Impact of microwave radiation on nitrogen removal and quantity of nitrifiers in biofilm[1]

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Abstract: The aim of this study was to determine the impact of microwave radiation on the efficiency of nitrification and on the percentage of ammonia-oxidizing bacteria in biofilm and to study the possibility of the occurrence of nonthermal effects caused by the interaction of microwaves and biofilm. Eight trickling filters with a biofilm were used in the experiment: four were exposed to microwave radiation, and four were heated with warm air as a control group. Microwave radiation at a frequency of 2.45 GHz was applied at an intensity of 18 W (0.01 $W \cdot cm^{-3}$ of the reactor packing), which increased the biofilm temperature by 6 °C compared with the ambient temperature. The hydraulic loading averaged 0.30 $m^3 \cdot m^{-2} \cdot h^{-1}$, and the organic loading equalled 1.93 g chemical oxygen demand (COD)$\cdot m^{-2} \cdot d^{-1}$. Microwave radiation had an effect on the concentration of nitrogen compounds in the biofilm, and microwave heating triggered alterations within the biofilm that increased the efficiency of both nitrification and denitrification and the percentage of ammonia-oxidizing bacteria.

Key words: nitrogen removal, wastewater, biofilm, trickling filter, microwave radiation, ammonia-oxidizing bacteria.

Résumé : Cette étude détermine l'impact du rayonnement micro-onde sur l'efficacité de la nitrification et sur le pourcentage de bactéries oxydant l'ammoniac contenues dans le biofilm; elle examine également la possibilité d'effets non thermiques causés par l'interaction entre les micro-ondes et le biofilm. Huit lits bactériens à biofilm ont été utilisés dans l'expérience : quatre ont été exposés au rayonnement micro-onde et quatre ont été chauffés à l'air chaud et servaient de groupe témoin. Le rayonnement micro-onde de 2,45 GHz fourni était de 18 W (0,01 $W \cdot cm^{-3}$ de garniture de réacteur); il a augmenté la température du biofilm de 6 °C par rapport à la température ambiante. La charge hydraulique était en moyenne de 0,30 $m^3 \cdot m^{-2} \cdot h^{-1}$, la charge organique était de 1,93 g DCO$\cdot m^{-2} \cdot j^{-1}$. Le rayonnement micro-onde a changé les composés azoteux. Le réchauffement aux micro-ondes a modifié le biofilm, ce qui a augmenté l'efficacité de la nitrification et de la dénitrification et a accru le pourcentage de bactéries oxydant l'ammoniac.

Mots-clés : élimination de l'azote, eaux usées, biofilm, lit bactérien, rayonnement micro-onde, bactéries oxydant l'ammoniac.

[Traduit par la Rédaction]

Introduction

The electromagnetic spectrum involves microwaves with wavelengths ranging from 1 mm to 1 m and with corresponding frequencies from 300 MHz to 300 GHz. The energy carried by a quanta of microwave radiation is too small to be absorbed by electrons or to excite them to be ejected. In addition, the interaction of microwave radiation with molecules does not result in any changes in their structure.

The literature attributes two types of interactions of microwaves, namely thermal and nonthermal (Porcelli et al. 1997). Thermal effects are related to generating heat as a result of the absorption of microwave energy by water or organic complexes with permanent or induced polarisation. Currently, little is known about the molecular mechanism related to the alleged nonthermal effect, which would require direct transfer of energy from the electromagnetic field to vibrating macromolecules, thus changing their molecular arrangement (Hong et al. 2004). Specific properties of microwaves, particularly their selectivity and ease of initiating and interrupting the process of heating, make it easy to use microwave radiation as a factor affecting the activity of biofilms. Through the use of plastic media and a metal casing for the bioreactor, microwave radiation freely penetrates the bioreactor media without radiating outside. Because the bioreactor is subjected to microwave radiation in intervals between the introduction of waste, radiation is absorbed only by the biofilm. Such an arrangement enables the precise imparting of energy directly to the biofilm.

Ensuring the optimum temperature of sewage treatment is of particular importance in the process of autotrophic nitrifi-

Received 5 September 2008. Revision accepted 19 November 2009. Published on the NRC Research Press Web site at cjce.nrc.ca on 30 April 2010.

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Written discussion of this article is welcomed and will be received by the Editor until 31 August 2010.

[1]A paper submitted to the Journal of Environmental Engineering and Science.

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cation. The relationship between a reaction rate and temperature is expressed by the Van't Hoff – Arrhenius equation. In modelling the processes of wastewater treatment, a transformed form of this equation is applied: $k_{T_1} = k_{20}\theta^{(T_1-T_{20})}$, where k_{T_1} is the reaction rate constant at temperature T_1 in variable units depending on the reaction order, k_{20} is the reaction rate constant at a temperature 20 °C, θ is the temperature index, and T_1 and T_{20} are temperatures. Kadlec and Reddy (2001) showed that the nitrification reaction is highly temperature sensitive and ceases at temperatures above 45 °C. This is also indicated by the values of the temperature index θ for the process, which range from 1.11 to 1.37 below 10 °C, from 1.07 to 1.16 between 10 and 15 °C, and from 1.06 to 1.12 between 15 and 20 °C. Grunditz and Dalhammar (2001) conducted a model study of pure culture of *Nitrosomonas* and *Nitrobacter* isolated from activated sludge. They analysed the oxidation rate for N-NH_4 and N-NO_2 and confirmed that *Nitrosomonas* and *Nitrobacter* are highly temperature sensitive. The highest *Nitrosomonas* activity was measured at 35 °C, and the highest *Nitrobacter* activity at 38 °C. The bacteria activity rose until the optimum temperature was reached; a further temperature increase caused it to drop rapidly. At the maximum temperature of 50 °C, no activity of either *Nitrosomonas* or *Nitrobacter* was measured. Ilies and Mavinic (2001) pointed out that autotrophic organisms, such as nitrifying bacteria, are more sensitive to a decrease in ambient temperature than heterotrophic bacteria. They claimed that nitrification is ineffective when the temperature drops below 14 °C. At 10 °C, its efficiency can range from 10% to 30%. Ilies and Mavinic pointed to temperature as the main nitrification factor.

This study was undertaken to determine the impact of microwave radiation on the efficiency of nitrification in biofilm. It is important to estimate the quantity of nitrifiers and to correlate it with the effectiveness of ammonia oxidation because such knowledge can help to maintain stable nitrification in wastewater treatment systems. Regarding the sensitivity of nitrifier to thermal conditions, we analysed if the change of heating method of the biofilm influences the activity of nitrogen reactions and the quantity of nitrifying bacteria. With the use of the fluorescent in situ hybridization technique (FISH) we determined the percentage of ammonia-oxidizing bacteria (AOB) in biofilm. This method was successfully used in the quantification of nitrifiers in biofilm, for example in nitrifying trickling filters (Persson et al. 2002). The additional aim of the research was to study the possibility of the occurrence of nonthermal effects caused by the interaction of microwaves and biofilm.

Materials and methods

Eight trickling filters with a biofilm were used in the experiment: four were located inside a microwave-exposed tube, and four were placed in a tube heated with warm air as a control group. The packing was maintained at the same temperature in each case. Microwave radiation at a frequency of 2.45 GHz was applied at an intensity of 18 W (0.01 W·cm^{-3} of the reactor packing), which increased the biofilm temperature by 5 °C compared with the ambient temperature. By analogy, the temperature in the control reactors (i.e., those exposed to conventional heating) was raised. The ambient temperature remained at 16 ± 1 °C, thus the temperature inside the reactors was maintained at 21 ± 1 °C. The temperature was measured using an HI 98804 thermometer with a K-type thermocouple probe (Hanna Instruments). These identical temperature conditions were a base to consider the microwave nonthermal effects (Fig. 1.)

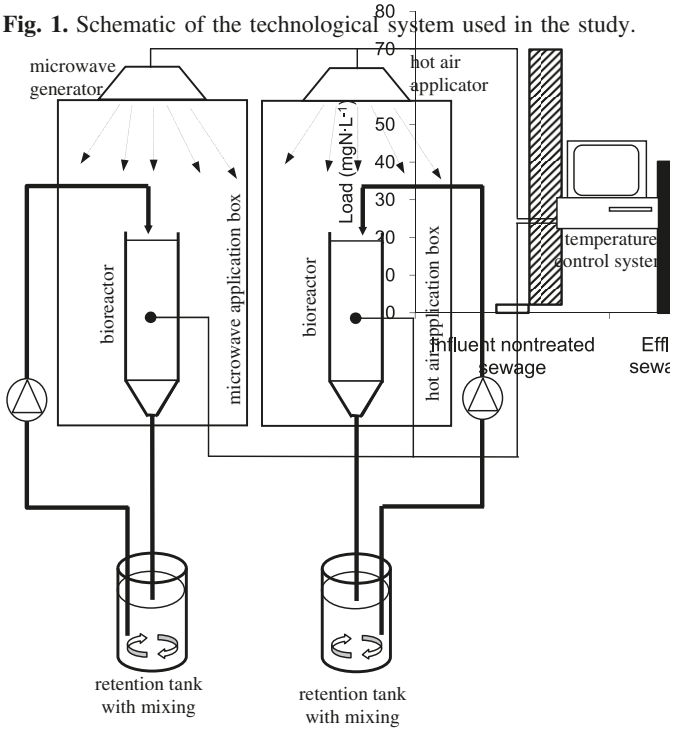


Fig. 1. Schematic of the technological system used in the study.

Table 1. Characteristic of the synthetic sewage.

Component	Concn. (mg·L^{-1})
Dry broth concentrate[a]	150
$NH_2CO{\cdot}NH_2$	30
CH_3COONa	10
$C_6H_{10}O_5$	50
$CaCl_2{\cdot}2H_2O$	7
$MgSO_4{\cdot}7H_2O$	50
NaCl	30
KCl	7

[a]Stock: meat concentrate, 0.40 g·L^{-1}; peptone, 4.00 g·L^{-1}; NaCl, 3.50 g·L^{-1}; peptone K, 5.40 g·L^{-1}; yeast concentrate, 1.70 g·L^{-1}.

According to the theory of microwave interactions, substances can be divided into permeable, absorbing, or reflecting microwaves depending on the dielectric properties. Plastic, as a substance with a low dielectric factor, is permeable to microwaves. The cover and packing of both reactors were made of plastic. Biofilm, containing mostly water, is highly absorbent of microwaves. The reactors active volume reached V = 565 cm^3 with the right surface of the packing of s = 202 m^2·m^{-3}, whereas the reactors theoretical active surface achieved F = 0.114 m^2. The most common source of microwaves is a vacuum lamp, called a magnetron, that transforms electric current to electromagnetic radiation. In our experiment, microwaves were transmitted to the reactors

Table 2. Probes used for hybridization.

Probe	Sequence (5′–3′)	Label	Formamide (%)	Specificity
EUB338	GCTGCCTCCCGTAGGAGT	FITC	20	Eubacteria
Nso190	CGATCCCCTGCTTTTCTCC	Cy3	55	Majority of AOB: *Nitrosomonas*, *Nitrosococcus*, *Nitrosolobus*, *Nitrosovibrio*, *Nitrosospira*

Fig. 2. Efficiency of organic compound (total organic carbon, TOC) removal at the hydraulic loading rate of 0.30 $m^3 \cdot m^{-2} \cdot h^{-1}$ and the organic loading rate of 1.93 g $COD \cdot m^{-2} \cdot d^{-1}$ in irradiated and control reactors.

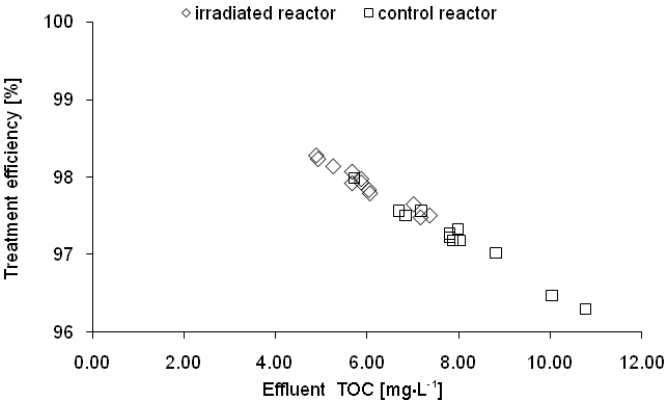


Fig. 3. Concentration of organic nitrogen, ammonia nitrogen, and N-NOx in the influent and effluents from irradiated and control reactors.

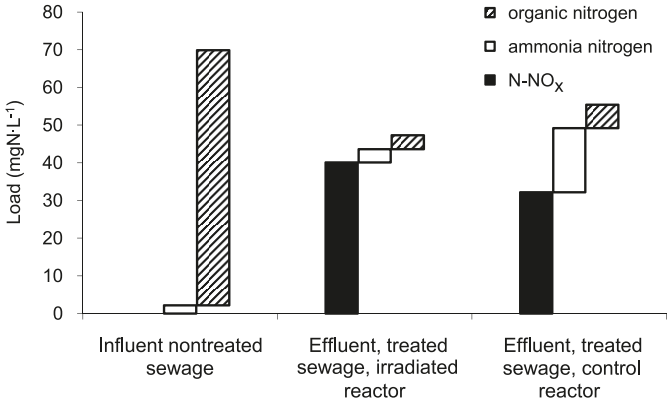
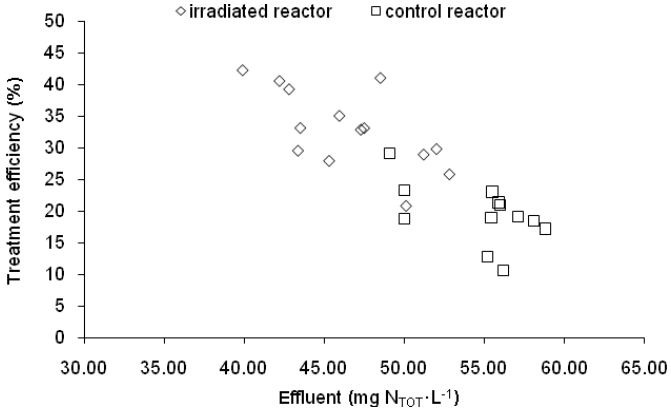


Fig. 4. Efficiency of nitrogen removal versus concentration of total nitrogen in the effluent in irradiated and control reactors.



through a waveguide. Table 1 presents the content of synthetic wastewaters used in the experiment. The ratio of chemical oxygen demand (COD) to biochemical oxygen demand (BOD) was calculated as 0.71 and is typical of that for highly biodegradable wastewaters. The biofilm depth ranged from 0.05 to 1.50 mm. The mean dry weight of the biofilm in the reactor was 0.8813 and 0.8825 g in the case of the microwave and conventionally heated reactors, respectively. The biofilm yield was 0.3856 g·g COD^{-1} in the microwave-heated reactors and 0.4261 g·g COD^{-1} in the control reactors. The reactors were operated on a time cycle, meaning that the bulk of the wastewater (V = 0.5 L) in the retention tank was changed once every 24 h. Wastewater was pumped from the retention tank to the biological reactor and then conveyed back to the retention tank (Fig. 1). The hydraulic loading used in the experiment achieved q = 0.30 $m^3 \cdot m^{-2} \cdot h^{-1}$, and therefore the bulk of the wastewaters flowed through the bioreactor 6.8 times every 24 h. The organic load (COD), calculated per total area of packing surface, was 1.93 $g \cdot m^{-2} \cdot d^{-1}$. Identical reactors were used in both types of experimental systems, i.e., in the case of both the microwave-exposed reactor and the control reactor. Identical activated sludge was embedded in each polyethylene packing by pumping it over the reactors for 24 h. This was followed by a 60 day period of reactor adaptation to the established technological parameters. Subsequently, 13 day technological tests were conducted, at the end of which samples for molecular analysis were collected. Treated sewage samples were collected daily to determine the concentration of organic compound total organic carbon (TOC) and total nitrogen. A Thermo Scientific model 1200 TOC analyzer was used to record the measurements. The method used to determine organic carbon content involves combustion of carbon in pure oxygen to carbon dioxide prior to its measurement in the infrared spectrum. The method of measuring total nitrogen was based on the combustion of nitric compounds to nitric oxide NO, and then a selective membrane connected with electrochemical cell was used. In addition, the concentrations of ammonia nitrogen and nitrogen oxidized forms (nitrates and nitrites) were calculated in the effluent. A Hach DR 2010 spectrophotometer and the company methodology were used in the measurement (Hach Company 1997).

The molecular analysis involved estimation of the percentage of AOB in the biofilm with the use of the FISH technique. Biomass used for FISH analysis was fixed immediately after sampling. Cells, after washing in 1× concentrated sodium phosphate buffer (PBS), were fixed by adding three volumes of fixation buffer (4% paraformaldehyde in PBS (pH 7.2)) to one volume of bacterial suspension (Amann et al. 1990). The sample prepared in this way was gently vortexed and then left at a temperature of 4 °C for 1 d. After centrifugation (3 min at 4000 rpm), the supernatant was removed and the sample was resuspended in the

mixture of 1× concentrated PBS and 96% ethanol (1:1 volume ratio of mixture components) and stored at a temperature of –20 °C for up to a few weeks. For analysis, 10 μL of sample was spread on the well of each glass slide (Marienfeld Laboratory Glassware), dried for about 10 min at 46 °C, and dehydrated by serial immersion of the slide in 50%, 80%, and 96% (*v*/*v*) ethanol (3 min each). Samples were air dried until the alcohol evaporated.

The composition of the hybridization buffer was as follows: 180 μL of 5 mol·L^{-1} NaCl, 20 μL of 1 mol·L^{-1} TrisHCl (pH 8.0), 550 μL of formamide, 250 μL of ultraclean water, and 2 μL of 10% (*w*/*v*) SDS. The washing buffer, containing 100 μL of 5 mol·L^{-1} NaCl, 1000 μL of 1 mol·L^{-1} TrisHCl (pH 8.0), 500 μL of 0.5 mol·L^{-1} EDTA (pH 8.0), 50 μL of 10% (*w*/*v*) SDS, and distilled water to complete the buffer to 50 mL, was preheated in a water bath to 48 °C.

Two molecular probes as suggested by Mobarry et al. (1996) were used for hybridization (Table 2).

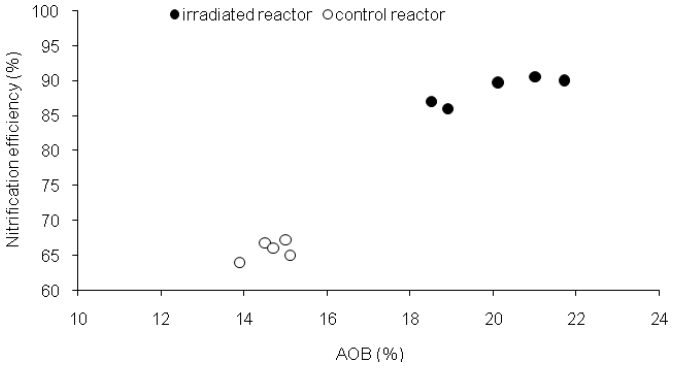
A mixture of 140 μL of hybridization buffer and molecular probes (10 pmol·μL^{-1}) was prepared. In each well, 12 μL of the mixture was placed, and then the slide was immediately transferred to the hybridization chamber. The slide was incubated in the hybridization oven at 46 °C for 3 h. After hybridization, the hybridization buffer was quickly rinsed from the slide with the washing buffer, and the slide was incubated in the washing buffer for 10 min in a preheated water bath at 48 °C. The washing buffer was removed from the slide with cold (4 °C) distilled water and dried.

Slides were mounted in the embedding medium Vecta-Shield (Vector Laboratories), which prevents rapid loss of fluorescence during microscopic examination, and a cover slip was placed on each slide. A Nikon Eclipse (Nikon) epifluorescence microscope under 100× objective was used for examination. For a reproducible and statistically correct result, about 30 images for each probe taken from 30 different positions of the slide were analysed (Hall et al. 2003).

The area covered by specific probe Nso190 and the area covered by the probe EUB338 in the same field of the glass slide, taken as images, were calculated using ImageJ software (US National Institutes of Health). The area ratios of the specific probe with the eubacterial probe were then calculated from the 30 sets of images. The occupation ratio of the area given by Nso190 to the area given by EUB338 gives a direct measurement of the number of active AOB (Rittmann et al. 1999).

In terms of the technological results, each data point in Figs. 2 and 4 is the arithmetic mean of the values obtained in four control reactors and four microwave-exposed reactors, operated in the same conditions. The data points in Fig. 5 are the arithmetic means of 30 measurements of the AOB occupation ratio for each sample. The data were tested for significance by one-way analysis of variance (ANOVA) at a significance level of $p < 0.05$. The normality of the distribution was confirmed by the Shapiro–Wilk test, and the hypothesis of the homogeneity of variances across the groups was verified on the basis of the Levene test. The differences between the mean values derived from particular groups were examined by Tukey's test.

Fig. 5. Relationship between the percentage of ammonia-oxidizing bacteria (AOB) in biofilm and nitrification in irradiated and control reactors.



Results

The efficiency of organic compound removal in reactors exposed to microwave radiation averaged 96.8%; the value was slightly lower in control reactors, 96.0% on average, but the difference was not statistically significant (Fig. 2). Organic compound concentration in the effluent did not exceed 7.3 and 10.7 mg TOC·L^{-1} in the microwave- and conventionally-heated reactors, respectively (Fig. 2). Such a low concentration of organic compounds in the effluent suggests that organic feed has been totally removed, and the biofilm likely contained autotrophic bacteria capable of acquiring energy from the oxidation of reduced mineral compounds such as ammonia. Such a claim was proven by the analysis of every single nitrogen compound in the effluent (Fig. 3). A significant difference in nitrogen compound concentration in the effluent was observed between the microwave-exposed and control groups. In the first case, the amount of nitrates and nitrites in the effluent remained at the level of 40 mg N-NO_x·L^{-1}, whereas the total amount of nitrogen compounds reached 46.3 mg N·L^{-1}. This proves almost total nitrification of the available ammonia nitrogen. In the case of conventionally heated reactors, the concentration of ammonia nitrogen in the effluent was significantly greater (Fig. 3), and the nitrification efficiency was lower despite identical technological conditions and analogous temperature. The amount of nitrates and nitrites in the effluent averaged 32.2 mg N-NO_x·L^{-1}, whereas the amount of ammonia nitrogen was 17 mg N-NH_4·L^{-1}, which made up nearly 30% of the total nitrogen in the effluent (Fig. 3).

The reduction in nitrogen oxidized forms (N-NO_x) was proven by the difference between the amount of total nitrogen in the raw wastewater and that in the treated wastewater. Denitrification performed by heterotrophic bacteria is of crucial significance. Under anoxic conditions, nitrates are used as electron acceptors, which results in nitrate reduction to either molecular nitrogen N_2 or nitric oxides N_2O. The gaseous products dissipate in the air, which reduces the concentration of total nitrogen in the effluent. The amount of total nitrogen was reduced by 32.3% on average in the case of microwave heating and by 18.9% in the case of conventional heating (Fig. 4).

Examination of biomass in the reactors using the FISH technique allowed determination of the occupation ratios of AOB in eubacteria of the biofilm. In the reactors exposed to microwave radiation, the percentage of AOB in the biofilm was about 20%, and the efficiency of ammonia removal from wastewater averaged 88.6% (Fig. 5). For comparison, in control reactors heated to the same temperature but in a conventional way, AOB occupied about 14.6% of the biofilm, which resulted in a nitrification efficiency of 65.8%.

Discussion

Although numerous studies have been published on the possible nonthermal effects of microwave exposure on many kinds of living systems, little is known about the modes of action of microwaves in those nonthermal cases (Mileva et al. 2003). However, alterations within the biofilm structure and its functions are pointed out, among other things, as a result of the effect microwaves exert on transport channels of calcium ions Ca^{2+}. An electromagnetic field is capable of inducing changes within biofilm on the basis of either a direct or an indirect influence on the properties of receptors that bind ligands such as Ca^{2+}, neurotransmitters, or hormones (Geletyuk et al. 1995). The influence microwave energy exerts on the biofilm seems to be less controversial than in the case of enzymatic systems, in which there are experimental difficulties in proper control and monitoring of the temperature. Numerous researchers have questioned the existence of microwave-induced nonthermal effects (Jeon and Kim 2000). Nonmeasurable and nonthermal effects on catalytic activity were observed in isolated enzymes exposed to in vitro irradiation. For instance, Parker et al. (1996) claimed that microwave-treated enzymatic reaction rates increased two to three times compared with those exposed to conventional heating. In contrast, other enzymatic systems such as protein thermophilic enzymes respond to various intensities of microwave field with an inhibition of activity (Porcelli et al. 1997).

Our results show an increase in the efficiency of nitrogen reactions in the reactors exposed to microwave radiation. Nitrification and denitrification were both observed to be more effective. In control and irradiated reactors, temperature was maintained at the same level. This suggests nonthermal effects of microwave heating on nitrogen removal. The changes in the contribution of AOB to the total number of bacteria in biofilm point out that an advantageous environment for autotrophic nitrifiers is created under the conditions of microwave radiation. Microwaves can influence bacteria activity. According to Woo et al. (2000), the impact of microwaves on microbial activity can be connected to the structure of the cellular wall. They examined the mechanisms of microbial cell inactivation by microwave heating and the differences in gram-positive and gram-negative bacteria. The electron microscopy approach revealed that the surface of *E. coli* cells was more sensitive than the surface of *Bacillus subtilis* cells. Furthermore, microwave-injured *E. coli* cells were easily lysed by SDS, in contrast with *B. subtilis* cells.

The FISH method with 16S rRNA-targeted oligonucleotide probes was used to quantitatively estimate AOB in biofilm, both in the reactors heated with microwave radiation and in the reactors heated conventionally, giving values of 20% and 14.6%, respectively. Such values are similar to those obtained by other authors. Biesterfeld et al. (2001) examined the nitrifying trickling filter in wastewater treatment plant treated sewage with an ammonia concentration in the influent of 23.3 ± 3.9 mg $N{\cdot}L^{-1}$. Ammonia oxidizers occupied 17% of the total biofilm. In the research by Persson et al. (2002), the proportions of AOB in the full-scale nitrifying trickling filter treating municipal wastewater varied from 16% to 45%. The aforementioned data are consistent with the observation of 5%–20% AOB in domestic wastewater systems (Okabe et al. 1999).

In our research, the use of microwave heating of the biofilm caused an increase in the percentage of AOB in the biomass of from 14.6% to 20.0% compared with that in conventionally heated biofilm. Consequently, the efficiency of ammonia removal from wastewater improved and averaged 88.6% for microwave-heated reactors and 65.8% for conventionally heated reactors . Ebie et al. (2002), quantitatively estimating the amount of AOB in an anaerobic–aerobic activated sludge process, claimed that when the ammonia concentration in the effluent decreased to below 1 mg $N{\cdot}L^{-1}$, the occupation ratio of AOB was 24%. When the effluent ammonia concentration increased to 30–40 mg $N{\cdot}L^{-1}$, the percentage of AOB was 2.7%. In our research, the concentration of ammonia in the effluent of the irradiated reactors was about 3.5 mg $N{\cdot}L^{-1}$, whereas it averaged 17 mg $N{\cdot}L^{-1}$ in the case of the control reactors. The increase in the percentage of AOB in the irradiated reactors can be explained by an excessive proliferation of ammonia oxidizers under such conditions. On the other hand, it cannot be proven that the percentage of AOB decreased drastically in the control reactors. Such results suggest that the decline in nitrification efficiency may not be caused by a decrease in the number of nitrifying bacteria but by a decrease in their activity or a change in dominant species (Ebie et al. 2002).

Denitrification changes induced by microwaves were affected to an even greater extent. The obtained data show that microwave radiation generated anoxic spheres in the biofilm, as a result of which nitrification and a reduction in oxidized forms of nitrogen occurred simultaneously. Microwave energy causes volumetric heating of a substance. When one takes into account a relatively small thickness of biofilm in comparison to the microwave penetration depth, it might be assumed that the biofilm was steadily heated in all of its cross section. At higher temperatures, oxygen was used more rapidly in the outer, more active layers of the biofilm, which resulted in anoxic spheres. The relation between anoxic spheres and the biofilm temperature has been confirmed by the experiment of Hao et al. (2002), who asserted that oxygen penetration depth increases as the temperature decreases.

Conclusions

The results obtained in this experiment prove undeniably that microwave radiation changes the concentration of nitrogen compounds in biofilm. Microwave heating triggered alterations within the biofilm that increased the efficiency of both nitrification and denitrification and the percentage of ammonia-oxidizing bacteria (AOB).

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List of symbols

F	theoretical active surface
k_{T_1}	reaction rate constant at temperature T_1 in variable units, depending on the reaction order
k_{20}	reaction rate constant at a temperature of 20 °C
q	hydraulic loading
s	right surface of the packing
T_1, T_{20}	temperature (K)
V	volume
θ	temperature index