeration and nutrient concentrations, which is caused by the water table – we found that 50% of the compared pairs of the microbial characteristics in the comparison of the upper vs. lower layer had changed, comprising 2 of the 5 pairs in the dry bed and 3 of the 5 pairs in the wet bed, whereas the pattern of the compared pairs of inflow vs. outflow side changed 30% – 1 of the 5 pairs in the dry bed and 2 of the 5 pairs in the wet bed. The pattern of the compared transects changed nearly

10%, comprising 1 of the 5 pairs in the dry bed and none of the 5 pairs in the wet bed.

These results indicate that in addition to having the strongest spatial differences, the vertical gradient of environmental conditions in the filter beds also had the lowest temporal stability.

3.2.2 The discussion of the changes in the microbial characteristics in the background of the results of the correlation analysis of microbial characteristics and the comparison of the layers in the dry bed and wet bed in 2002

The correlation analysis in the upper and lower layers of dry and wet beds (see: section 3.2.1.1.1 and Table 2. in Publication III) gave significant negative correlations of biomass C and biomass C:N ratio with immobilized N in the layers of the Kodijärve HSSF CW, except for the lower layer of the wet bed. Biomass C was positively correlated with the biomass C:N ratio in the upper layer of the dry bed and in the lower layer of the wet bed. These correlations indicate that the decrease in immobilized N was related to the increase in the proportion of the microbes that were available for the rapid degradation of easily degradeable C resource. At the same time, there were negative correlations of soil nutrients with biomass C:N ratio and biomass C (except for soil C with biomass C), positive correlations of soil nutrients with immobilized N and soil respiration; and soil respiration was also positively correlated with immobilized N and negatively correlated with the biomass C and biomass C:N ratio. These correlations show that soil sites with higher nutrient content had supported the maintenance of the part of the microbial community that was proportional to immobilized N and had decreased after the decrease in the organics in the inflowing wastewater. We measured the biomass C with two parallel methods in 2003. The biomass C:N ratio, which was detected using the SIR method, was 26.4. At the same time, the fumigation-extraction method gave a 7.8-fold lower biomass C:N ratio and biomass C content. These results, and the fact that the biomass N:P ratio did not change after the environmental conditions were altered, indicate that the microbial biomass had fallen in the Kodijärve HSSF CW and in 2002 was proportional to immobilized N and not SIR-based biomass C. The decrease in microbial biomass is related to the low buffering ability of the soil of the Kodijärve HSSF CW. Due to its sandy substrate and short history, the soil of Kodijärve HSSF has low humus content in comparison to natural soils. It destabilizes the microbial community, which depends merely on inflowing organics and is more open to external influences.

We conclude that in the changing environmental conditions, such as steeply changing aeration and feeding conditions, as in the comparison of the biomass C content of the soil of HSSF CW-s, the SIR-based biomass C can hardly be

used as the indicator of actual biomass C. We used SIR-based biomass C as an indicator of the direction and nature of microbial processes or as the characteristic of the state of the community in the sites of the specific system.

The rest of the correlations that were related to the increase in the measured biomass C, biomass C:N ratio and the decrease in immobilized N include positive correlations of NO_2-N with biomass C and the biomass C:N ratio in the upper layer of the dry bed, positive correlations between the biomass C:N ratio and PS-C, and negative correlations between immobilized N and PS-C in the upper layer of the wet bed. These correlations in the upper layer of the dry bed may be caused by the spoiled balance between the different stages of nitrogen transformation due to the altered consistence of the microbial community. The detected relationships in the upper layer of the wet bed show that more clogged sites also had a stronger decrease in immobilized N and actual biomass. This may be related to the lower hydraulic conductivity, which decreases the availability of nutrients. The positive correlation between the biomass C:N ratio and PS-C may be related to the negative correlation between PS-C and immobilized N, because immobilized N had a strong negative correlation with the biomass C:N ratio in that layer. In the analysis of the correlations of the upper layer in the wet bed in Publication III, we found that the negative correlation between immobilized N and biomass C:N ratio, as the only correlation related to biomass C and immobilized N, indicates the loose relationships between these characteristics in that layer. We proposed that these relationships were modified by the variation in environmental factors such as aeration conditions and the presence of less degraded soil C. We herein also propose that microbes' ability to give a respiration response after glucose amendment was influenced by both abundance and the aerobic ability of bacteria. The importance of aeration conditions is indirectly supported by the negative correlation of biomass C with PS-C and the positive correlation with potential nitrification in the lower layer of the wet bed. The exact reasons for or mechanisms of the supposed increase in the proportion of r-strategist heterotrophic microbes remains unclear in the light of the present data.

The comparison of the upper and lower layer in parallel beds of the Kodijärve HSSF CW revealed the changes in the distribution of the patterns of microbial characteristics in 2001 vs. 2002 1) for biomass C and potential nitrification in the dry bed; 2) for immobilized N, potential nitrification and the biomass C:N ratio in the wet bed (Table 1. in: Nurk et al., submitted; Publication III). The comparison of the changes in the values of microbial characteristics in the layers in 2001 vs. 2002 (Fig. 4. in: Nurk et al., submitted; Publication III) gave a significant growth in respiration only in the upper layers of both beds, and a highly significant growth in biomass C only in the upper layer of the dry bed, whereas the other microbial characteristics did not reveal differences in the changes of the layers in 2001 vs. 2002.

The change in the distribution of the pattern of biomass C between the layers of the dry bed in 2002 resulted in greatly higher values in the upper layer. The

great increase in biomass C in this layer is related to better aeration conditions due to the lower water table of the dry bed and the higher growth of aeration in the upper layers of the wetland in 2002. The upper layer of the dry bed, which had higher respiration compared to the lower layer in both studied years and a greater increase in respiration compared to the lower layer, similarly to that of the wet bed, probably had the greatest impact of all of the changed environmental conditions in 2002. We assume, based on the discussion in Publication III, that enhanced aeration and increased soil respiration in the upper layers is attributable mainly to the impact of the extremely dry autumn. We supposed in Publication III that higher biomass C in the upper layer of the dry bed was related: 1) to the enhanced aerobic ability of the microbial community due to enhanced aeration, 2) to the enhanced availability of easily degradeable C due to higher plant biomass production in 2002 and the lower proportion of hard (recalcitrant) plant tissues compared to that of the wet bed. We also assumed that the lowering of the BOD7 had a greater effect in that layer due to the damage of water-conducting biofilms. The latter assumption is partially supported by the greater decrease in the potential nitrification in the upper layer of both beds, resulting in the loss of differences between the layers in 2002, which could be related to the greater change in the microbial community in the upper layers. There was also a change in the pattern of immobilized N and biomass C:N ratio in the wet bed. These changes are attributable to the greater increase in aeration in the upper layer of the wet bed. In contrast to the dry bed, higher aeration supported the re-immobilization of N due to the higher amount of less degraded soil C, which probably supported the partial maintenance of the microbial community, whereas the anaerobic lower layer, which supposedly depended mainly on the anaerobic degradation of BOD7, changed at a greater extent.

3.2.3 The comparison of the inflow and outflow sides of both beds in 2002

The majority of microbial characteristics did not differ between the inflow vs. outflow sides in 2001 or 2002. The differences between the values of the microbial characteristics in the inflow vs. outflow sides in 2002 and changes of the pattern of microbial characteristics in 2002 compared to 2001 were detected for soil respiration, which in 2002 was higher near the inflow than near the outflow of the dry bed ($p<0.01$, paired t-test) and for immobilized N, which in 2002 was higher near the inflow than near the outflow of the wet bed ($p<0.05$, Wilcoxon Matched Pairs test). There was also the change in the pattern of potential nitrification, which in 2001 was higher near the inflow of the wet bed ($p<0.05$, Wilcoxon Matched Pairs test), in contrast to 2002.

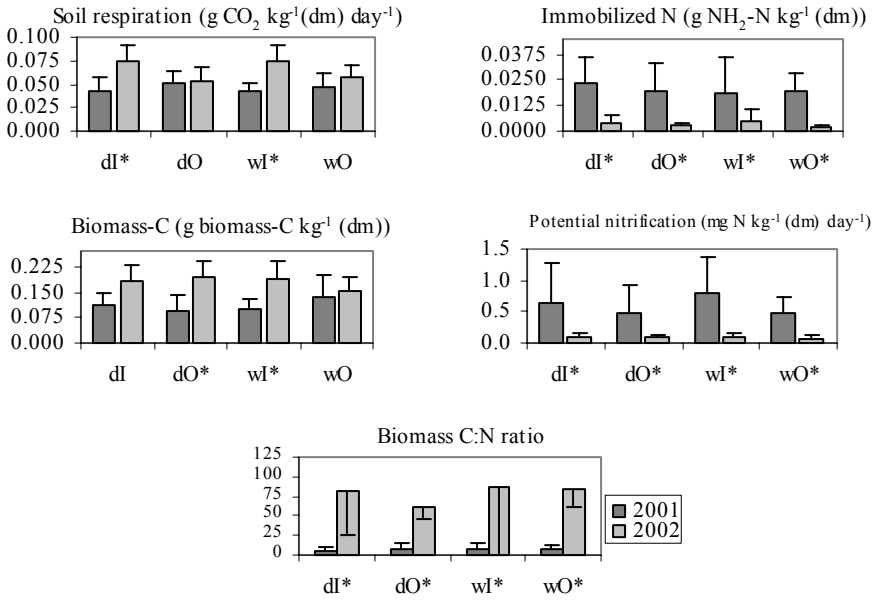

Figure 5. The average and standard deviation values of soil respiration (g CO_2 kg $^{-1}$ (dm) d^{-1}), biomass-C (g biomass-C kg^{-1} (dm)), immobilized N (g NH_2 kg^{-1} (dm)), potential nitrification (mg N kg $^{-1}$ (dm) d^{-1}) and biomass C:N ratio in the inflow side (I) and outflow side (O) in the dry bed (d) and wet bed (w) of the Kodijärve HSSF CW in 2001 and 2002. The detected significant differences of the respective values of the years (paired t-test for soil respiration, for the rest – Wilcoxon Matched Pairs tests) are indicated as a * for $p<0.05$.

These changes indicate that stronger influences of aeration near the inflows have resulted in different reactions from the microbial community in the different beds. These differences are probably caused by the already previously relatively higher content of aerobic bacteria and relatively recalcitrant soil C resources (e.g. the lower content of easily degradable soil C) near the inflow to the dry bed compared to that near the inflow to the wet bed. Thus only the microbial community near the inflow of the wet bed had sufficient resources of soil C to cause significant N immobilization (re-immobilization) in the presence of enhanced concentrations of oxygen. The lack of a difference between the respiration in the inflow vs. outflow sides of the wet bed in 2002 was probably caused by the highest content of available less degraded soil C near the outflow to the wet bed, which was utilized for soil respiration during measurement, and was not necessarily connected with the high aerobic ability of the microbes.

The latter explanation is supported by the results of the comparison of the changes in microbial characteristics in 2002 vs. 2001, which provided stronger evidence of the growth of respiration in the inflow sides of both beds (Fig. 5).

The comparison of the changes in biomass C in the dry bed demonstrated significant growth only near the outflow side, whereas in the wet bed there was significant growth only near the inflow side. We assume that the most aerobic conditions of the inflow side of the dry bed had caused the most rapid change in the microbial community, and the peak of the growth of supposed r-strategist microbial community was probably already over due to the depletion of the available resources, whereas near the inflow of the wet bed, the growth of biomass C was supported by the higher amounts of less degraded soil C, which became available due to the enhanced aeration.

3.2.4. The comparison of three transects in both beds in 2002

The microbial characteristics did not differ between the transects of the dry bed in 2002, whereas there was a change in the pattern of the potential nitrification, which had differences between the transects in 2001 ($p<0.05$, Kruskal-Wallis ANOVA). There were no differences between the transects and the changes in the pattern for any of the studied indicators in the wet bed ($p>0.05$, Kruskal-Wallis ANOVA). In 2001 there was a gradual growth in potential nitrification ($p<0.05$, ANOVA), when moving from inflow towards outflow (the first transect had lower potential nitrification than the third transect ($p<0.05$, Duncan test)) of the dry bed, and higher respiration in the second transect than in the first and the third transects ($p<0.05$, Duncan test), and lower potential nitrification in the second than in the third transects of the wet bed ($p<0.05$, Duncan test) (Nurk *et al*., 2005; Publication I).

The change in the pattern of potential nitrification in the dry bed may be caused by the effect of the pretreatment with vertical filter, which had lowered the hypothetical influence of the toxic pollutants. The comparison of the changes of potential nitrification after the change in wastewater consistence gave no evidence of the lowering of potential nitrification in the first and second transects of the dry bed, whereas the third transect of the dry bed and the transects of the wet bed demonstrated lowering of potential nitrification (Fig.6). We assume that the second transect of the dry bed had a different structure of microbial community, which was formed by fluctuations in water level (similarly to the lower layer of the dry bed), and the first transect was already less influenced by the change in microbial community due to the low availability of organics in 2001.

The comparison of the changes in respiration after the change in wastewater consistence yielded the growth in the third transect of the wet bed and in the second transect of the dry bed (Fig. 6). The growth in the respiration in the third transect of the wet bed could be caused by the relatively greater amount of less degraded soil C that became available during the measurements of respiration, whereas the soil C resources of the second transect and the first transect were more degraded and less available for respiration. The second transect of the dry

bed had the most intensive improvement of aerobic conditions, because it was inside the zone of water level fluctuations and was most affected by the dry year.

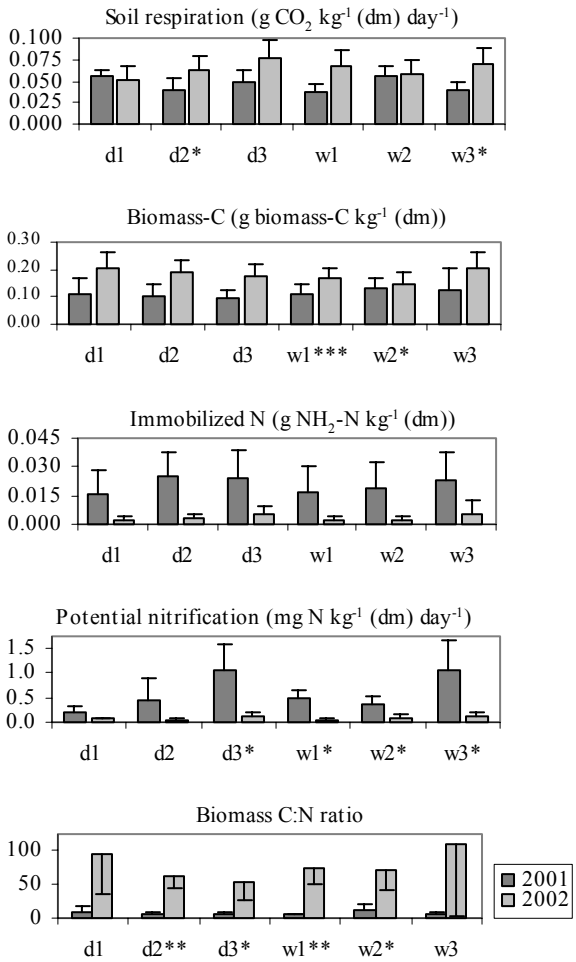

Figure 6. The average and standard deviation values of soil respiration (g CO_2 kg^{-1} (dm) d^{-1}), biomass-C (g biomass-C kg^{-1} (dm)), immobilized N (g NH_2 kg^{-1} (dm)), potential nitrification (mg N kg^{-1} (dm) d^{-1}) and biomass C:N ratio in the first (1), second (2), and the third transect (3) in the dry bed (d) and wet bed (w) of the Kodijärve HSSF CW in 2001 and 2002. The detected significant differences of the respective values of the years (Wilcoxon Matched Pairs test for immobilized N, for the rest – paired t-tests) are indicated as * for $p<0.05$, ** for $p<0.01$ and *** for $p<<0.01$.

The evidence of the growth of biomass C in the second transect and strong evidence of the growth in the first transect of the wet bed (Fig. 6) could be caused by the better aeration and lower availability of soil C, which enabled faster change in the consistence of the microbial community by the r-strategic heterotrophic microbes compared to the third transect, where the soil C resources were relatively less degraded and offered protection for the previous microbial community.

The comparison of the changes in the biomass C:N ratio provided stronger evidence in the transects where the extinction of the microbial community, which depended mainly on the organics of the inflowing wastewater and accumulated soil organics, was combined with the relatively higher growth of aeration, such as in the second transect of the dry bed and in the first transect of the wet bed (Fig. 6). We assume that both of the transects had the highest growth of the aeration compared to the other transects of both beds (the growth of soil respiration in the third transect of the wet bed was caused by the higher amount of less degraded carbon, which became available during the measurements). The higher growth of aeration was caused by the lower water table in the first transect of the wet bed and also by the relatively higher hydraulic conductivity compared to the other transects of the wet bed, which enabled better availability of inflowing aerated wastewater. The studied sampling sites of the second transect of the dry bed were situated inside the zone of the fluctuating water level, which had became entirely aerobic. The first transect of the dry bed, which had no evidence of the growth of biomass C:N ratio, was already above the zone of water level fluctuations before the dry year, and hence almost aerobic and less affected by the impact of the dry year and the change in the consistence of the wastewater, whereas the third transect of the wet bed demonstrated no evidence of the growth in the biomass C:N ratio, because it remained mainly aerobic even in 2002 due to the lowered hydraulic conductivity and enhanced amounts of less degraded organics in relation to clogging. The other transects – the third transect in the dry bed and the second transect in the wet bed – exhibited weaker evidence of the growth of biomass C:N ratio due to the weaker growth of aeration.

3.3. Molecular assessment of the microbial community structure in the dry bed in 2002

The multivariate randomization test of the bacterial community-level DNA fingerprints showed significant structural differences in the microbial communities between the upper and lower layers ($p<0.01$). Significant differences, although weaker ($p<0.05$), were also found between the inflow and outflow sides of the filter bed. These results coincide with results of the analysis of microbial characteristics, which also revealed the biggest differences between upper and

lower layer and for the second, between the inflow and outflow side. The bacterial community structure based on DGGE fingerprints was related to the C and N values of microbial biomass, as well to soil respiration activity. Highly significant correlations were found between respiration and bacterial community structure ($p<0.01$, $R = -0.75$, Spearman correlation) which was assessed with the 328f–GC/535r primer pair common to all bacteria, and also between the respiration and ammonia oxidizing bacteria ($p<0.01$, $R = -0.75$, Spearman correlation). Highly significant correlations were also found between biomass-C and bacterial community structure ($p<0.01$, $R = -0.89$, Spearman correlation), which was assessed with the 968f–GC/1401r primer pair common to all bacteria, and also between the biomass-C and ammonia monooxygenase genes ($p<0.01$, $R = -0.82$, Spearman correlation). These findings show that the values of respective microbial characteristics were strongly related to the presence of specific groups of microbes (Truu et al., 2005; Publication II). This consideration is fundamental support for the hypothesis about the activities of r-strategist microbes and shows that community structure can play a crucial role in determining the intensity of water purification processes.

3.4. Immobilized N as component in the N budget of the Kodijärve HSSF filter in 2001

The most important flux in the N budget in 2001 was in N_2 emission (12.4 kg a^{-1} or 41.7%), followed by microbial immobilization (8.7 kg a^{-1}), accumulation in soil (4.2 kg a^{-1}), plant assimilation (4.1 kg a^{-1}), and N_2O emission (0.35 kg a^{-1}; Fig. 7; Mander et al., 2003; Publication IV). Thus the proportion of microbial immobilized N was 29.2% of total amount of N retained in the soil filter.

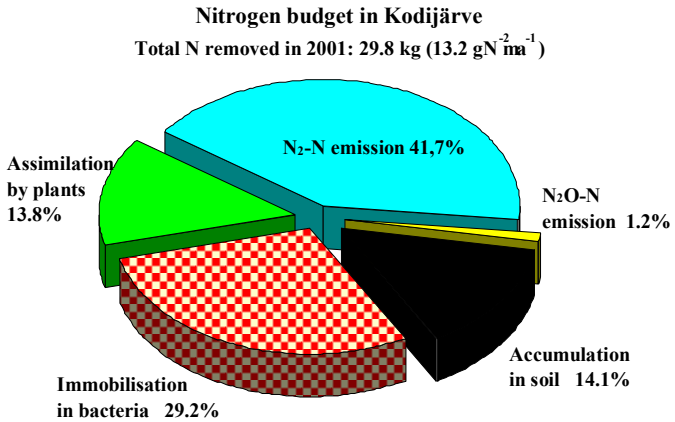

Figure 7. Nitrogen budget in Kodijärve HSSF CW (Mander et al., 2003; Publication IV).

This number is, however, probably overestimated, due to the relatively high amount of microbial biomass during the senescence and degradation of plants, which could enhance microbial N immobilization. The microbial nutrient cycling is tense and during most of the growing season the active biomass is not that high. In the case of "normal" soils, it is reasonable to assume that the proportion of immobilized N is mostly much lower (see Schinner et al., 1996). In the Kodijärve HSSF filter material (coarse sand), the initial concentration of organic (incl. microbial) material was close to zero, so this relatively high immobilized N value could be related to biofilm formation or the rapid accumulation of bacterial material in the filter media, also observed in other CWs (Tanner, 1998; Nguyen, 2000, 2001).

Average annual N removal from the system was 13.2 g m^{-2} a^{-1}. This value can be significantly higher for N (Vymazal et al., 1998). The main problem is the lack of oxygen in the filter system, which does not allow the nitrification of ammonia into nitrates. This problem was, however, partly solved by the establishment of a pretreatment VSSF filter in 2002 (Noorvee et al., 2005).

4. CONCLUSIONS

The microbial characteristics studied showed remarkable variability on both spatial and temporal levels, depending on changes in environmental conditions. As feedback, the change in microbial communities influences the efficiency of wetland purification.

- In 2001, the microbial community of the wet bed had higher diversity and metabolic activity than the dry bed. These differences were probably related to the enhanced availability of nutrients in the studied layers of the wet bed due to the higher water table. We suppose that differences in the structure of the microbial community between the beds is caused by the differences in the aeration conditions and the availability of nutrients.
- In 2001, the lower layer of the dry bed had lower microbial metabolic activity. There were also greater structural differences between the microbial communities of the soil samples in the dry bed than in the wet bed. We assume that the greater structural differences in the dry bed and the difference in metabolic activity between the layers of the dry bed are related: 1) to the differences in the availability of the nutrients between the soil sites due to the lower water table, 2) to the different sources of the nutrients – the microbial community of the upper layer most likely depended mainly on the decomposition of plant litter, and 3) to the greater differences in the aeration conditions between the soil sites in the upper and lower layers of the dry bed than in the wet bed.
- The lowered BOD_7 value after the building of the VSSF pretreatment filter in August 2002, and enhanced aeration in autumn of 2002 resulted in a change in the structure of the microbial community. The proportion of the r-strategists within the microbial community increased, which allowed for the rapid degradation of the easily degradable carbon source.
- The intensity of soil respiration and also the values of biomass-C are strongly related to the structure of the microbial communities. The latter fact is positive evidence that enhanced biomass-C in 2002 was related to the dominance of r-strategist microbes in the microbial community.
- The sandy filter material with low humus content makes the buffering system quite poor, and destabilizes selected characteristics of the microbial community, which depends merely on inflowing organic material and is open to external influences. The deposits of accumulated carbon, nitrogen, and phosphorus offered protection against changes in the microbial community: in sites with higher C, N and P concentrations, the structure of microbial communities had changed at the lower extent.
- The lowered BOD_7 value and enhanced aeration mainly changed the distribution of the values of microbial characteristics in the vertical gradient within filter beds.

- We assume that due to lower nutrient resources and weaker aeration, the microbial community of the lower layers was more vulnerable than that of the upper layers. This resulted in the weaker re-immobilization of N in the lower layers.
- We assume that two months after the establishment of the VSSF filter, the microbial community in the anaerobic sites of the HSSF was still in a state of change and not balanced with the new environmental conditions.
- We assume that the main reasons for the greatest change in the microbial community in the upper layer of the dry bed were:
  - the faster microbiological processes due to the enhanced aeration;
  - the higher concentration of easily degradable organics from plant remains, because the vegetation of the dry bed in 2002 – *Scirpus sylvaticus* and *Urtica dioica* – had less hard tissues and became more easily available to the microbial community than the *Phragmites australis* of the wet bed;
  - the damage of the water-conductive biofilms between the sand particles, which caused a greater decrease in the availability of organic sources than in the other layers.
- The effect of increased aeration by the VSSF pretreatment filter on the microbial community was limited, because the oxygen was already consumed in the inflow sides of both beds.
- The changes in the pattern of the potential nitrification between the transects in both beds is probably caused by the lowered influence of the toxic pollutants on the nitrifying microbes due the pretreatment of the wastewater.
- The clogging enhances the local water table due to the stronger capillary forces compared to the less clogged sites, and accelerates the rate of accumulation of organic matter until the flow pattern changes.
- In 2001, the proportion of microbial immobilized N was 29.2% of the total amount of N retained in the soil filter.

We conclude that the higher purification efficiency of the dry bed and most of the differences in the selected microbial chracteristics and parameters of the microbial community are related to the differences in the aeration conditions between the beds. The lower water table enables better contact between the soil air and the wastewater and increases the rates of nutrient transformation and transfer processes. Further investigations concerning the effects of different media and flow regimes on wastewater aeration and nutrient transfer with respect to microbial processes are crucial for the understanding and optimisation of wastewater purification in horisontal subsurface flow constructed wetlands.

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SUMMARY IN ESTONIAN

Mikroobsete näitajate ja keskkonnatingimuste vahelised seosed horisontaalvoolulises heitveepuhastus-tehismärgalas

Heitveepuhastus tehismärgalades on arenev alternatiivne meetod konventsionaalsetele reoveepuhastusmeetoditele, mille puhul kasutatakse taastuvaid energiaallikaid ja mille rajamise mõju ümbritsevale keskkonnale on minimaalne. Reovee puhastumine süsiniku- ja lämmastikuühendeist toimub märgalas põhiliselt mikroobide elutegevuse käigus. Selleks, et reovee puhastumise protsessi optimeerida, on vaja teada kuidas märgala ehituslikud parameetrid ja kasutatav substraat mõjutavad mikroobide elutegevusele olulisi keskkonnatingimusi tehismärgalades. Käesolevas töös uuriti Kodijärve (Kambja vald, Tartumaa) horisontaalvoolulisest taimestik-pinnasfiltrist 2001. ja 2002. aasta hilissuvel ja sügisel kogutud pinnaseproovide mikrobioloogilisi näitajaid. Töö eesmärkideks olid:

1) määrata mikroobse lämmastiku, mikroobse biomassi (mõõdetud substraadi poolt indutseeritud hingamise meetodiga), mulla hingamise, potentsiaalse nitrifikatsiooni väärtusi ning mõõta mikroobikoosluste metaboolset profiili Biolog-plaadi andmete põhjal Kodijärve horisontaalvoolulises taimestik-pinnasfiltris, 2) leida, kuidas mikroobikooslus reageeris sissevoolava reovee aeratsiooni ja BHT_7 väärtuse muutusele ning suurenenud evapotranspiratsioonile märgalast 2002. aasta hilissuvel ja sügisel (võrrelduna 2001.a. sama perioodiga), 3) määrata mikroobikoosluse funktsionaalsed erinevused märgala eri osades, 4) leida seoseid uuritud mikroobikoosluse funktsionaalsete näitajate ja koosluse geneetilise struktuuri vahel ning 5) anda hinnang mikroobide biomassi seotud lämmastiku osale märgala üldises lämmastikubilansis.

Kodijärve horisontaalvooluline taimesik-pinnasfilter puhastab lähedaloleva umbes 40 hoolealusega hooldekodu reovett. Kodijärve tehismärgala koosneb kahest jämeda liivaga täidetud vannist, mis mõlemad on mõõtmetega 25*6.25*1 m. Enne märgalasse sisenemist läbib heitvesi kahekambrilise septiku. Märgala vannide liiv on erineva konsistentsiga, mis tingib erinevused hüdraulilistes tingimustes, veetasemes ja sellest tingituna ka töövõimes. Kuivas vannis, milles kasvas metskõrkjas ja kõrvenõges, oli veetase 20–50 cm madalamal kui märjas vannis, milles domineeris pilliroog.

Uurimise tulemusena selgus, et mikroobikoosluse struktuuri ruumiline jaotus erines kuivas ja märjas vannis. Mikroobikoosluse suurem mitmekesisus ja kõrgem metaboolne aktiivsus märjas vannis on tõenäoliselt tingitud toitainete paremast kättesaadavusest võrreldes kuiva vanniga. Kuivas vannis olid suuremad mikroobikoosluse struktuursed erinevused ja kõrgem metaboolne aktiivsus ülemises kihis – viimaste järeldustega on kooskõlas ka mikroobsete näitajate väärtuste suurem kihtidevaheline erinevus kuivas vannis. Me oletame, et need

erinevused tulenesid toitainete kättesaadavuse ja kvaliteedi ning aeratsiooni erinevustest kuiva vanni eri piirkondades. Kuiva vanni ülemise kihi mikroobikoosluse kõrgem metaboolne aktiivsus ja mitmekesisus olid ilmselt tingitud taimejäänustest tulenevast kõrgemast orgaaanikasisaldusest selles kihis.

Sissevoolava reovee BHT sisalduse ja aeratsiooni muutumine pärast vertikaalvoolulise õhustusfiltri ehitamist märgala sissevoolu ette ning oluliselt suurem evapotranspiratsioon 2002. aasta suvel põhjustasid kõigi uuritud mikroobsete näitajate väärtuste olulise muutuse. Mullaproovides määratud hingamine tõusis 1,4 korda ja mikroobse biomassi süsiniku hulk suurenes 1,6 korda, samas kui immobiliseeritud lämmastiku hulk vähenes 5,8 korda ja potentsiaalne nitrifikatsioon vähenes 7,2 korda. Kuiva ja märja vanni mikroobsete näitajate väärtuste vahel 2001 ega 2002 aastal erinevusi ei olnud. Mikroobsete karakteristikute väärtuste jaotuse muutus ilmnes eelkõige vertikaalsihis. Toimus mikroobikoosluse struktuuri muutus, mis väljendus r-strateegide osakaalu kasvus koosluses. Kodijärve horisontaalvoolulise tehismärgala filtermaterjal on tulenevalt madalast huumusesisaldusest väikese puhverdusvõimega, see põhjustab mikroobikoosluse ja mõõdetud mikroobsete näitajate väärtuste ebastabiilsust. Pinnases akumuleerunud toitained kaitsevad mikroobikooslust muutuste eest.

Vertikaalfiltri poolt põhjustatud sissevoolava heitvee hapnikusisalduse tõus horisontaalvoolulise filtri uuritud kihtides oli võrreldes kuivast aastast tingitud aeratsiooni tõusuga suhteliselt madal. Märgalasse siseneva heitvee varasemast suurema hapnikusisalduse mõju mikroobsetele protsessidele oli piiratud horisontaalfiltri sissevooluga, kus heitvees lahustunud hapnik ära tarbiti.

Kokkuvõttes võib öelda, et mikroobsed protsessid tehismärgalas on suurel määral sõltuvad märgala füüsikalis-keemiliste ja bioloogiliste näitajatega määratud keskkonnatingimustest. Nende vastastikuste mõjude lähem uurimine avab võimalusi heitveepuhastusprotsessi optimeerimiseks tehismärgalades.

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Impact of enhanced aeration and changed wastewater quality on the microbial characteristics of a horizontal subsurface flow planted soil filter for wastewater treatment

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ABSTRACT

We analysed selected microbial characteristics (soil respiration, biomass-C, immobilized N, potential nitrification, biomass C:N ratio) of the filter material (coarse sand) in a two-bed horizontal subsurface flow (HSSF) constructed wetland (CW) in Kodijärve, Estonia, before and after the establishment, in August 2002, of a vertical subsurface flow (VSSF) filter as a pretreatment system. Significant changes in microbial characteristics between the two beds and 2 depth layers in both wet and dry HSSF beds in 2001 and 2002 have been found. The average value of the respiration of the soil samples enhanced 1.4 times and the average value of the measured biomass C increased 1.6 times. At the same time, the immobilized N decreased 5.8 times and potential nitrification 7.2 times. No significant differences in microbial characteristics were found between the wet and dry bed. The lowered BOD_7 value and enhanced aeration after the establishment of the VSSF changed the distribution of the values of microbial characteristics mainly in the vertical gradient. The microbial community of the lower layers was more vulnerable than that in the upper layers. It resulted in a weaker re-immobilization of N in the lower layers.

Key words: Biomass C and C: N ratio, Horizontal subsurface flow filter, Immobilized N, Potential nitrification, Soil respiration, Vertical subsurface flow filter, Wastewater treatment

1. INTRODUCTION

The efficiency of water purification in constructed wetlands is determined in large by the dimensions of constructional parameters and optimal flow regime for better conditioning of microbiological and geochemical nutrient transformation and retention processes.

The majority of the data about different CW-s describe wetland performance in terms of nutrient flows, accumulation and transformations for entire individual systems. The great variability of wastewater purification efficiency and durability of CW-s indicates that it depends not only on the hydraulic load and pollutant concentrations, but also on the inner environmental conditions that vary between the different wetland types. This has led on one hand to the creation and study of wetland systems, where different types of CW-s are combined to cover the needs of the different stages of nutrient transformation (Cooper et al., 1999) and on the other hand stresses the need for a deeper understanding about the limitations and possibilities of nutrient transformation in different wetland media.

The C and N retention and transformation in CW-s are mediated mainly by microbes. Microbial metabolism is responsible for the transformation of organic and inorganic C and N forms and causes the nutrient transfer between the pools of wetland ecosystems and the loss through the gaseous emissions. Microbial C and N pools are interdependent and in dynamic balance with the other C and N pools. These balances are influenced by the environmental factors that modify nutrient cycling inside the wetland ecosystem and losses from the wetland ecosystem.

The pools of microbial biomass C and biomass N are formed by the processes of C assimilation and N immobilization and are interdependent because the balance of microbial C and N are genetically determined. The balance between the soil C and soil N with microbial C and N depends on the intensity of the soil C utilization through microbial C assimilation and respiration and soil N mineralization and immobilization and the processes that drain the soil N such as subsequent nitrification and denitrification and on the other hand it also depends on the inflow of C and N to the system (Reddy, D'Angelo, 1997; Hart et al., 1994). The intensity of the microbial processes of C mineralization, assimilation and coupled N immobilization are related to the amount of active microbial biomass and are controlled by the availability of O_2 or alternative electron acceptors, temperature, quality of soil organic matter –such as the ratio of easily degradable soil C to recalcitrant soil C and by the availability of soil N (Reddy, D'Angelo, 1997; Magill, Aber, 2000; Whalen et al., 2000). Different microbial functional groups, which are specialized to different stages of organic matter decomposition, have different needs for N availability for C mineralization (Henriksen, Breland, 1999)

The aeration and the redox potentials are the most important factors influencing nutrient transformation processes in constructed wetlands. The

differentiation of the microbial processes, such as nitrification and denitrification, along the O_2 and redox gradient is related with both vertical zonality and microzonal growth of the microbes (Reddy, D'Angelo, 1997). The microzonal growth in biofilms and detrital agregates allows for the better exchange of essential metabolites (Paerl, Pinckney, 1996) and anaerobic activities to occur in aerobic habitats (Storey et al., 1999). Thus the microzonal growth is space-effective because of short distances for diffusion of the metabolites and can be space-effective also in terms of wastewater purification.

Microzonal growth needs substrate for the attachment of microbes and is thus supported in CW systems, which have large specific contact areas between water and substrate such as in subsurface flow systems. The higher specific contact area between the water and substrate in these systems results in generally higher water purification efficiency compared to free-water systems. At the same time, these systems lack sufficient aeration of wastewater. According to Kadlec and Knight (1996), the oxygen diffusion in flooded soils is nearly 10,000 times slower than in aerobic soils and a low dissolved oxygen level results in the accumulation of organic matter in wetland soils, because of a reduced level of microbial activity and organic decomposition. Thus, the studies of the relationships between the microbial characteristics and the substrate in HSSF CW systems have focused so far on the organic matter accumulation and clogging of these systems. Tanner et al. (1998) and Nguyen (1999) focused on clogging and maturation of gravel-bed HSSF CW-s and relationships of different organic matter fractions with microbial respiration, microbial biomass C and biomass N.

We studied the horizontal subsurface flow (HSSF) constructed wetland (CW) in Kodijärve, Estonia, 2 months after the establishment of a vertical subsurface flow (VSSF) filter as a pretreatment system for better aeration of wastewater in August 2002, after an extremely dry summer. The different water tables and resulting different aeration of the wastewater cause significantly different purification efficiencies in the parallel beds of Kodijärve HSSF (Noorvee et al., 2005). We were interested how the zonal differences of environmental conditions and functional differences of microbial community were related. The main objectives of this study were: 1) to find out, how the microbial community reacted to the aeration of the inflowing wastewater, to the higher evapotranspiration from the wetland and to the lowered BOD_7 value of the wastewater, 2) to determine functional differences of the microbial community in the different layers of the CW. We measured the values of microbial characteristics inside CW and specified the zonal differences of microbial characteristics in 2002 and analyzed these patterns with respect to the parameters of Kodijärve CW and the results of the analyses of 2001 (Nurk et al., 2005).

2. MATERIAL AND METHODS

2.1.Study site

A detail site description of the Kodijärve CW system is given by Mander et al. (2005). The Kodijärve HSSF consists of two parallel beds (each 25*6.25*1 m), filled with coarse sand. The wastewater is shared in equal flow rates between the beds. The beds have different hydraulic conductivity and are called a dry bed and a wet bed due to the different water levels therein. Eighteen water sampling wells, 9 in each bed, are distributed evenly throughout the sand filter. The HSSF system was established in 1996, it treats effluent from a hospital (about 40 PE). In the summer of 2002, the VSSF part, as a pretreatment system for better aeration of the wastewater, was added to the HSSF system.

<Fig. 1>

2.2. Soil and water sampling

Soil samples for microbiological and parallel soil samples for chemical analyses and water samples for standard water quality analysis (APHA; 1998) from inflow; outflow and 18 wells were collected in November 2002. Soil samples were collected randomly around a 1 m radius of the water sampling wells near the inflow (samples around wells No 1, 4, 7 in the dry bed and 12, 15, 18 in the wet bed) and outflow (samples around wells No 3, 6, 9 in the dry bed and 10, 13, 16 in the wet bed) in both dry and wet beds at a depth of 0–10 cm ("upper layer") and 20–30 cm ("lower layer") (Fig. 1).

2.3. Microbiological methods

All microbial characteristics were measured and calculated according to Schinner *et al*. (1996). For the measurement of soil respiration (g CO_2 kg^{-1} (dm) d^{-1}), respired CO_2 was trapped in NaOH solution and determined by titration with HCl after the addition of BaCl. Biomass C (g C kg^{-1} (dm)) was measured by the SIR method, which is based on the measurement of initial respiratory response (CO_2 outcome as for the detection of soil respiration), after glucose addition into the soil sample. Immobilized N (g NH_2-N kg^{-1} (dm)) was measured by fumigation-extraction technique coupled with ninhydrin-reactive nitrogen (NH_2-N of the proteins and nucleic acids) detection. For the measurement of potential nitrification (mg N kg^{-1} (dm) d^{-1}), the soil samples were incubated for 5 h at 25§C in 20 ml of 1 mM ammonium sulfate solution in a shaking water bath so as to saturate the solution with oxygen. Further nitrite

oxidation to nitrite was inhibited by adding 0.1 ml 1.5 M sodium chlorate and the nitrite was detected colorimetrically.

2.4. Meteorological data

Air and soil temperature, wind velocity, solar radiation and precipitation evapotranspiration were measured using a DAVIS Groweather automatic weather station installed close to the CW. The summer of 2002 was significantly warmer and dryer than 2001 (Mander et al., 2005).

2.5. Statistical analysis

The data were transformed with Microsoft Excel and statistical analyses were done with *Statistica* 6 (StatSoft Inc.) software. We compared dry and wet beds of the wetland with t-test or Mann-Whitney U-test, upper and lower layers in both beds with paired t-test or Wilcoxon Matched Pairs test. Respective parts of the wetland in 2001 vs. 2002 were compared with paired t-test or Wilcoxon Matched Pairs test.

We determined Pearson and Spearman Rank Order correlation coefficients between the microbiological characteristics, soil chemical parameters in the upper and lower layers and water quality indicators from the water sampling wells in the dry bed and wet bed.

3. RESULTS AND DISCUSSION

3.1. Comparison of microbiological characteristics of entire soil filter in 2001 and 2002

Enhanced aeration and lowered BOD_7 of the inflowing wastewater reported by Noorvee et al. (2005) and an extraordinarily dry year with higher temperatures and evapotranspiration influenced the values of all studied microbial characteristics, which differed significantly between 2001 and 2002 ($p<<0.01$, paired t-test, Wilcoxon Matched Pairs test, Fig. 2).

<Fig. 2>

These changes increased the average value of the respiration of the soil samples 1.4 times, and the average value of the measured biomass C of the soil samples 1.6 times. At the same time, the immobilized N decreased 5.8 times and potential nitrification 7.2 times.

The 5.8-fold decrease of immobilized N resulted in an extremely high biomass C:N ratio in 2002, when the average biomass C:N ratio was 10.4-fold higher than in 2001 (Fig. 2) and the ratio of the average biomass C to average immobilized N was 9.4 times higher than in 2001. The biomass N:P ratio of the entire soil filter varied much less in comparison: the average immobilized N versus average biomass P ratio was 2.6 in 2001 and 3.0 in 2002. We considered that lowered organics of the wastewater caused death and lysis of the previous microbial community, which was responsible for N immobilization. Whereas a higher biomass C was related to the growth of the proportion of r-strategic heterotrophic microbes (see Lebuhn et al., 1994; Sharma et al., 1998), which allow for a fast turnover of easily degradable organics from dead microbial matter with low coupled immobilization of N. (We checked this hypothesis with further correlation analysis.)

3.2. Comparison of the dry and wet bed

The average values of microbial characteristics of the dry and wet bed were not different in 2002 ($p>0.05$; for the soil respiration and biomass C, t-test; for the rest of microbial characteristics, Mann-Whitney U-test) and comparable to the results of the respective analysis in 2001 by Nurk *et al.* (2005). Whereas there was stronger evidence of the growth of biomass-C and weaker evidence of the growth of respiration in the dry bed than in the wet bed (Fig. 3).

<Fig. 3>

We assume that the studied layers of the dry bed were, due to the lower water table, more aerobic and also had, due to the poorer availability of wastewater, less soil organics for respiration already in 2001, which caused the weaker effect of aeration and lower organics of the wastewater in 2002. The weaker growth of biomass C in the wet bed was caused by the slower degradation of organics by the heterotrophic population that was adapted to anaerobic conditions and was less capable of the fast degradation of easily degradable carbon compared to the aerobes.

The correlation analysis between microbial characteristics, soil chemical indicators and water quality parameters in the upper and lower layers of the filter beds gave 26 significant correlations in the dry and 6 significant correlations in the wet bed in 2002 (both beds had 160 possible correlations) (Table 2). In 2001, there were 14 significant correlations in the dry bed and 24 in the wet bed.

A large decrease in the number of significant correlations in the wet bed indicates that the changes in the conditions were more fundamental in the wet than in the dry bed. This was probably caused by the stronger influences of the lowered organics and enhanced aeration, because the layers of the wet bed

depended more on the changes of the water quality than the layers of the dry bed. We assume that the lower number of correlations of the wet bed was caused also by low aeration – the growth of aeration was limited with the inflow of the wet bed and was not sufficient to make it as aerobic as the dry bed and the new balances were still being established.

The higher number of correlations in the wet bed than in the dry bed in 2001, there were 14 significant correlations in the dry bed, indicate that the microbial characteristics of the wet bed were earlier well balanced with the environmental conditions and depended on the multitude of factors that had equal importance. The altering aeration conditions of the dry bed did not allow for the establishment of balances between the multiple factors.

The growth of the number of significant correlations in the dry bed can be explained by the relative stability and fast changes due to the high aeration, which had the same direction as the processes before the change of the conditions and allowed for the establishment of new balances within 2 months.

<Table 1>

3.2.1. Comparison of the upper and lower layer in the dry bed and wet bed

3.2.1.1. Correlation analysis of the layers of the dry bed and wet bed

The correlation analysis between microbial characteristics, soil chemistry indicators and water quality parameters in the upper and lower layer gave 21 significant correlations in the upper layer and 6 significant correlations in the lower layer of the dry bed, whereas in 2001 there was 6 significant correlations in the upper layer and 8 significant correlations in the lower layer of the dry bed, respectively. The wet bed had 3 significant correlations in the upper layer and 3 significant correlations in the lower layer, and in 2001 there were 17 significant correlations in the upper layer and 7 significant correlations in the lower layer, respectively (every compared layer had 80 correlations). The upper layer of the dry bed had stable conditions for the longest period, already before the building of the VSSF pretreatment filter, because the water level was constantly low and the upper layer had been dry due to the lack of the rain. This layer had changing conditions in 2001, which also explains the lower number of correlations compared to the wet bed in 2001, which had stable conditions at that time. The relatively higher number of correlations in the upper layer of the wet bed in 2001 indicates that this layer had stable conditions and more factors that influenced the microbial characteristics than in the anaerobic lower layer or the layers of the dry bed, where altering aeration and feeding conditions did not allow specific relationships, but only general relationships between the indicators.

<Table 2>

3.2.1.1.1 Analysis of the dry bed

Upper layer

Detected significant correlations of soil respiration (Table 2) indicate that: 1) soil respiration was controlled by the availability of the soil nutrients instead of the aerobic ability of the respiring microbial community, 2) the respiring microbial community differed from the part of microbial community responsible for the growth of biomass C. Negative correlations of biomass C with soil P, soil N and immobilized N indicates that the growth of this part of microbial community was not limited by the availability of soil nutrients, except soil C, and was connected with the lowering of immobilized N, whereas positive correlation with biomass C:N ratio shows that the lowering of immobilized N enhanced the biomass of these microbes. We believe that these microbes belonged to the functional group of bacteria described by Hendriksen and Breland (1999), and are connected to the initial phases of organic matter decomposition and are responsible for the degradation/turnover of easily degradable organics. The positive correlations of soil nutrients with immobilized N and negative correlations with biomass C:N ratio and strong negative correlation between immobilized N and biomass C:N ratio, whereas biomass C lacked the negative correlation with soil C, indicates that these bacteria depended on the easily degradable fraction of soil C, which became available after death and lysis of the older microbial community.

We assume that the positive correlation between NO_2-N of the wastewater with biomass C and biomass C:N ratio shows that decline of the previous community had spoiled the balance between the different stages of nitrogen transformation.

Lower layer

Missing correlations of biomass C with other indicators, whereas there was negative correlation between biomass C:N ratio and immobilized N, shows that the growth of r-strategist heterotrophic microbes was in the initial phase. We assume that the reasons were: 1) slower processes due to lower aeration, 2) better availability of organics from the wastewater, 3) the different structure of the microbial community already in 2001 (Nurk et al., 2005). Positive correlations of the biomass C:N ratio with PO_4-P and total P of the wastewater indicates that degradation of biofilms enhanced the concentrations of soluble P, which was earlier immobilized or absorbed on the surface of sand particles, which were covered with biofilms. There is also the possibility that readily available PO_4-P for ATP generation speeded up the growth of the r-strategist heterotrophic microbial community that was suppressed by the relatively low aeration. The positive correlation between soil respiration and soil P and lack of correlations of soil respiration with soil C and soil N supports earlier hypothesis of the different structure of the microbial community of that layer – there was the transition zone between aerobic and anaerobic conditions. This zone had a

fluctuating water level and it supported the exhaustion of soil C and soil N by the gaseous emissions from the biofilms. Remaining recalcitrant fractions of soil N and C did not support the soil respiration during the measurements. The positive correlation of potential nitrification with soil C is attributable to heterotrophic nitrification of these sites. It also shows that higher soil C protected microbial consortia against death and lysis. This explanation is supported by the findings of the analysis of 2001 (Nurk et al., 2005), which revealed that the N immobilization had been lower in the sites, where there was a higher soil C:N ratio. We consider that the sites with lower immobilized N and higher soil C:N ratio had, due to lower aerobicity, a higher ratio of coupled nitrification-denitrification vs. aerobic respiration. We consider that these sites also had better protection against the changes of the microbial community due to more anaerobic conditions, and the better availability of wastewater should have supported the survival of nitrifiers and counteracted the exhaustion of the soil C resources.

3.2.1.1.2 Analysis of the wet bed

Upper layer
A negative correlation between the biomass C:N ratio and immobilized N, where biomass C was not negatively correlated with immobilized N and positively correlated with biomass C:N ratio, indicates that the growth of biomass C was not necessarily connected with the decrease of immobilized N and the growth of biomass C:N ratio, but the sites that experienced the growth of biomass C:N ratio also had a lowered immobilized N. We suppose that the loose relationships of the growth of r-strategic microbial community with the lowering immobilized N of the older community were caused: 1) by the high content of relatively less degraded soil C, 2) by the high variability of aeration conditions and availability of wastewater, 3) by the enhanced aeration, which influenced the availability of the less degraded soil C. A negative correlation of PS-C of the wastewater with immobilized N shows that the clogging and decreased hydraulic conductivity counteracts with the needs of N immobilization (immobilized N had negative correlations with PS-C in the upper layer of the wet bed also in 2001), whereas a positive correlation of PS-C with biomass C:N ratio shows that enhanced availability of less degraded soil C of the PS-C due to the higher aeration after enhanced evapotranspiration promoted the growth of r-strategist heterotrophic microbes.

Lower layer
A positive correlation of biomass C with biomass C:N ratio and the negative correlation with PS-C indicates that the change of the microbial community was more intensive in the less clogged soil sites, where better flow conditions also allowed better aeration by the aerated wastewater.

The strong positive correlation of potential nitrification with biomass could be caused by the effect of enhanced aeration after the building of the vertical filter, because the growth of both indicators is favored by the enhanced oxygen concentrations in the studied sites.

3.2.1.2 Comparison of the layers of the dry bed and wet bed

The pattern of respiration in 2002 compared to 2001 did not change. The respiration was higher in the upper layer of the dry bed and had no differences between the layers of the wet bed (Table 1). At the same time, the comparison of the changes of the layers gave evidence about the growth of respiration in the upper layers of both beds (Fig. 4).

<Fig. 4>

The difference between the patterns of the beds was caused by the different water tables, which was too high to allow sufficient aeration that could enable significantly higher respiration in the upper layer of the wet bed. The higher respiration in the upper layer of the dry bed was caused by the lower water table that enabled constant aeration of the soil water by the soil air through the soil pores above the water table, in contrast to the upper layer of the wet bed, where the soil pores were filled mainly with water. Evidence of the increase of respiration only in the upper layers of both beds indicates that the effect of the vertical filter was relatively low, compared to the effect of the enhanced aeration, which was caused by the enhanced evapotranspiration in the extraordinarily dry summer of 2002. We assume that the aeration from the vertical filter is limited with the amount of oxygen that can be dissolved and transported with the wastewater, whereas the zone of the vertical gradient of oxygen concentrations in the horizontal filter offers better aeration due to the big specific contact area between the soil water and soil air, which enables the constant diffusion of oxygen into the water.

The biomass C was higher in the upper layer of both beds and the pattern of biomass C in 2002 differed from the pattern of biomass C in 2001, when there were no differences between the layers of the dry bed (Table 2). We found also that there was evidence of the growth of biomass C only in the upper layer of the dry bed (Fig 1). These findings indicate that the consistence of the microbial community had changed towards the ability of rapid aerobic utilization of easily degradable carbon source at the largest extent in the upper layer of the dry bed. The data of 2001 show, that the microbial community of the upper layer of the dry bed depended largely on plant remains such as decaying roots, leaves and stems (Nurk et al*.,* 2005). We suppose that the significant growth of biomass C in the upper layer of the dry bed has three main interconnected reasons. The first reason is the enhanced aerobic ability due to the dry summer and autumn –

enhanced evapotranspiration had dried the upper layer, which was in 2001 less aerobic due to the moisture of the rain water and due to the rise of the wastewater by the capillary forces in the biofilms between the sand particles. The second reason could be the higher concentration of organics from plant remains due to the higher productivity in 2002 (Mander et al., 2005). The vegetation of the dry bed differed from the wet bed – it consisted mainly of the *Urtica dioica* (nettle) *Scirpus sylvaticus* (wood club-rush), and *Epilobium hirsutum* (hairy willow-herb).All these plantshave less hard tissues and therefore degrade easily, compared to the vegetation of the wet bed, which was dominantly reed. The latter fact also supports the hypothesis, that part of the growth of biomass C should be caused by plant organics. The third reason is that the lowering of the BOD_7 of the wastewater had, due to the influences of the enhanced evapotranspiration, a stronger effect in the upper layer of the dry bed. It is possible that the dry summer and autumn with enhanced evapotranspiration and without rain had caused drying and enhanced aeration that led to the gradual damage and destruction of water-conducting biofilms between the sand particles and in the dead root channels, leading eventually to the enhanced effect of the lowering of the BOD_7.

The immobilized N and potential nitrification significantly decreased whereas the biomass value increased in both layers of the dry and wet bed. The immobilized N was higher in the upper layers of both beds. The pattern of immobilized N had changed in the wet bed, where no differences between the layers in were found in 2001 (Table 2). We suppose that the microbial community of the upper layer of the wet bed, having more aerobic conditions than in the autumn of 2001, had used the resources of soil C, that became available for coupled C mineralization and re-immobilization of N, which compensated the effects of the lowering of BOD_7 value. The potential nitrification had no differences between the upper and lower layers in the both beds and different patterns compared to the year 2001, when there was higher potential nitrification in the upper layers of both bed. The loss of differences between the layers could be caused by the more complete change of the consistence of the microbial community in the upper layers of both beds that have resulted also in the extinction of nitrifiers.

The biomass C:N ratio had no differences between the layers of the dry bed and was lower in the upper layer of the wet bed. The change of the pattern of the biomass C:N ratio after the change of conditions had been towards a greater increase of the values in the lower layers of the wet bed (Table 2). This trend is caused by the stronger influence of the lowering of BOD concentrations of the wastewater in the lower layer of the wet bed, which had, in contrast to other studied layers, weaker re-immobilization and higher vulnerability of the previous microbial community.

4. CONCLUSIONS

The lowered BOD_7 value resulted in the extinction of the previous microbial community of the studied sites. The previous microbial community was replaced by the r-strategist heterotrophic microbial community, which allowed for the fast degradation of easily degradable carbon source that became available after death and lysis of the microbial cells.

The sandy filter material with low humus content makes the buffering system rather poor and destabilizes selected characteristics of the microbial community, which depends merely on inflowing organic material and is open to external influences.

The deposits of accumulated carbon, nitrogen, and phosphorus offered protection against the changes in the microbial community consistence: in sites with higher C, N, and P concentrations all characteristics of microbial communities decreased.

The lowered BOD_7 value and enhanced aeration changed the distribution of the values of microbial characteristics mainly in the vertical gradient.

Due to smaller nutrient resources, and weaker aeration, the microbial community of the lower layers was more vulnerable than that in the upper layers. It resulted in the weaker re-immobilization of N in lower layers.

Two months following the establishment of the VSSF filter, the microbial community in the anaerobic sites of the HSSF was still in a state of change and not balanced with new environmental conditions.

The main reasons for the greatest change of the microbial community in the upper layer of the dry bed were:

– the faster microbiological processes due to the enhanced aeration;
– the higher concentration of easily degradable organics from plant remains, because the vegetation of the dry bed in 2002 – *Scirpus sylvaticus* and *Urtica dioica,* had less hard tissues and became easily available for the microbial community, when compared to the *Phragmites australis* of the wet bed;
– the damage of the water-conductive biofilms between the sand particles, which caused a greater decrease of the availability of organic sources, when compared to the other layers.

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Table 1. Comparison data of upper vs. lower layer (paired t-test, Wilcoxon Matched Pairs test) inside either bed in 2002 and in 2001. Non-parametric tests are indicated with (w) and the detected significant differences of the average values of compared parts are indicated as * for $p< 0.05$, ** for $p<0.01$.

The beds and compared parts	Soil respiration		Biomass C		Immobilized N		Potential nitrification		Biomass C:N ratio	
	2001	2002	2001	2002	2001	2002	2001	2002	2001	2002
Dry bed, upper vs. lower layer	higher/ lower * (w)	higher/ lower * (w)	---	higher/ lower **	higher / lower * (w)	higher/ lower * (w)	Higher /lower *(w)	--- (w)	--- (w)	---(w)
Wet bed, upper vs. lower layer	---(w)	---(w)	higher / lower *	higher/ lower *	---(w)	higher/ lower * (w)	higher/ lower *(w)	--- (w)	--- (w)	lower/ higher * (w)

Table 2. Significant Pearson correlations ($p<0.05$; *– $p<0.01$) and Spearman correlations ((S); $p<0.05$) between the microbial characteristics, water parameters and soil parameters in the upper layer and in the lower layer of the dry bed and wet bed (underlined values) of the Kodijärve HSSF CW

Upper layer					
	Soil respiration	Biomass C	Immobilized N	Potential nitrification	Biomass C:N ratio
Biomass C	–0.87				
Immobilized N	0.89 (S)	–0.89 (S)			
Biomass C:N ratio	–0.92*	0.97*	–1.00(S)/ –1.00(S)		
PS-C			–083 (S)		0.90
BOD_7				0.84	
NO_2-N		0.85			0.81
NO_3-N				–0.89 (S)	
Soil N	0.94*	–083 (S)	0.89 (S)		–0.84
Soil P	0.87	–0.92*	0.94 (S)		–0.91
Soil C	0.81		0.83 (S)		–083 (S)

Lower layer					
	Soil respiration	Biomass C	Immobilized N	Potential nitrification	log Biomass C:N ratio
Potential nitrification		0.94*			
log Biomass C:N ratio			–083		
atan Biomass C:N ratio		0.82			
PS-C		–083 (S)			
PO_4-P					0.82
Total P					0.82
Soil P	0.81				
Soil C				0.92*	

CAPTIONS OF FIGURES

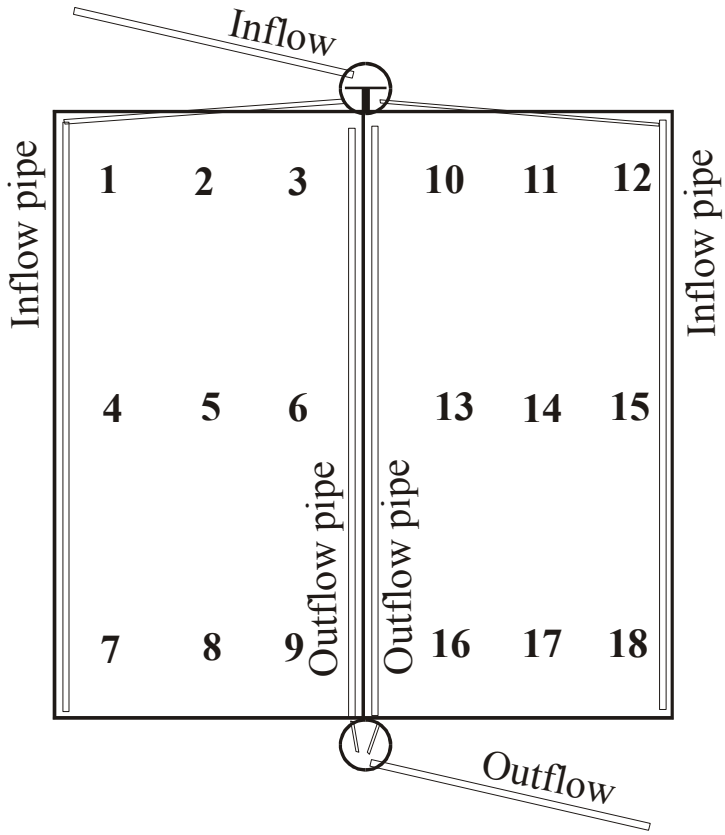

Fig. 1. Schematic overview of the Kodijärve HSSF CW. Numbers indicate water-sampling wells.

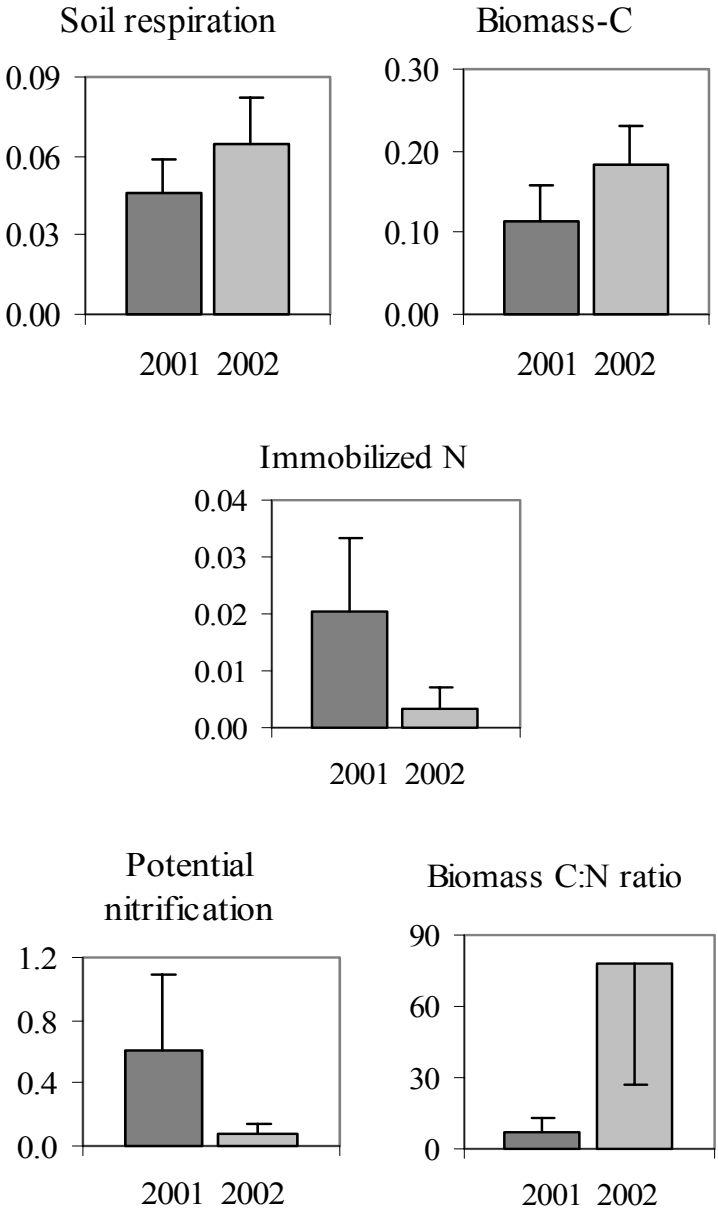

Fig. 2. The average and standard deviation values of soil respiration (g CO_2 kg^{-1} (dm) d^{-1}), biomass C (g kg^{-1} (dm)), immobilized N (g NH_2-N kg^{-1} (dm)), potential nitrification (mg N kg^{-1} (dm) d^{-1}) and microbial biomass C:N ratio in the Kodijärve HSSF CW in 2001 and 2002. $p << 0.01$, soil respiration and biomass-C were analyzed with paired t-test, the other microbial characteristics were analyzed with Wilcoxon Matched Pairs Test.

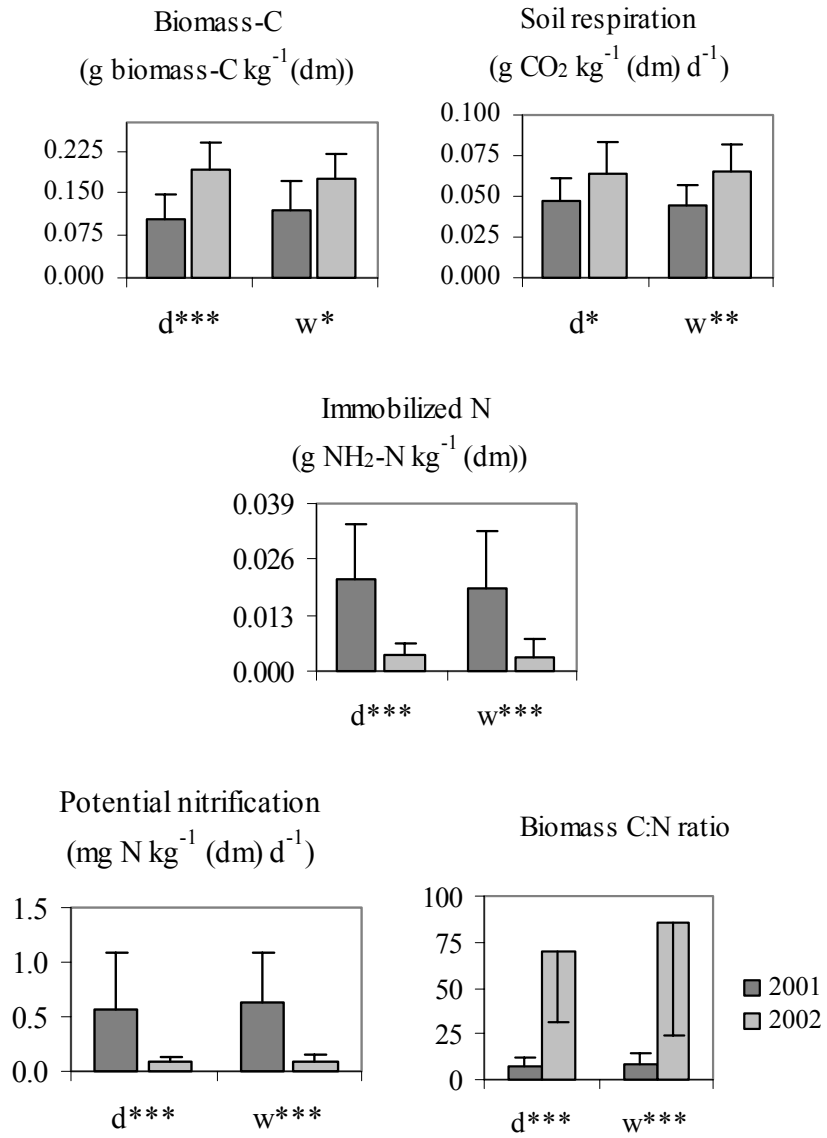

Fig. 3. The average and standard deviation values of microbial characteristics in the dry bed (d) and wet bed (w) of the Kodijärve HSSF CW in 2001 and 2002. The detected significant differences of the respective values of the years are indicated as * for $p<0.05$, ** for $p<0.01$ and *** for $p<<0.01$. For the soil respiration and biomass-C the paired t-test, for the other microbial characteristics the Wilcoxon Matched Pairs Test was used.

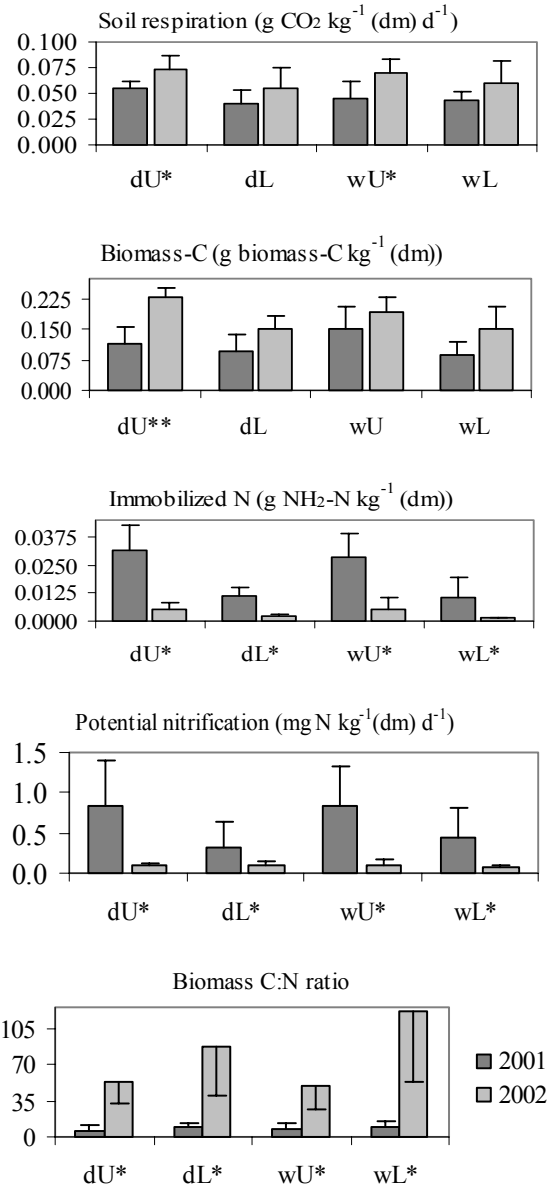

Fig. 4. The average and standard deviation values of the soil respiration (g CO_2 kg^{-1} (dm) d^{-1}), biomass-C (g biomass-C kg^{-1} (dm)), immobilized N (g NH_2 kg^{-1} (dm)), potential nitrificaton (mg N kg^{-1} (dm) d^{-1}) and biomass C:N ratio in the upper (U) and lower (L) layer in the dry bed (d) and wet bed (w) of the Kodijärve HSSF CW in 2001 and 2002. The detected significant differences of the respective values of the years (paired t-test for biomass C, for the rest – Wilcoxon Matched Pairs tests) are indicated as * for $p<0.05$ and ** for $p<<0.01$.

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