Desalination and Water Treatment
www.deswater.com

doi: 10.5004/dwt.2018.22840

134 (2018) 188–198
December

Evaluation of seasonal activity of various bacteria in a constructed wetland using AT4 and TTC tests

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Received 2 March 2018; Accepted 22 July 2018

ABSTRACT

The article presents the results of research studies aimed at the application of AT4 (respiration activity) and TTC (dehydrogenase activity) tests to assess the seasonal variability of the efficiency of a constructed wetland wastewater treatment plant. The research was carried out on the site, serving a single-family house inhabited by three people. The test results clearly showed that the wastewater treatment plant complies with the legally required quality requirements for discharged treated wastewater, yielding an average of 98% removal efficiency of organic biodegradable compounds (BOD_5) and 93% of organic pollutants expressed in the COD parameter. The efficiency of biogen removal was relatively low, in the range characteristic for this type of systems 13%, respectively, for total nitrogen and total phosphorus. The use of the polishing pond allowed in summer to reduce the total nitrogen content in treated sewage by nearly 50%. In the winter, the polishing pond played mainly a retention role, not contributing to increasing the efficiency of the entire system. The microbiological activity tests in relation to the system effectiveness were based on respiration (AT4) and dehydrogenase activity (TTC). A proprietary solution was used to enable non-invasive sampling of soil samples from the interior of the filtering filtration material, which allowed the obtention of an image of the stratification of microbial activity. The oxygen demand of the filtration material in the summer was about 50% higher than in the autumn and winter period. The obtained results showed a seasonal change in the distribution of microbial activity inside the filtration material, reflecting the adaptive abilities of the filtration material's microfauna to the variability of thermal conditions. It was found that in the winter period, the wetland filtration material, which was originally a system implementing oxygen processes, goes into anaerobic state, without reducing the efficiency of organic compound removal.

Keywords: Constructed wetlands; TTC – dehydrogenase activity; AT4 – static respiration index; Microbiological activity

1. Introduction

Household sewage treatment plants are gaining more and more popularity, and the technologies offered on the market are very diverse in terms of price and the achieved environmental effect. The main criterion for choosing the system is the price and issues related to the maintenance of the object. In recent years, it is noted that the popularity of the well-known constructed wetland system for its conditions of low price, reliability and simplicity of operation has been growing.

Three basic construction and technological solutions for wetlands were developed based on the mechanisms present in wetland ecosystems, but differing in the way of supplying and distributing wastewater in the plant–soil system. The treatment plants can be constructed to ensure the flow of sewage over the surface of the plant–soil system (Free Water

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Presented at the 13th Conference on Microcontaminants in Human Environment, 4–6 December 2017, Czestochowa, Poland.

Surface Constructed Wetland – FWSCW) or under its surface (Vegetated Submerged Beds – VSB) (Figs. 1(A) and (B)).

In FWSCW systems, wastewater forms a water table on the surface of a channel or a reservoir inhabited by an appropriate species of hydrophytic vegetation. It is a system that is a full mapping of the processes of self-cleaning of water reservoirs additionally supported by the appropriate selection of flora. The filtration mechanism in this technology is limited to the minimum, giving way to sedimentation. Most systems maintain forced sewage flow. The parameter that is of key importance for the efficiency of operation of the surface treatment plant is retention time and ambient temperature [2,3].

Another solution is the vertical flow constructed wetland (VFCW) vertical flow construction plant (Fig. 1(C)), which uses wastewater filtration mechanisms, that intensifies the processes of separation of solid particles from wastewater and biological treatment by creating a more favorable environment for the formation of biological membranes. The filtration layers can be fed in a vertical or horizontal system, which leads to different conditions of removing particular impurities. Forcing the flow of sewage in the direction from top to bottom of the filtration layer, relieves the contact surface, but above all it ensures simultaneous oxygenation of wastewater, which affects the intensification of the nitrification process and improves the process of aerobic decomposition of organic compounds [2].

An extremely important role in the process of wastewater treatment in constructed wetland treatment plants is performed by microorganisms that colonize the filtration material. Constructed wetlands, considered as one system are characterized by enormous biodiversity, which is largely due to the fact that in a single purification system there is a whole range of environmental conditions. Depending on the technical solutions used in wetlands treatment plants, it is possible to obtain a high variability of oxidation–reduction conditions, and the potential of Eh varies in the range from +700 to –400 mV [4]. The value of this indicator is largely determined by the degree of water saturation, the type of filling medium and the quality of sewage, which determines the presence of electron acceptors such as NO_3, SO_4 and O_2. In the case of constructed wetlands, it can be assumed that the limit of oxidation conditions (Eh = from 250 to 700 mV) and reduction (Eh = from –400 to 250 mV) determine the level of saturation of the system. The factors differentiating the stratigraphic distribution of the population inside the filtration material are, furthermore, temperature and pH as well as the availability of nutrients.

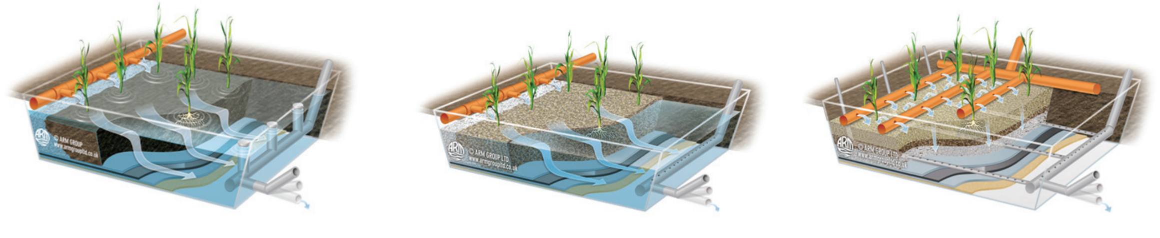
In the surface layer 10 cm thick bed, microorganisms constitute 95% of the total amount. The dominating population is fungi that do not actually affect the biodegradation of pollutants [5]. The mass fraction of bacteria increases with depth, which becomes dominant only at a depth exceeding 20 cm. In the case of classic sand filters, a depth of 20 cm is also a conventional boundary for the effective removal of organic compounds, above which significantly enzymatic activity with respect to nitrogen compounds (urease) and phosphorus (phosphatase) is noted.

Factors affecting microbiological activity in wetlands are primarily the organic matter and nutrient content of sewage, the presence of filling materials with a porous structure ensuring adequate microbial development and temperature [4]. Other factors such as oxygenation of the bed or the degree of saturation of the filtration material with sewage lead to further selectivity of the population, defining the system's strict orientation toward the purification of selected pollutants. A significant part of microbial activity occurs in the rhizosphere zone; hence, the interactions between plants and the bacterial flora of the filtration material can significantly affect the efficiency of the treatment plant [6–8].

In the case of the VFCW treatment plant, the population of bacteria is dominated by the type of proteobacteria, with a much smaller population of bacteria such as *Cytophaga-Flavobacterium*, Actinobacteria and Firmicutes. Nitrifying bacteria (e.g., *Nitrosococcus oceani*, *Nitrosococcus halophilus*, *Nitrosomonas*, and *Nitrosospira* spp.) [9] and to a lesser degree the denitrifying (e.g., Bacillus, Enterobacter, Micrococcus, Pseudomonas, and Spirillum) [10] are crucial for the wastewater treatment processes. The research also shows the presence of anammox in the filtration bed [11]. In the VFCW type of treatment plants, the presence of extremophiles from the group of acid bacteria was also observed [12]. Taking into account the microbiological diversity of wetlands, they can compete with activated sludge systems [12,13].

In recent years, new challenges are put forward on how to enhance the efficiency of wastewater treatment technology. Microbiological activity as well as temperature is important for biological wastewater treatment on account of its effect on biochemical reactions in many ways, such as reaction rates or reaction pathway [14–16].

Microbiological tests in wetlands are carried out in terms of optimizing design assumptions so as to create environmental conditions ensuring maximum microbiological activity. The directions of research concern the species composition, microbiological activity and the interaction of the population of microorganisms and environmental



(a) (b) (c)

Fig. 1. (A) Free water surface constructed wetland. (B) Horizontal flow constructed wetland. (C) Vertical flow constructed wetland [1].

conditions. Depending on the orientation, microbiological tests are carried out on the basis of classical breeding methods or molecular methods. In the case of diagnostic tests aimed at determining the presence of a given species, the methods of direct measurement (counting microorganisms in the direct preparation or counting microorganisms in the Thoma chamber) or breeding methods are sufficient.

Analysis of microbial activity requires the use of molecular analysis methods (e.g., based on DNA extraction) allowing the quantification of metabolic or enzymatic activity and its targeting, as well as detailed interactions between given populations [17].

The determination of mitochondrial metabolism and the activity of the respiration chain of cells in response to environmental conditions is most often performed using colorimetric tests using tetrazolium salts (TTC, INT, MTT, FDA, etc.) [17,18]. Also popular in this area are methods allowing to analyze the intensity and volume of gas emissions (CO_2, N_2, CH_4) that are the products of cellular respiration processes [19,20]. The course of the transformation path of individual pollutants can be carried out by means of isotopic labeling, especially in relation to organic compounds and nitrogen. Behavior and characteristics of specific populations are analyzed using CLPP (metabolic profile analysis), PFLA (analysis of phospholipid fatty acids), FAME'S (analysis of fatty acid methyl esters) or PCR (analysis of polymerase chain reactions) [14,21].

As a valuable indicator to evaluate the microbial viability, dehydrogenase was largely reported on the important role in environmental remediation. TTC has also been used as an indicator of the health of the microbial community [8,14,17].

The article presents the results of research studies aimed at the application of AT4 and TTC tests to assess the seasonal variability of the efficiency of a constructed wetland wastewater treatment plant. The research was aimed at expanding knowledge about microbial activity in hydrophyte fields operating under moderate climate conditions. Determining the changes taking place in the filter bed will allow for optimal design, taking into account the characteristics of filtration fillings dedicated to the removal of selected pollutants. The test results can be used to develop a new design method, different from traditional rules based on a constant size of the infiltration surface regardless of the filling material used.

2. Research methodology

2.1. Researched object

The research was carried out in a vertical constructed wetland treatment plant designed based on a patented solution [22]. The household sewage treatment plant consists of a system connected in series: a polyethylene settler with a capacity of 2 m^3 (1), a pumping station with a submerged pump (2) and an elevated root filter connected (3) by a drainage system with a polishing pond (4) (Fig. 2). The root filter was made in a system of three filtration layers. The 20 cm thick top layer is chipped pinus bark. The middle, 50 cm thick layer was filled with medium sand and successively 20 cm with a layer of river gravel rinsed with a grain size of 20–50 mm. The surface of the filter is overgrown with the sedge (*Carex nigra*). The filter construction was raised 90 cm above the ground level, allowing the gravitational drainage of treated wastewater to the polishing pond. The filter bed was isolated from the ground with a 2 mm thick foil. The entire installation covers a total of 49 m^2, of which the filter construction is 31.5 m^2, and the joint is 17.5 m^2 (Fig. 3).

Based on the readings of water consumption in the household, the average daily flow of the treatment plant was 0.4 m^3/d or calculated per 1 m^2 of the filtration material of 12.7 L/m^2·d. The filter bed is supplied in the pressure system with wastewater subjected to pre-treatment in a settling tank. Wastewater dosing is carried out irregularly, depending on the filling of the pumping station, with the pump being switched on each time after filling the tank with approximately 75 L. Thus, the filtration material is supplied from five to six times a day.

In the assessment of the results, the water balance of the object covering atmospheric precipitation and evapotranspiration was not taken into account, because it can be assumed that the values of these parameters are comparable:

Fig. 3. View on the constructed wetland system (Photo by F. Bydałek).

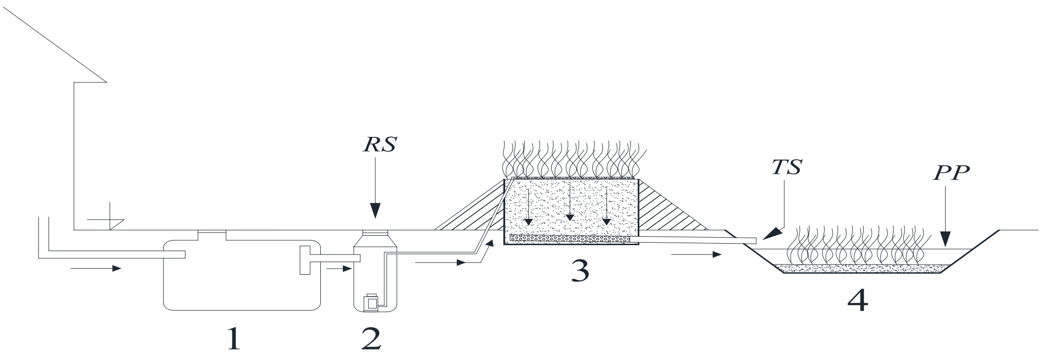


Fig. 2. Cross section of treatment system analyzed in the research ((1) – settler, (2) – pumping station, (3) – root filter, (4) – polishing pond, RS – raw sewage, TS – treated sewage, PP – sewage from polishing pond).

- Average size of atmospheric precipitation for the area of the treatment plant [23,24], approximately 575 mm/m^2·y, which at the surface of the water treatment plant (bed, clarifying pond) of 49 m^2, gives approximately 29 m^3/y of precipitation, which goes to the surface of the treatment plant;
- Evaporation from the surface of water varies greatly depending on the region of Poland and temperature. In the literature [23,24], a range from 500 to 1,000 mm/m^2·y is given. For the location of the research facility (Lubuskie Voivodeship), this value is 500 mm/m^2·y. However, evaporation from surfaces covered with hydrophytes takes values from 1,000 to 2,000 mm/m^2·y [24]. Thus, assuming the minimum values of evapotranspiration (500 and 1,000 mm/m^2·y), the evapotranspiration determined for the analyzed wetlands treatment plant is about 33 m^3/y.

2.2. Sewage sampling and efficiency analysis

The cleaning efficiency characterizing the bed-purifying pond system was determined based on the results of analytical studies in the sewage quality monitoring system at three measurement points: raw sewage (RS), sewage treated in the filtration material (TS) and sewage purified in the pond (PP) (Fig. 2). During the sampling, the temperature of sewage at each measurement point was measured, as well as the air temperature and temperature at a depth of 30 cm inside the filter bed. In total, 16 samples were taken within 6 months.

The samples of sewage are COD (PN-ISO 6060: 2006), BOD_5 (respiration method), TKN (PN-EN 25663), ammonium nitrogen (PN-C-04576-4), nitrate nitrogen (PN-82/C-04576.08) and general phosphorus (EN-ISO 6878: 2004). The total nitrogen content was calculated as the sum of nitrogen compounds present in the wastewater.

The effectiveness of wastewater treatment was calculated taking into account the characteristics of raw sewage and the next measurement point.

2.3. Collection of filtrating material's samples

In order to determine the microbiological activity characterizing individual filtration layers in a given layer system, samples were taken from the lower part of each filter layer. Due to the dynamics of changes in microbial activity that take place in the bed profile, it was necessary to establish a collection point that would be easy to relocate repeatedly. The presence of an isolation foil in the bottom of the filter bed precludes the possibility of drilling for the purpose of sampling. For the needs of the study, a solution was developed that allows for quick and reproducible sampling from the required points determined in the bed profile. A modular piezometer was constructed, consisting of three disjoint segments – each with a length corresponding to the given filtration layer. The performance of the walls from the permeable mesh allowed to maintain the contact conditions between the filtration material in the piezometer and the surrounding material of the filtering material. Thus, it was possible to obtain reliable values of microbial activity in the piezometer reflecting the conditions prevailing in the filtration material. For the needs of the study, three piezometers were made that allowed the sampling of differentiated wastewater loads. The collected samples were averaged, packed into hermetically sealed plastic bags and transported to the laboratory in conditions similar to the temperature prevailing in the filter bed. Humidity, respiration activity and dehydrogenase activity were determined in the samples.

2.4. Research on microbiological activity

2.4.1. Respiration activity – AT4 test

The study of the respiration activity of the bed of the constructed wetlands was carried out based on the AT4 indicator using the OxiTop Control system. The OxiTop Control system allows a manometric measurement of oxygen consumption. The sample reacts biologically of decomposition of organic compounds with the release of CO_2 i H_2O. Mineralization of organic matter is the result of dissimilatory metabolism. Released in the breathing process, carbon dioxide is absorbed by sodium hydroxide solution (1 M NaOH). Thus, in closed conditions, that is, the measuring vessel, there is a pressure drop, which is then registered by a manometric sensor. Pre-weighed samples of 100 cm^3 materials were used for the measurement. At the same time, the moisture content of the material was measured. The samples were incubated in a thermostatic cabinet.

On the basis of the manometric sensor readings, the respiration activity index expressed in mgO_2/g dm was calculated during 4 d [25]:

$$\mathrm{BA} = \frac{\mathrm{M_R(O_2)}}{R \cdot \mathrm{T}} \cdot \frac{V_{\mathrm{fr}}}{m_{\mathrm{BT}}} \cdot \Delta p \tag{1}$$

where BA – respiration rate of the sample (mgO_2/g dm); $M_R(O_2)$ – molar mass of oxygen (3,200 mg/mol); V_{fr} – gas volume (L); R – gas constant (83,141 mbar mol^{-1} K^{-1}); T – temperature (K); m_{BT} – dry mass of the sample in the measuring set (g dm); Δp – pressure drop in the measuring set (hPa). where V_{fr}:

$$V_{\mathrm{fr}} = V_{\mathrm{ges}} - V_{\mathrm{AG}} - V_{\mathrm{AM}} - V_{\mathrm{Bf}} \tag{2}$$

where V_{fr} – gas volume (L); V_{ges} – total volume of the vessel (L); V_{AG} – the capacity of the absorbent vessel (L); V_{AM} – absorbent capacity (L); V_{Bf} – volume of the wet sample (L).

2.4.2. Dehydrogenase activity – TTC test

Dehydrogenases are indicators of microbial activity, because they are intracellular enzymes present only in the cells of living organisms [26], additionally not accumulating in the environment. Dehydrogenases form a very broad subclass of oxidoreductases, characterized by high specificity and selectivity.

The TTC method developed on the basis of the methodology of Casida et al. [27] was used in the research. Six grams of the material sample was incubated at 30°C for 20 h in the presence of 2,3,5-triphenyltetrazolium chloride (TTC). TTC acts as an acceptor of electrons and protons, replacing other naturally occurring acceptors, that is, mainly NO_3 and CO_2 (the material is flooded with water). During incubation, TTC is enzymatically reduced to the color form of triphenylformazan (TPF). The dehydrogenase activity in the sample is measured as the amount of formazan produced by the mass unit of the sample per unit of time. Formazan measurement is performed spectrometrically at a wavelength of 485 nm, after previous ethanol extraction. At the same time, control tests are carried out without the addition of TTC. The dehydrogenase activity can be calculated according to the formula [28] given below:

$$\mathrm{TTC} = \frac{(\mathrm{S} - \mathrm{C}) \cdot \mathrm{V} \cdot 100}{6 \cdot 300.4 \cdot \%\mathrm{d.m.}} \tag{3}$$

where TTC – dehydrogenase activity, amount of triphenylformazan produced by 1 g dm samples within 20 h (μmol TPF/(g dm·20 h); S – TPF concentration in the test sample (μg/cm^3); C – reading from the standard curve for the control sample without TTC (μg/cm^3); V – total volume of added solutions (i.e., TTC solution, water and alcohol) (cm^3); 6 – weight of filling material sample (g); 300.4 – molecular weight of TPF; 100% dm^{-1} – conversion factor for the dry mass of the sample.

For respiration and dehydrogenase activity, on the basis of the unit values of these indicators per gram of dry matter of the filling material, the cumulative activity for each layer was determined.

3. Test results

3.1. Seasonal variability of meteorological conditions

The research was carried out from August to January. Thus, the obtained results reflect the work conditions of the treatment plant in the summer-autumn-winter transition cycle. Measured values of air temperature at the ground are reflected in seasonal fluctuations of thermal conditions in which the treatment plant operated. In the summer period (from August to September) the average value of the ambient temperature was 25.8°C, in autumn (from October to December) 8°C, and in winter –2.7°C (until the end of January). In summer, temperature fluctuations in the filtration material were recorded (measurement in the filter at a depth of 30 cm) in the range from 17.5°C to 23°C (average 19.8°C), autumn in the range from 6°C to 13°C (mean 9.4°C), while in winter from 2.3°C to 3.1°C (average 2.6°C). The temperature of raw sewage in the pumping station ranged from an average value of 23°C in the summer to 8°C in the winter. The course of seasonal variability of air temperature, raw sewage, treated sewage, water of the polishing pond and temperature in the filter prevailing at a depth of 30 cm is shown in Fig. 4.

3.2. Effectiveness of wastewater treatment

Effectiveness of sewage treatment is shown in Table 1.

There were no statistically significant relationships between the load of pollutants brought to the treatment plant and the quality of treated sewage, which proves the high retention capacity of the system. In the winter season, the highest values of COD indicator were recorded in treated sewage, despite the reduced COD load delivered to the filtration material. The winter period was characterized by a decrease in efficiency to below 90%, with the average value still being satisfactory (88%). In the summer and autumn period, the efficiency of work remained at a level exceeding 90%, with an average of 95%.

Biodegradable organic compounds accounted for 70% of the pollutant load expressed in COD, the ratio being subject to irregular changes ranging from 50% to 90%. In the polishing pond, an increase in pollution expressed by BOD_5 to the value of 100 mgO_2/L was noted. Regardless of the season,

Table 1
Effectiveness of sewage treatment

Parameter	Effectiveness (%)			
	Average	Minimum	Maximum	Standard deviation
COD	93	88	99	0.0442
BOD_5	98	96	99	0.0102
Total nitrogen	13	–5	33	0.0989
Total phosphorus	13	4	26	0.0522

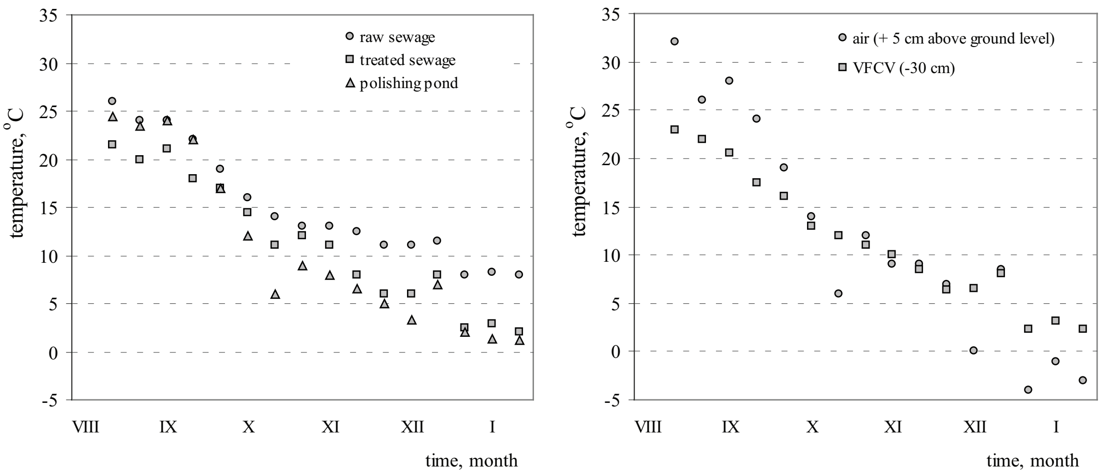


Fig. 4. Seasonal variability of temperature measured for influent, effluent and water in polishing pond as well as air temperature and thermic conditions 30 cm inside the filter bed.

the treatment plant provided at least 96% efficiency of BOD_5 removal, with an average value of 98%.

The lowest values of the total nitrogen were recorded in the winter. The average efficiency of this parameter removal was 13%. The effectiveness of the treatment plant was irregular. The lowest values of total nitrogen concentrations in the examined water from the polishing pond were recorded in the summer period (55–75 mgN/L), which gradually increased in autumn, stabilizing at 80–90 mgN/L in the winter period.

The average content of total phosphorus in treated wastewater was 19.9 mgTP/L, which is a 13% reduction compared with the content in raw sewage. The presence of phosphorus in treated sewage did not show seasonal dependence. There was no case of phosphorus release from the filtration material, which would indicate the depletion of the sorption capacity of the system. In a few cases, the phosphorus content in the water of the pond was higher than in the treated wastewater. These results could indicate an intensification of the biomass decomposition process from the pond, as a result of release of phosphorus previously accumulated in the tissues.

3.3. Moisture of the material

The top filtration layer was characterized by the highest moisture (average 66%) due to the specificity of easily absorbable material (bark) (σ = 0.04). Moisture of the bottom filtration layers was in the range of 6% to 15% and from 2% to 6% for sand and gravel, respectively (Table 2).

3.4. Respiration activity – AT4 test

The determined values of respiration activity of the tested materials were characterized by a large discrepancy (Table 3). The average AT4 value for bark was 4.64 mgO_2/g dm (σ = 2.13), and the measured values ranged from 2.31 to 9.15 mgO_2/g dm. Respiration activity measured in the sand and gravel layer was on average about 10 times smaller than in the bark layer and amounted to 0.61 mgO_2/g dm, and 0.34 mgO_2/g dm, respectively, in the ranges from 0.20 to 1.39 (sand) and from 0.07 to 0.77 mgO_2/g dm (gravel). The skewness factor, which maps the characteristics of the distribution of measurement results, is comparable, which suggests the absence of extreme factors that could disrupt the seasonal course.

Table 2
Water content of layers material

Material	Average	Standard deviation	Minimum	Maximum	Skewness
Bark, %	66	0.04	59	73	0.04
Sand, %	9	0.02	6	15	1.56
Gravel, %	3	0.01	2	6	1.34

3.5. Dehydrogenase activity – TTC test

As in the case of respiration activity measurements (AT4), the obtained values of the dehydrogenase activity indexes differed significantly (Table 4). The organic material, which is bark, was characterized by an average value of 31.71 μmol TPF 20 h^{-1} g^{-1} dm while maintaining a relatively homogeneous distribution over the measurement period (σ = 5.97) ranging from 23.41 to 42.54 μmol TPF 20 h^{-1} g^{-1} dm. Measurement of the TTC activity of mineral materials, that is, sand and gravel, showed that the unit value was 50–100 times smaller. The average value measured for sand was 0.73 μmol TPF 20 h^{-1} g^{-1} dm with a large seasonal inequality (σ = 0.28). A further decrease in value was obtained for gravel, with an average of 0.30 μmol TPF 20 h^{-1} g^{-1} dm with a high standard deviation of the measured samples (σ = 0.20).

4. Discussion

Seasonal temperature variability affected the respiration activity of bark, gravel and sand to the same extent, reducing in the autumn and winter the oxygen demand by about 50% in each case (Fig. 5). Since the quality of raw sewage did not change significantly, it should be assumed that the thermal conditions prevailing in the filtration material were the determining factor. Interesting is the lack of differences in respiration activity (AT4) measured in the autumn (October – December) and winter (January). The average daily winter air temperature is close to 11°C lower than in the autumn. On the other hand, the temperature inside the filter measured at a depth of 30 cm showed that in autumn-winter season the average temperature dropped by approximately 7°C,

Table 3
Results of respiration activity measurement (AT4)

Material	Average	Standard deviation	Minimum	Maximum	Skewness
Bark, mgO_2/g dm	4.64	2.13	2.31	9.15	1.02
Sand, mgO_2/g dm	0.61	0.36	0.20	1.39	1.17
Gravel, mgO_2/g dm	0.34	0.22	0.07	0.77	0.85

Table 4
Results of dehydrogenase activity measurement (TTC)

Material, μmol TPF/20 h·g dm	Average	Standard deviation	Minimum	Maximum	Skewness
Bark	31.71	5.97	23.41	42.54	0.40
Sand	0.73	0.28	0.43	1.46	1.44
Gravel	0.33	0.20	0.14	0.85	1.61

while in the winter there were always conditions above 0°C inside the filter, despite the negative air temperatures. This testifies to the good thermal insulation of the filtration material. The source of heat was not only the wastewater supplied (in the winter the average temperature of raw sewage in the pumping station was 8°C), but also exothermic processes of decomposition of organic matter contained in wastewater. It is therefore visible that the use of an appropriate insulation layer results in a more stable system operation.

The full picture of the respiration activity of microorganisms was obtained after converting the unit specific values for the bedding material into the total activity of a given layer (Fig. 6; Table 5).

The accumulated values of respiration activity of the filtering material reflect the tendency observed for unit indices (Fig. 6(B)). In summertime, cumulative respiration activity was on average two times higher than in autumn and winter. It is significant, however, that the proportion of the respiration activity of individual layers has not changed over the 7 months of monitoring. Irrespective of the season, microorganisms inhabiting the bark layer were responsible for 20%–30% of the total oxygen demand in the filtration material (Fig. 6(A)), and the sand layers for 50%–60%. It should be noted that among the three layers of the hydrophyte filter, this is the layer characterized by far the largest retention time, due to both the thickness of the layer and the filtration coefficient,

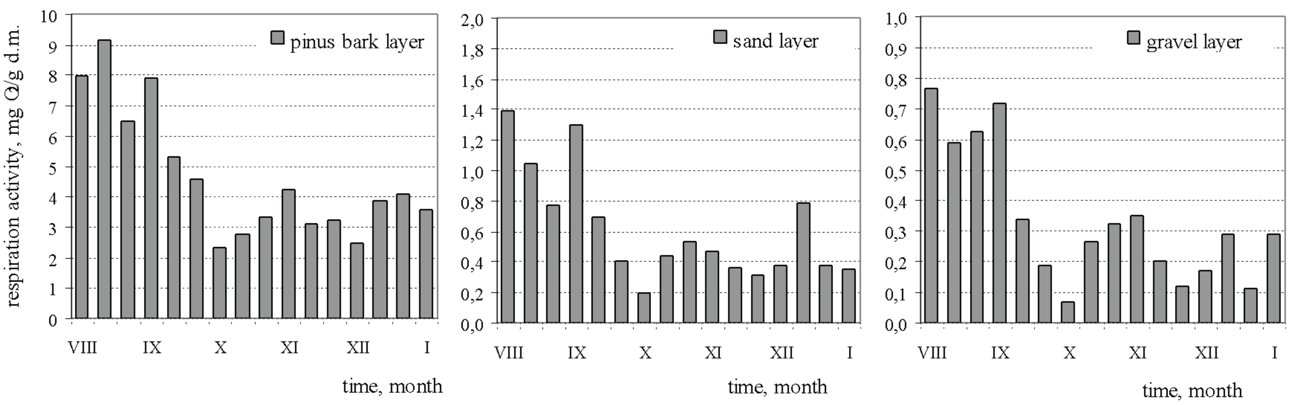


Fig. 5. Seasonal changes in respiration activity of layers materials.

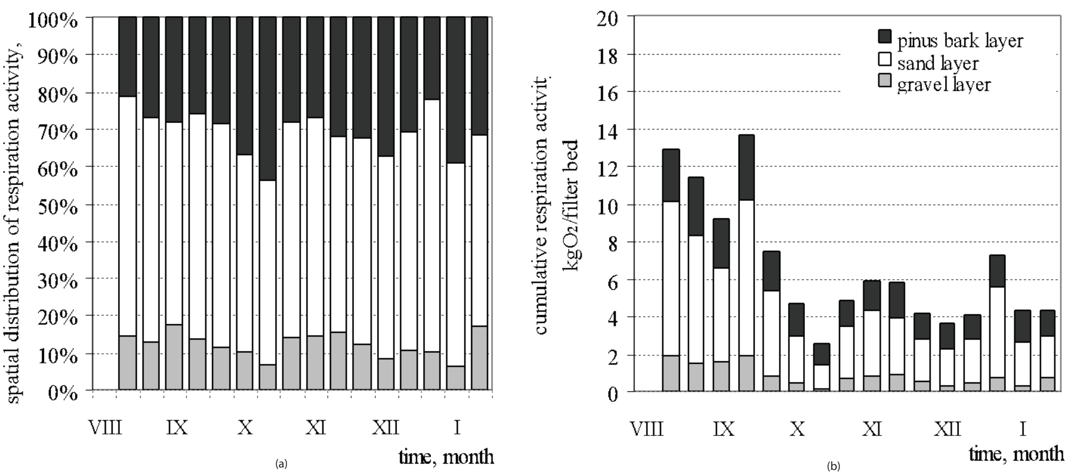


Fig. 6. Spatial distribution of respiration activity (A) and cumulative respiration activity of filter bed (B).

Table 5
Cumulative respiration activity for filter bed layers

Material, kgO_2/layer	Average	Standard deviation	Minimum	Maximum	Skewness
Filtration material	6.63	3.44	2.54	13.69	1.05
Bark	1.90	0.73	1.11	3.54	1.14
Sand	3.87	2.23	1.26	8.29	1.07
Gravel	0.86	0.55	0.17	1.89	0.82

which for the layer with a fine-grained structure will be many times larger than in the case of bark or gravel layer. Thus, conditions are ensured for the availability of food as well as the stability of environmental conditions, which is preferred for increased microbiological activity. Interestingly, despite the highest exposure to unfavorable weather conditions, microorganisms inhabiting the bark layer in the winter period still maintained a constant respiration activity. Even respiration activity, regardless of the season and temperature changes, confirms that vertical hydrophilic purification plants create good conditions for maintaining the aerobic activity of microorganisms in a large part of the bed profile. The mechanism of oxygen supply to the interior of the treatment plant is associated with molecular diffusion and gas exchange through the rhizomes and roots of plants [29,30]. In the case of the tested sewage treatment plant, in the winter the plant cover was cut, triggering another important process – air injection through the stems of dead plants as a result of the Venturi effect [31,32]. In addition, the installation of drainage pipes in the bottom, connected to the exhaust fireplace and the open outlet pipe, additionally caused the oxygenation of the particularly bottom filtration layer, that is, gravel.

The values of the static respiration index (AT4) reflect the total microbial activity in the bed, a method that is not selective for populations of microorganisms oxidizing organic compounds. Using a measurement of enzymatic activity focused on bacterial populations related to the oxidation of organic compounds, a much fuller picture of the microbial activity in the treatment plant was obtained. Dehydrogenase activity (TTC) (Fig. 7) showed significant variability in the seasonal approach, in contrast to observation of the AT4 index, but this course was different for individual materials. In the summer and autumn periods, the unit activity of dehydrogenases measured for the bark layer was maintained in the range from 12 to 16 μmol TPF 20 h^{-1} g^{-1} dm. In the winter period, the measured values decreased by 50%. On the other hand, for sand and gravel, a stable dehydrogenase activity was observed in the summer and autumn period, and in the winter it doubled. Such a situation should be explained by the stratification of microbial activity in vertical filtration material. The most effective oxygen degradation of organic matter takes place in the surface layer of 40 cm of the profile [33,34]. Partial freezing of the bark in the winter caused a decrease in the microbiological activity of this layer, which resulted in a reduction in the efficiency of organic compounds removal in the first 20 cm of the bed profile. Thus, the lower filtration layers received a greater amount of nutrients which stimulated the growth of heterotrophic bacteria associated with the oxidation of organic compounds, what is reflected in the increase in the activity of the sand and gravel layer.

The dehydrogenase activity should be analyzed in relation to the cumulative values of the activity of individual layers (Fig. 8). It is characteristic to obtain stable activity of the entire filtration material, which was maintained at the same level regardless of the season and temperature. This fact also explains the year-round stability of the treatment plant in terms of removing organic compounds expressed in BOD_5, and partly COD. The increase in the enzymatic activity of dehydrogenases in the lower filtration layers was accompanied by a decrease in respiration activity recorded during the winter period. Thus, it can be hypothesized that in the analyzed period, the importance of anaerobic organic compound removal processes increased. The efficiency of degradation of organic compounds under anaerobic conditions is several times lower than that of aerobic bacteria, which in turn explains the decrease in the efficiency of removing organic compounds expressed in COD, which include difficult biodegradable high molecular organic compounds whose biological degradation is very energy consuming. The occurrence of periodic anaerobic conditions was also confirmed during sampling, when the layers of sand at a depth below 0.5 m were colored black, which is a characteristic of the environment with reduction conditions (mainly sulfur).

The results of the research allowed to assess the impact of the variability of microbiological activity on the efficiency of the treatment plant. The carbon cycle in the constructed wetland plant reflects not only the mechanisms of removing organic compounds but also other non-organic biodegradable compounds, in particular nitrogen compounds. The presence of coal, largely coming from sewage, affected processes such as breathing, fermentation, denitrification, iron and sulfur reduction, or methanogenesis [4].

Constructed wetlands, due to the specificity of the environmental conditions, thanks to their design, enable the launch of a number of nitrogen removal mechanisms. However, in contrast to the removal of most organic compounds, phosphorus or heavy metals, the transformation process of nitrogen compounds takes place in several stages, which requires a high flexibility of systems, consisting in the variability of oxygenation conditions of the filtration material.

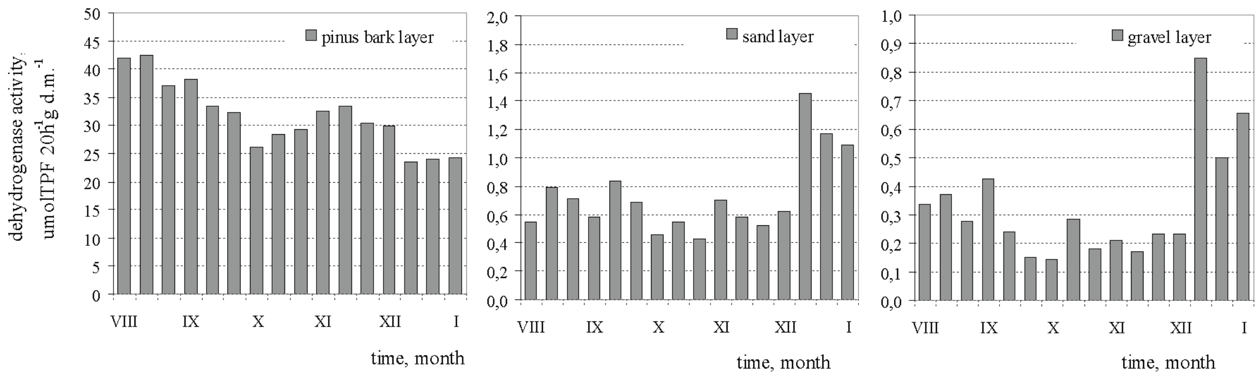


Fig. 7. Seasonal changes of dehydrogenase activity of layers materials.

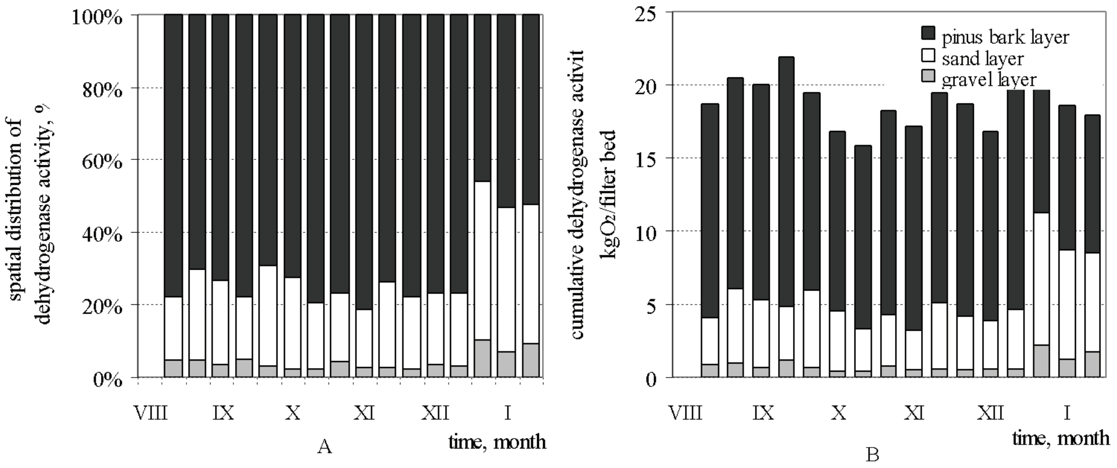


Fig. 8. Spatial contribution of dehydrogenase activity for different filtration layers (A) and cumulative dehydrogenase activity measured in filter bed (B).

The tests confirmed that the VFCW system solutions do not provide effective nitrogen removal due to the lack of appropriate conditions to run the full nitrification and denitrification mechanism. These observations are in line with the results of tests carried out in field and laboratory conditions [35,36].

Among the several nitrogen transformation mechanisms in VFCW type wastewater treatment plants, nitrification is the dominant process [35]. In vertical flow systems, the nitrification process is often inhibited by the highly effective mineralization of organic matter, which consumes the majority of available oxygen [37]. In practice, effective nitrification takes place only at a depth of less than 40 cm [33], because in the upper part of the profile the processes of oxidation of organic compounds predominate. VFCW systems are characterized by a high degree of aeration, but when they are fed into wastewater containing a large amount of organic matter [38] or in the conditions of filter clogging [39], there is a significant change in oxygen consumption.

The filling of the filtration material in the tested object consisted of materials lacking high sorption capacity and ion exchange capacity, which is the potential of a given system to remove the phosphorus compound [35]. According to the literature data, the sorption capacity of gravel is dependent to a large extent on the calcium content and ranges from 3 to 47.5 gP/kg, which allows to achieve, in the case of domestic sewage, efficiency in the range of 0%–60% [40,41]. The quartz sand used is characterized by much lower rates, sometimes reaching values close to zero (0.058 gP/kg [42]). Therefore, the tested treatment plant was characterized by only 13% efficiency of phosphorus removal, which is not surprising in the light of the values quoted above. It is a value comparable with systems of similar construction, where the emphasis is on the effective removal of organic compounds. Lack of legal requirements regarding the removal of nitrogen and phosphorus discharges in home sewage treatment systems, promotes the use of technologically simpler solutions.

The inclusion of the polishing pond as part of the wastewater treatment chain is a common practice in large installations. Not infrequently, also hydrophyte systems with surface flow are used as the third stage of wastewater treatment [43]. Due to the location in the technological line and the characteristics of the VFCW treatment plant, the pond was to play the role of a denitrifying factor [22,44]. As research has shown, in the summer period, an effective nitrogen removal process was indeed recorded. The content of total nitrogen in the pond decreased by 39% in relation to purified sewage, while the concentration of nitrate nitrogen was reduced by 50%. In the autumn, more than twofold decrease in efficiency compared with the summer period was noted, with the pond still showing a clearly positive impact. No impact was observed only in the winter period, where the reduction of nitrates in medium terms did not occur, while the removal of total nitrogen took place at 6% efficiency. Studies have also shown an increase in the content of organic substances. The BOD_5 value increased on average by 44.7 mgO_2/L, which corresponded to a fivefold increase in the initial value, while the content of organic substances measured with the COD parameter increased by an average of 80.7 mgO_2/L (a twofold increase). The increase in biodegradable substances is the result of an increased activity of plants and microorganisms inhabiting the pond – with presence of numerous nutrients (mainly nitrogen and phosphorus). This may be proved by the fact that the BOD_5 content rapidly decreases in the winter period, approaching the value of the parameter obtained in treated wastewater.

During the monitoring of the sewage treatment plant, no statistically significant dependences between the effectiveness of removing organic compounds (BOD_5 and COD) and respiration activity, expressed as AT4, were found.

Analysis of the dependence of the ammonium removal efficiency and the bark layer dehydrogenase activity showed a significant correlation (Fig. 9; Table 6).

In the course of the research, a significant decrease in the efficiency of NH_4 removal in the winter period was observed. Literature data indicate that the efficiency of nitrification in classic systems of wetlands purifies rapidly under conditions below 10°C [10,45,46]. However, there are hydrophyte systems, adapted to work in low temperature conditions, achieving high (over 70%) nitrogen removal efficiency [47–50], which confirms the possibility of maintaining biological mechanisms for removing nitrogen compounds at low temperatures. Therefore, it cannot be unequivocally indicated

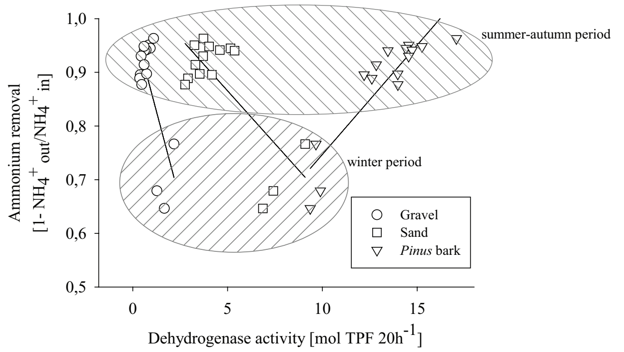


Fig. 9. Impact of dehydrogenase activity on the removal effectiveness of ammonium nitrogen.

Table 6
Correlation of ammonium effectivenes removal and dehydrogenase activity in different filtration layers

Material	Pearson correlation coefficient	Significance
Bark	0.889	0.0000002
Sand	–0.721	0.00242
Gravel	–0.676	0.00562

that the drop in air temperature in the dominant way resulted in a reduction in the efficiency of ammonium ions removal.

Statistical analysis showed a significant linear correlation of the decrease in the efficiency of ammonium ion removal with the increase of dehydrogenase activity in the lower filtration layers. The reason is the shift of the border of microbial activity into the profile, which was dictated by the occurrence of stress factors (temperature) in the top layer. In addition, attention should be paid to reducing the availability of oxygen in the filtration material, resulting from the reduction of the gas exchange area as a result of freezing part of the bark layer and covering the filter with a biomass layer. Reduced plant activity also contributed to lowering the amount of oxygen delivered to the system. Despite unfavorable environmental conditions (low temperature in the filtration material), populations of microorganisms in the layer of sand and gravel were activated due to an increased amount of nutrient loads flowing in, whose transport in summer conditions was limited due to the activity of the bark layer. The structure of the sand layer causing increased sewage holding time increases the time of sewage contact with the biological membrane, which allows to intensify the oxidation of organic compounds. The increase of the oxidation activity of organic compounds in the layer, which was the zone of activity of nitrifying bacteria, leads in the further sequence to the reduction of the depth of the effective nitrification zone, pushing the nitrifying bacteria away from the oxygen saturation zone.

Thus, it can be seen that the seasonal variability of dehydrogenase activity in individual layers reflects the seasonal shifts of the activation boundary of the nitrification process in the filtration profile.

5. Conclusion

Research on the microbiological activity of the filtration material showed that the constructed wetland wastewater treatment plant has a high biological potential. Obtained results of respiration activity also enabled observation of seasonal variability of aerobic species population activity, which determine the ability of the treatment plant to effectively remove organic compounds. The application of the AT4 method to assess the activity of the constructed wetlands has no reference in scientific research, with the unit values obtained being consistent with the results of tests on respiration activity of stabilized landfills. The population of microorganisms inhabiting the filtration material showed great flexibility in adapting to thermal conditions, which was reflected in the dynamics of changes in dehydrogenase activity. The obtained results indicate that the activity of dehydrogenases in the constructed wetlands can be similar to the values found in the activated sludge chambers, as well as systems for purifying gray water in the "green roof" system. It was found that in the case of the assessment of respiration activity, the dominant role was played by microorganisms inhabiting the sand layer, while the dehydrogenase activity was predominantly concentrated in the bark layer.

Simultaneous changes in enzymatic activity along with changes in thermal conditions in the reservoir confirm that these systems can successfully operate in winter conditions, maintaining high biological activity, without the need for additional energy inputs in the form of aeration or reheating.

Literature data clearly indicate that VFCW type treatment plants, with the unsaturated filtration material, are dominated by aerobic microorganism species, while anaerobic species play a marginal role, and their excessive presence can serve as an indicator of reservoir dysfunction. The obtained results indicate, however, that during the winter there was a shift in the treatment of wastewater from groups of aerobic bacteria to anaerobic, which despite unfavorable temperature conditions were able to maintain very high efficiency of the bed for removing organic compounds. An adaptive mechanism of the bed's microfauna was also observed, consisting in lowering the center of microbial activity into the profile in the winter. The fact of occurrence of seasonal migration to the layers distant from the freezing zone indicates , that for the treatment efficiency is very important the mulch layer, the bark in this case.

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