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Antimicrobial activity of commercial zeolite A on *Acinetobacter junii* and *Saccharomyces cerevisiae*

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ABSTRACT

The influence of three samples of commercially produced zeolite A (named A, M and R) in water medium on the bacterium *Acinetobacter junii* and yeast *Saccharomyces cerevisiae* was investigated. These microorganisms were used in the bioassay and are not specifically related to the use of zeolite A. All zeolite samples showed the negative influence on the survival and physiological status of *A. junii* and *S. cerevisiae*. The EC_{50} values for the inhibition of CFU of *A. junii* were 0.328, 0.138 and 0.139 g l^{-1} for zeolite sample A, M and R, respectively. The EC_{50} values of tested zeolites for *S. cerevisiae*, estimated by fermentation and fluorescence microscopy assay, ranged from 2.88 to 5.47 g l^{-1}. The genotoxic effect of three samples of zeolite to *S. cerevisiae* was shown by the alkaline comet assay. When assuming all the aspects of zeolite toxicity to bacterium and yeast, the zeolite sample R appeared to be less toxic than the samples A and M. The hydrolysis of zeolite crystals, amorphous aluminosilicate and unreacted gel fraction in water medium and consecutive dissolution and leaching of aluminium and silicon in the form of aluminosilicate molecules (700–1300 Da) was detected.

1. Introduction

Zeolite A (LTA) is a synthetic microporous mineral, which has no natural analogue. Its crystal unit cell can be described with general oxide formula [1]: $48Na_2O \times 48Al_2O_3 \times 96SiO_2 \times 216H_2O$. The 3-dimensional framework of zeolite consists of aluminium and silicon atoms (tetrahedron coordination) connected via common oxygen atoms to form system of channels and caves. Negative charge of framework is compensated with exchangeable sodium cations. Due to their crystal structure and chemical resistance, zeolites are widely used in daily life, such as sorbents (gases, liquids), cation exchangers (detergents), molecular sieves (membranes for small molecules) and catalysts in many industrial processes (e.g. oil hydrocracking). The largest use can be seen through annual consumption of zeolites in the European detergent market of around 650,000 tons [2]. Typical concentrations of zeolite A in laundry detergents is in range from 20 to 34% weight.

Zeolite A is safe compound in general. Its apparent safety was determined in relation to the standard governmental testing assays. Synthetic zeolite A is not absorbed by oral application in the diet and is not toxic to metazoan [2]. In vivo tests on rats and mice did not indicate a genetic toxicity of zeolite A. Studies on rats, hamsters and monkeys did not reveal a potential of zeolite A to induce carcinogenicity [2]. Zeolites A were not teratogenic in experiments with rats, mice, rabbits and hamsters [2]. Few studies [2] showed no genetic toxicity of zeolite A to *Salmonella typhimurium*, *Escherichia coli* and *Saccharomyces cerevisiae*. Zeolite A is biologically nondegradable material and can be accumulated in environment such as water, sediment, terrestrial compartments and wastewater treatment plants. Presently, very little information is available about the ecological properties of commercial zeolite A and its influence on microorganisms. This artificial mineral has a potentially hazardous effect on microorganisms and therefore some consideration in future regulations should address to this issue.

The commercially produced zeolite A consists of cubic microcrystals of different size and morphology. They agglomerate partially during the spray-drying procedure to bigger particles, which may disintegrate in water [2]. The impurities in the material may consist of amorphous aluminosilicate, unreacted or non-transformed gel, iron-oxide/hydroxide/oxyhydroxide nanoparticles, clusters or agglomerates on the external surface of particles. The amorphous (non-crystalline) and unreacted gel phase remained in the product after synthesis increase the solubility of

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commercial product in water media [3]. The chemical composition and crystalline structure of commercial zeolite A are almost identical, but the individual samples have different purity resulting in different behaviour in water media. A different solubility of material in water media can result in different degree of toxicity against microorganisms. The aim of this study was to investigate the inhibitory influence of three samples of commercially produced zeolite A on the prokaryotic and unicellular eukaryotic microorganisms.

2. Materials and methods

2.1. Zeolite A

Three samples of commercially produced (Silkem d.o.o., Slovenia) zeolites A were used in this study, named as A, M and R. Chemical composition of this crystalline sodium aluminosilicate varies due to the synthesis conditions and its average values (in weight %) are: 17–19 Na_2O, 28–30 Al_2O_3, 31–34 SiO_2, 18–22 H_2O. All materials had purity level of approximately 99%, while trace impurities were consisted of heavy metals (<0.26%), amorphous aluminosilicates and unreacted gel. The cubic microcrystals with rounded corners and edges had $D_{0.5}$ (size value under which is 50% of total mass of the sample) of 3.550 μm in sample A, 3.436 μm in sample M and 3.403 μm in sample R. The 5% slurry had pH level of 11–12. There was no appreciable difference in material data sheets between A, M and R and all materials are registered under same CAS number 1318-02-1. The main application of studied samples of zeolite A is as follows: sample A in production of detergents and chemical industry; sample M in production of molecular sieves and chemical industry; sample R in production of detergents and plastics. Materials upon receipt were sterilized by drying at 105 °C in oven for 16 h before the experiments were to commence. The concentration levels of zeolite used in experiments were in the range starting from the first inhibition effect to the concentration where almost complete inhibition of microorganisms was observed (0.1–6.0 g l^{-1}).

2.2. Tested microorganisms

As a prokaryotic microorganism the bacterium *Acinetobacter junii* strain DSM 1532 was tested. This Gram-negative bacterium is normally present in wastewater and in the activated sludge biomass. The most widely studied physiological characteristic of this bacterium is the ability to accumulate the soluble phosphate (P) present in the wastewater in the form of intracellular nonsoluble poly-P granules [4]. *S. cerevisiae* strain ATCC 64252 was used as unicellular eukaryotic test organism. This yeast was chosen since it is prescribed for the application in determination of water toxicity, and its physiological state is easy to follow through fermentation [5].

2.3. Experimental procedure for treatment of bacteria

The *A. junii* was pregrown on the nutrient agar (Biolife, Italy) for 20 h at 30.0 ± 0.1 °C. Thereafter the biomass was suspended (Kartell TK3S) in sterile 0.3% NaCl. One ml of suspended biomass was inoculated into 100 ml of autoclaved simulated wastewater (composition in mg l^{-1} of distilled water: Na-propionate 300; peptone 100; $MgSO_4 \times 7H_2O$ 10; $CaCl_2 \times 2H_2O$ 6; KCl 30; yeast extract 20; KH_2PO_4 88; pH 7.0 ± 0.2). The initial concentration of *A. junii* in such prepared flasks was $9.52 \pm 3.37 \times 10^9$ CFU l^{-1}. Into each flask the zeolite A (A, M or R) in the concentration range of 0.1–2.0 g l^{-1} was added. The bottles for negative control were left without zeolite addition. The flasks were sealed with a sterile gum cap with a central hole through which the aeration with filtered air (1 l min^{-1}) was provided. The flasks were incubated at 30.0 ± 0.5 °C in a water bath (Memmert WNB22) with stirring (70 rpm) during 24 h of experiment. All experiments were carried out in triplicate tests. The P ($P\text{-}PO_4^{3-}$) concentration in wastewater was measured after filtration through the Whatman filter units of pore diameter 0.2 μm in a DR/2500 Hach spectrophotometer by the molybdovanadate method (Hach method 8114). The number of viable bacterial cells was determined as colony-forming units (CFU) grown on the nutrient agar after incubation at 30 ± 0.1 °C for 72 h.

2.4. Experimental procedure for treatment of yeast

Three samples of zeolite A (A, M and R) were added to 100 ml of distilled water in the concentration range of 2.0–6.0 g l^{-1}. Bottles were stirred at 70 rpm at 30.0 ± 0.5 °C for 24 h and thereafter the pH of the solution was adjusted to 7.0 ± 0.1 by dropwise addition of 1 M HCl (Merck, p.a.) with magnetic stirring in order to eliminate the negative influence of alkaline pH on yeast. Such prepared suspensions of zeolite were tested for the toxicity against yeast.

The *S. cerevisiae* was pregrown on YM agar (Difco 0712) at 30.0 ± 0.1 °C for 12 h to obtain a log-phase culture. The biomass was suspended (Kartell TK3S) in sterile 0.3% NaCl and the density of the cell suspension was adjusted to an absorbance of 3.0 at 550 nm against the distilled water as blank. A 0.5 ml of yeast suspension was inoculated into 24 ml closed glass bottles containing the 4 ml of autoclaved liquid nutrient medium (composition in g per 100 ml of distilled water: sucrose 4.0; peptone 2.0; yeast extract 1.7; pH 7.0 ± 0.2). According to the previous procedure [5], the tested bottles set up in triplicate were filled up with 20 ml of prepared suspensions of zeolite, while the bottles serving as negative control were filled up with distilled water. The initial concentration of *S. cerevisiae* in such prepared bottles was 10^5 cells ml^{-1}. To allow the exhaustion of the gas produced during the fermentation of sucrose, an 18 G needle was stuck to its end through the rubber bung into the liquid medium in each bottle and the open syringe (10 ml) was stuck on the needle. Inoculated bottles were incubated in the dark at 28.0 ± 0.1 °C for 16 h. All experiments were carried out in triplicate tests. From such prepared set of bottles three types of toxicity tests were performed (fermentation test, fluorescence microscopy of dead cells and alkaline comet assay).

2.4.1. Fermentation test

The fermentation test was performed according to protocols described by Hrenovic et al. [5]. In brief, this test is based on the fact that the yeast *S. cerevisiae* is able to ferment sucrose to carbon dioxide. This fermentation takes place in a closed bottle with liquid medium. Gas produced during the fermentation process of sucrose presses out the equivalent volume of liquid to the open syringe. If sucrose is combined with some toxicants that influence the yeast and hinder the fermentation, the amount of created carbon dioxide is reduced in comparison with the control, or it is not formed at all. By measuring the volume of liquid pressed out, the amount of gas produced and the intensity of fermentation could be indirectly estimated. The higher the toxicity, the higher the reduction of produced gas will be. The results were expressed as a percent of inhibition of fermentation in sample bottles when compared to the negative control. The EC_{50} (effective concentration) of zeolite suspension which inhibits the fermentation for 50% when compared to negative control was calculated from these results.

2.4.2. Fluorescence microscopy of dead cells

To examine the dead of yeast cells in fermentation bottles, a dye exclusion method was performed according to protocols described by Jajte et al. [6]. The cells were stained with two fluorescent binding dyes, where the ethidium bromide does not penetrate the plasma membrane in viable cells and stains only dead cells, while

acridine orange penetrates the plasma membrane without permeabilisation and stains viable and dead cells. When visualised by fluorescence microscopy, live cells appear green while dead cells orange.

Aliquots of 1 μl of acridine orange (100 μg ml^{-1} of PBS) and ethidium bromide (100 μg ml^{-1} of PBS) were placed in a glass tube. A 25 μl of the yeast suspension from the bottles where the fermentation took place was added in tube. A 10 μl of mixture was placed on the microscopic slide, covered with coverslip and examined using the fluorescence microscope (Zeiss, Germany) equipped with appropriate filter. Encrypted slides were evaluated by the single observer. A minimum of 200 of total cells per sample were scored. The results were expressed as a percent of dead cells among total cells in sample when compared to the negative control. The EC_{50} of zeolite suspension which caused a death of 50% of cells when compared to negative control was calculated from these results.

2.4.3. Alkaline comet assay

The comet assay was previously established as an initial indicator of general, non-specific DNA damage/genotoxicity and an effective biomarker for environmental monitoring [7,8]. The interaction of genotoxic agents with DNA forms strand breaks, alkali labile adducts and other modifications, which due to enzymatic removal of damaged nucleotides can contribute to an increased level of DNA strand breaks that could be sensitively detected by the alkaline comet assay. Due to the fact that it involves the analysis of single cells, inter-cell variability in response may be also investigated in the course of the assay. Comet assay has been gaining importance in ecotoxicology, especially within the last few years when it was successfully applied in a range of phylogenetically disparate groups of organisms [9]. A few of comet assays studied included organisms with lower level of organisation, such as *Euglena gracilis* [10,11], *Chlamidomonas reinhardtii* [12,13] and marine diatoms [14]. However, only a few comet assay studies were focused on yeast as a model system for studying genotoxicity [15–18]. The single cell gel electrophoresis or comet assay was for the first time applied on *S. cerevisiae* strain ATCC 64252. There is no literature data on the application of comet assay to study the genotoxicity of commercial zeolites.

Yeast cells from the bottles where the fermentation took place were collected by centrifugation at 865 g for 3 min, washed with distilled water, and resuspended in S-buffer (1 M sorbitol, 25 mM KH_2PO_4, pH 6.5). The alkaline comet assay was performed according to protocols described by Miloshev et al. [15] and Lah et al. [16] with minor modifications. Chemicals and reagents used to perform comet assay were of analytical grade and were purchased from Sigma–Aldrich Chemical Co. (St. Louis, USA) unless otherwise noted.

Two fully frosted microscopic slides per sample were prepared. Each slide was covered with a sandwich gel: 1% and 0.6% normal melting point agarose. Aliquots of yeast suspension (5 μl; approximately 5×10^4 cells in control) were mixed with 0.7% low melting point agarose containing 2 mg ml^{-1} of the enzyme zymolyase 20T (Seikagaku Corp.) and spread over the slides. Slides were covered with cover glasses and incubated at 30 °C for 20 min to disintegrate the yeast cell wall and obtain spheroplast. The enzyme was inactivated on the icy cold surface (4 °C) for 5 min and slides were covered with the fourth layer of 0.5% low melting point agarose. After solidification of agarose, slides were immersed for 75 min in freshly prepared lysing solution (30 mM NaOH, 1 M NaCl, 0.1% N-laurylsarcosine, 100 mM DMSO (Kemika) and 1% Triton-X 100) to lyse the spheroplasts. The slides were then rinsed three times during 20 min with a freshly prepared buffer (30 mM NaOH, 2 mM Na_2EDTA, pH 12.4) to unwind the nuclear DNA. Electrophoresis was carried out in the same buffer for 5 min at 25 V and 300 mA at 1.0 V/cm. Neutralisation was performed using 0.4 M Tris–HCl buffer (pH 7.5) for 15 min. Slides were stained with ethidium bromide (20 μg ml^{-1}), covered with a coverslip and stored at 4 °C in humidified sealed containers before the beginning of analysis. Microgels for the alkaline comet assay were prepared from treated yeast cells and corresponding negative and positive controls. Slides for the positive control were prepared in the same way as others, but before lysis they were treated with 10 μM hydrogen peroxide for 10 min at 4 °C.

Analysis of comet slides was performed using an image analysis system (Comet Assay II, Perceptive Instruments Ltd., U.K.) attached to a fluorescence microscope (Zeiss, Germany), equipped with appropriate filters. Encrypted slides were evaluated by the single observer. Altogether 200 comets per sample (100 comets/slide) were scored. Comets were randomly captured at a constant depth of the gel, avoiding the edges of the gel, occasional dead cells, cells near or in a trapped air bubble and superimposed comets. The parameters selected for the quantification of DNA damage were: comet tail length and tail intensity (% DNA) as calculated by the software. The extent of DNA damage, as recorded by the alkaline comet assay, was analyzed considering the mean (±standard error of the mean), median and range of the comet parameters measured.

2.5. Experimental procedure for behaviour of zeolite A in water

Three samples of zeolite A (A, M and R) were added to 100 ml of distilled water in the concentration range which was used for assessment of toxicity against bacteria and yeast (0.1, 0.2, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0 and 6.0 g l^{-1}). Bottles were stirred at 70 rpm at 30.0 ± 0.5 °C for 24 h. Such prepared suspensions were centrifuged at 33,000 × *g* for 10 min. The Al and Si concentration in the supernatant were determined by standard atomic absorption spectrometry, using the Perkin-Elmer A Analyst 200 instrument.

2.6. Experimental procedure for mass spectrometric method

A 2.0 g of tested zeolite A samples (A, M and R) were added to 100 ml of deionised water to obtain saturated suspensions. The suspensions were divided into three parts. They were adjusted to pH 6.0, 7.0, and 8.0 by a dropwise addition of 36% HCl (Merck, p.a.) with magnetic stirring. The pH was measured with a Mettler Toledo MP 220 pH meter using a Mettler Toledo InLab 410 Ag/AgCl-electrode calibrated with FF-Chemicals buffer solutions with pH values of 4.00 and 7.00. Samples were constantly stirred by magnetic stirrer for 24 h at 70 rpm and 30 ± 2 °C in order to obtain dynamic equilibrium. The samples were filtered through 0.45 μm membrane filter prior to the recording of the mass spectra.

The ESI TOF mass spectra were recorded by a Micromass LCT mass spectrometer equipped with a Z-spray electrospray interface. The solutions were introduced into the spectrometer by a Harvard Apparatus Model 11 syringe pump at flow-rates of 10 μl min^{-1}. Several different sample cone voltages were tested, but the best spectra were obtained by 70 V. The capillary voltage was 3500.0 V. Different RF-lens values were tested as well and 200 V was chosen. Other operating conditions of the mass spectrometer were: extraction cone voltage 5 V; desolvation temperature 150 °C; source temperature 120 °C; resolution 4000.0; cone gas flow 300 l h^{-1}; desolvation gas flow 802 l h^{-1}; mass range *m*/*z* 70–1500. All ESI mass spectral data were acquired using the Masslynx NT software (version 3.4). The interpretation of the spectra were done using the simulation of isotopic spectrum of silicates, where silicon (^{28}Si) has two isotopic signals 5% (^{29}Si) and 3% (^{30}Si) in one and two unit parts from the main signal (100%). Unfortunately sodium ^{23}Na and aluminium ^{27}Al are monoisotopes and thus their amount in complexes could not be verified by mass spectrometric methods. In accordance with the MS spectrum, no chlorine was involved in silicon containing species.

2.7. Statistical analyses

Statistical analyses were carried out using Statistica Software 8.0 (StatSoft, Tulsa, USA). Raw data obtained by comet measurements and numbers of bacterial CFU were logarithmically transformed beforehand to normalize distribution and to equalize variances of the measured parameters. The comparisons between samples were done using the one-way analysis of variance (ANOVA) and subsequently the post hoc Duncan test was performed for the calculations concerning pair-wise comparisons. The correlation between variables was estimated by Spearman correlation analysis. Statistical decisions were made at a significance level of $p < 0.05$.

3. Results

3.1. Influence of zeolite A on bacterium A. junii

The results of influence of the three tested samples of zeolite A against P-accumulating bacterium *A. junii* are shown in Table 1. The addition of zeolite A resulted in a dose-dependent increase of the final pH level of wastewater with the maximum difference between the reactors with zeolite addition and control reactors of 0.55 pH units. All three zeolite samples displayed a dose-dependent inhibition of final CFU. The percent of CFU inhibition showed significantly ($p < 0.05$) positive correlations for the zeolites A, M and R ($R = 0.941$–0.990). Based on the percent of inhibition, the sample A had significantly higher EC_{50} value ($0.328\ g\ l^{-1}$) than the samples M and R (EC_{50} 0.138 and $0.139\ g\ l^{-1}$). Inter-group comparisons (Table 1) showed that zeolite samples were not significantly toxic at concentration of $0.1\ g\ l^{-1}$ when compared to negative control, while at higher concentrations the final log CFU was significantly lower. When comparing the CFU of *A. junii* in reactors with different samples of zeolite by the same concentration (Table 1) it can be seen that by the zeolite concentration of $2.0\ g\ l^{-1}$ the final difference in log CFU was four orders of magnitude. Decreased number of physiologically active viable bacterial cells resulted in a dose-dependent lower percent of P removal from wastewater (Table 1). When assuming all the aspects of zeolite toxicity, the overall toxicity of three samples of zeolite A to *A. junii* was in the order: M > A > R.

3.2. Influence of zeolite A on yeast S. cerevisiae

3.2.1. Fermentation test

The results of the yeast fermentation test are shown in Fig. 1. All three tested zeolites A inhibited the fermentation activity in zeolite dose range of 2.0–6.0 $g\ l^{-1}$. The toxicity of the three tested zeolites did not differ significantly at the concentration of 2.0 and $3.0\ g\ l^{-1}$, while at the higher concentrations of 4.0–6.0 $g\ l^{-1}$ zeolite R was less toxic than A and M. The sample A with the EC_{50} value of $3.32 \pm 0.11\ g\ l^{-1}$ and M with EC_{50} of $3.43 \pm 0.08\ g\ l^{-1}$ appeared to be much more toxic to yeast than sample R with EC_{50} of $5.47 \pm 0.50\ g\ l^{-1}$.

3.2.2. Fluorescence microscopy of dead cells

The percentage of dead cells in samples with zeolite dose range within 2.0–6.0 $g\ l^{-1}$ obtained by fluorescence microscopy is given in Fig. 2. The sample A appeared to be the most toxic with the EC_{50} value of $2.88 \pm 0.35\ g\ l^{-1}$, followed by M with EC_{50} of $3.43 \pm 0.07\ g\ l^{-1}$ and R with EC_{50} of $3.54 \pm 0.21\ g\ l^{-1}$. The difference in the toxicity of three examined zeolites was lower than in the fermentation test, while the toxicity curves followed the similar trend. The toxicity profile in fermentation test and fluorescence microscopy showed the significantly positive correlation ($R = 0.873$ for A, 0.956 for M and 0.948 for R).

Table 1
Inhibition effect of three samples of zeolite A (A, M and R) in the concentration range 0.1–2.0 $g\ l^{-1}$ on the *Acinetobacter junii* when compared to corresponding negative control [t_0 CFU (10^9 CFU l^{-1})] = 9.52 ± 3.37; [t_0 P-PO_4 ($mg\ l^{-1}$)] = 20.02 ± 0.44. Significantly different values: a—compared to negative control; b—compared to concentration 0.1 $g\ l^{-1}$; c—compared to concentration 0.2 $g\ l^{-1}$; d—compared to concentration 0.5 $g\ l^{-1}$; e—compared to concentration 1.0 $g\ l^{-1}$; f—compared to concentration 1.5 $g\ l^{-1}$.

Parameter	Concentration of zeolite ($g\ l^{-1}$)						
	0	0.1	0.2	0.5	1.0	1.5	2.0
Sample A							
Final pH	7.70 ± 0.03	7.77 ± 0.02	7.84 ± 0.02^{a}	$7.88 \pm 0.03^{a,b}$	$7.95 \pm 0.01^{a,b,c}$	$7.99 \pm 0.02^{a,b,c,d}$	$8.09 \pm 0.11^{a,b,c,d,e,f}$
Phosphate removal (%)	51.73 ± 1.54	45.07 ± 4.48^{a}	$39.40 \pm 2.67^{a,b}$	$25.25 \pm 5.90^{a,b,c}$	$6.89 \pm 0.48^{a,b,c,d}$	$5.03 \pm 0.50^{a,b,c,d}$	$1.00 \pm 0.52^{a,b,c,d,e}$
Total cells (CFU l^{-1})	$1.84 \pm 0.43 \times 10^{11}$	$1.62 \pm 0.34 \times 10^{11}$	$1.12 \pm 0.23 \times 10^{11}$	$6.63 \pm 2.50 \times 10^{10a,b,c}$	$2.67 \pm 1.16 \times 10^{8a,b,c,d}$	$6.73 \pm 1.42 \times 10^{7a,b,c,d,e}$	$6.00 \pm 1.84 \times 10^{6a,b,c,d,e,f}$
Inhibition of CFU (%)	0.00	11.83 ± 2.05^{a}	$38.84 \pm 2.08^{a,b}$	$64.36 \pm 8.85^{a,b,c}$	$99.84 \pm 0.09^{a,b,c,d}$	$99.96 \pm 0.01^{a,b,c,d}$	$100.00^{a,b,c,d}$
EC_{50} ($g\ l^{-1}$)				0.328 ± 0.051			
Sample M							
Final pH	7.67 ± 0.02	7.69 ± 0.01	7.71 ± 0.03	$7.78 \pm 0.07^{a,b}$	$7.84 \pm 0.04^{a,b,c}$	$7.89 \pm 0.008^{a,b,c,d}$	$8.02 \pm 0.05^{a,b,c,d,e,f}$
Phosphate removal (%)	42.84 ± 1.98	35.50 ± 4.85^{a}	$13.77 \pm 4.00^{a,b}$	$8.58 \pm 0.64^{a,b,c}$	$6.95 \pm 0.82^{a,b,c}$	$4.70 \pm 1.00^{a,b,c}$	$0.33 \pm 0.29^{a,b,c,d,e,f}$
Total cells (CFU l^{-1})	$1.47 \pm 0.47 \times 10^{11}$	$1.06 \pm 0.27 \times 10^{11}$	$6.56 \pm 3.70 \times 10^{9a,b}$	$5.23 \pm 4.23 \times 10^{5a,b,c}$	$3.53 \pm 2.72 \times 10^{5a,b,c}$	$1.02 \pm 0.32 \times 10^{5a,b,c,d}$	$6.10 \pm 2.21 \times 10^{4a,b,c,d,e}$
Inhibition of CFU (%)	0.00	27.31 ± 4.59^{a}	$94.66 \pm 4.21^{a,b}$	$100.00^{a,b,c}$	$100.00^{a,b,c}$	$100.00^{a,b,c}$	$100.00^{a,b,c}$
EC_{50} ($g\ l^{-1}$)				0.138 ± 0.007			
Sample R							
Final pH	7.65 ± 0.08	7.70 ± 0.07	7.74 ± 0.05^{a}	$7.83 \pm 0.07^{a,b,c}$	$8.01 \pm 0.02^{a,b,c,d}$	$8.09 \pm 0.02^{a,b,c,d}$	$8.20 \pm 0.02^{a,b,c,d,e,f}$
Phosphate removal (%)	42.77 ± 1.94	33.55 ± 1.76^{a}	$12.37 \pm 1.19^{a,b}$	$9.45 \pm 1.11^{a,b,c}$	$6.34 \pm 1.17^{a,b,c}$	$5.36 \pm 0.79^{a,b,c,d}$	$2.70 \pm 0.58^{a,b,c,d,e}$
Total cells (CFU l^{-1})	$1.70 \pm 0.74 \times 10^{11}$	$1.27 \pm 0.57 \times 10^{11}$	$4.36 \pm 1.81 \times 10^{9a,b}$	$1.17 \pm 0.98 \times 10^{9a,b,c}$	$5.31 \pm 2.29 \times 10^{8a,b,c}$	$4.10 \pm 2.03 \times 10^{8a,b,c}$	$2.16 \pm 0.56 \times 10^{8a,b,c,d}$
Inhibition of CFU (%)	0.00	26.05 ± 1.42^{a}	$97.14 \pm 1.36^{a,b}$	$99.34 \pm 0.45^{a,b,c}$	$99.69 \pm 0.05^{a,b,c}$	$99.76 \pm 0.06^{a,b,c}$	$99.86 \pm 0.04^{a,b,c}$
EC_{50} ($g\ l^{-1}$)				0.139 ± 0.002			

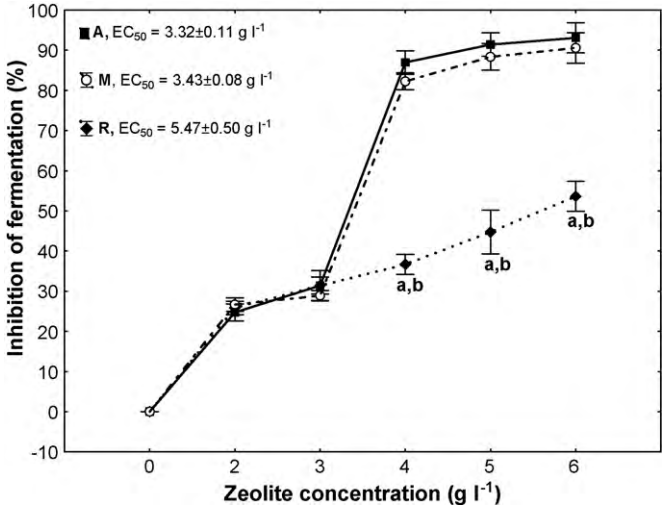

Fig. 1. Inhibition of fermentation estimated by yeast toxicity test for three samples of zeolite A (A, M and R) in the concentration range of 2.0–6.0 g l^{-1} when compared to corresponding negative control. Volume of produced gas in negative control was 12.0 ± 0.2 ml. Significantly different values: a—compared to zeolite A; b—compared to zeolite M.

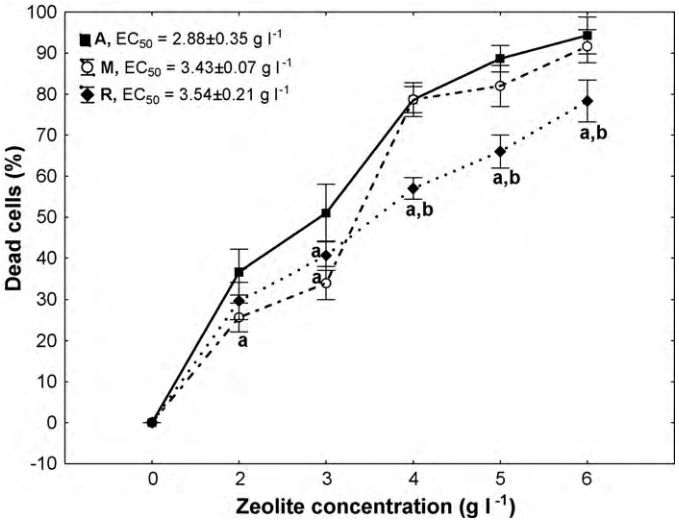

Fig. 2. Percentage of dead yeast cells estimated by fluorescence microscopy for three samples of zeolite A (A, M and R) in the concentration range of 2.0–6.0 g l^{-1} when compared to corresponding negative control. Parameters of the fluorescence microscopy were evaluated by counting at least 200 cells per sample. Percent of dead cells in negative control was 4 ± 2%. Significantly different values: a—compared to zeolite A; b—compared to zeolite M.

3.2.3. Alkaline comet assay

The results of the alkaline comet assay performed on yeast cells following *in vitro* exposure to zeolites A, M and R are summarized in Table 2 and Fig. 3. The alkaline comet assay had adequate sensitivity to assess the levels of primary DNA damage in yeast cells. The values of comet parameters measured in control sample indicate the low level of spontaneous DNA damage: mean tail intensity was 0.16 ± 0.04% and mean tail length 3.06 ± 0.05 μm.

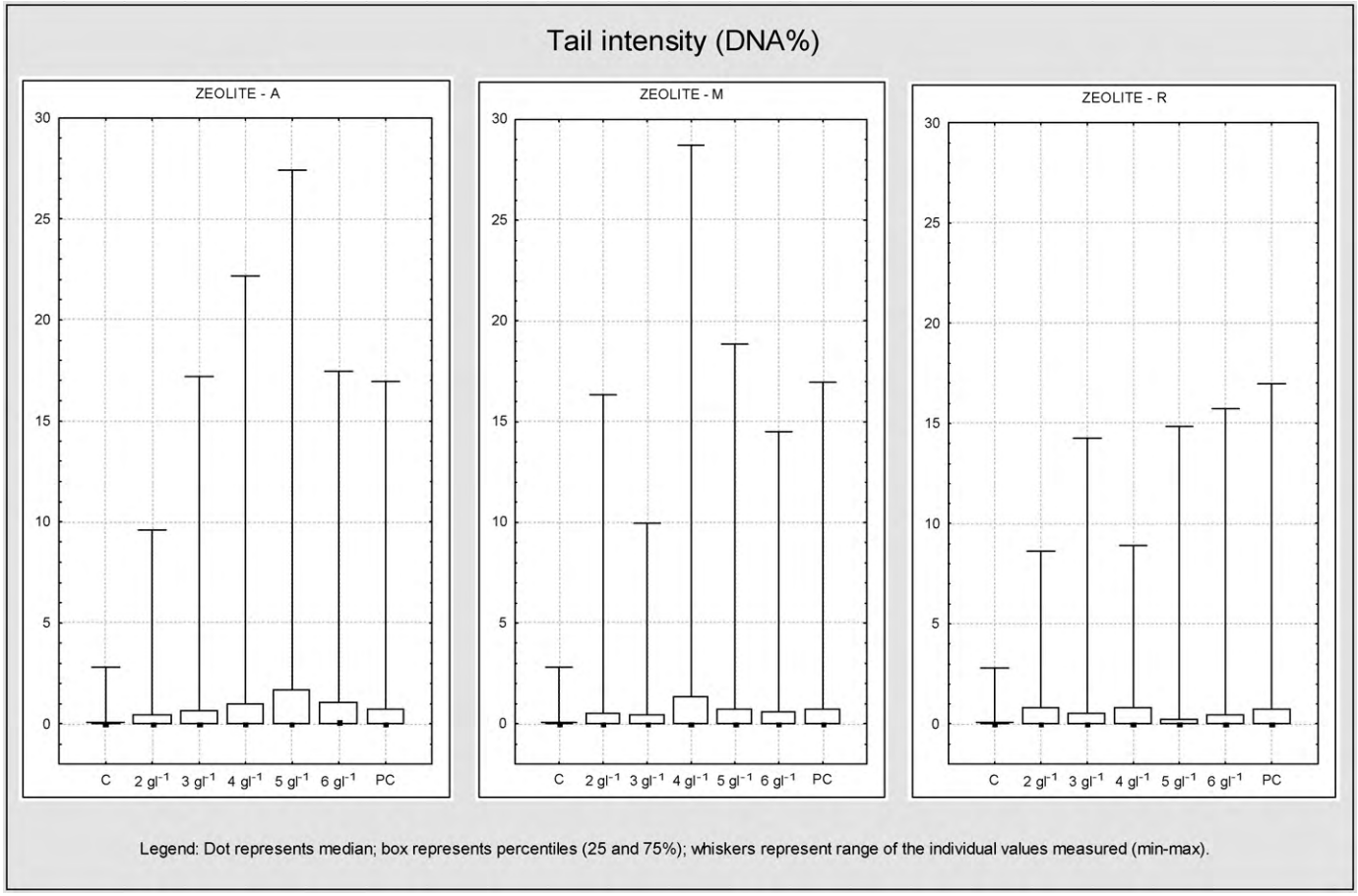

Fig. 3. Distribution of individual values of tail intensities measured in yeast cells exposed to three samples of zeolite A (A, M and R) in the concentration range of 2.0–6.0 g l^{-1} and corresponding negative (C) and positive control (PC; 10 μM hydrogen peroxide). Parameters of the alkaline comet assay were evaluated by measuring 200 comets per sample.

Table 2
Results of the alkaline comet assay expressed as mean ± SE of tail intensity and tail length, measured in yeast cells exposed to three samples of zeolite A (A, M and R) in the concentration range of 2.0–6.0 g l^{-1}.

Concentration of zeolite (g l^{-1})	Sample A	Sample M	Sample R
Tail intensity (DNA %)			
2.0	0.65 ± 0.11^{a}	0.86 ± 0.16^{a}	0.74 ± 0.11^{a}
3.0	0.96 ± 0.16^{a}	0.44 ± 0.07^{a}	0.70 ± 0.13^{a}
4.0	1.64 ± 0.40^{a,b}	1.30 ± 0.24^{a,c}	0.75 ± 0.11^{a}
5.0	1.81 ± 0.31^{a,b,c}	0.96 ± 0.16^{a,c}	0.76 ± 0.15^{a}
6.0	1.28 ± 0.20^{a,b}	0.88 ± 0.15^{a,c}	0.75 ± 0.14^{a}
Tail length (μm)			
2.0	2.99 ± 0.04^{d,e}	2.52 ± 0.04	2.70 ± 0.04^{d,e,f}
3.0	2.96 ± 0.05^{e}	2.57 ± 0.04	2.69 ± 0.04^{d,e,f}
4.0	2.76 ± 0.04	2.47 ± 0.04	2.42 ± 0.03
5.0	2.71 ± 0.04	2.54 ± 0.04	2.38 ± 0.04
6.0	3.00 ± 0.04^{d,e}	2.68 ± 0.04^{b,c,d,e}	2.36 ± 0.04

Parameters of the alkaline comet assay were evaluated by measuring 200 comets per sample. Matched negative and positive controls were studied in parallel. The value of mean tail intensity in negative control was 0.16 ± 0.04% and in positive control (10 μM hydrogen peroxide) 1.00 ± 0.18%. Corresponding values of tail length were 3.06 ± 0.05 and 2.59 ± 0.03 μm. Significantly increased values: a—compared to negative control; b—compared to concentration 2 g l^{-1}; c—compared to concentration 3 g l^{-1}; d—compared to concentration 4 g l^{-1}; e—compared to concentration 5 g l^{-1}; f—compared to concentration 6 g l^{-1}.

Three explored samples of zeolite had DNA damaging potential. All treatments pronounced a statistically significant increase of tail intensity in yeast cells as compared to negative control (Table 2). In the case of sample A, a positive dose-dependent increase of tail intensity in dose range 2.0–5.0 g l^{-1} was observed. However, yeasts treated with the highest concentration of sample A showed slightly decreased tail intensity (Table 2). This decrease might be related to reduced cell viability, e.g. the most damaged cells died and therefore escaped from detection. After treatment with zeolite M, no clear dose-dependent increase of tail intensity was observed (Table 2, Fig. 3). The mean values of tail intensity recorded after treatment with zeolite R were similar, but the range of the values recorded in individual samples points to the dose-dependent response (Table 2, Fig. 3).

Based on the obtained results, treatments with all three samples of zeolite affected more the tail intensity than tail length. It is possible that treatment with zeolites lead to cross linking between DNA strands that might slow down the migration of yeast DNA during the electrophoresis in alkaline conditions. At the lowest concentration tested zeolite M was the most genotoxic to yeast cells. However, our results indicate that the overall genotoxicity of zeolites when they were applied in higher doses was in the range: A > M > R.

The results obtained using the fermentation test, assessment of cell viability and alkaline comet assay were evaluated by the Spearman correlation analysis. Statistically significant correlations were recorded for the zeolites A and R, while for zeolite M correlations were not significant. The inhibition of fermentation and percentage of dead cells were in positive correlation with comet tail intensity for sample A ($R = 2.959$; $p = 0.042$) and sample R ($R = 3.105$; $p = 0.036$). These results indicate that increasing concentrations of zeolites lead to the increase of DNA damage in yeast cells, which was also accompanied by the inhibition of fermentation and reduced viability of treated cells.

3.3. Interaction of zeolite A with water

It is likely that the chemical composition of commercially produced zeolite A and its degree of dissolution and leaching in the water medium contributed to the toxicity observed. Therefore, the behaviour of three samples of zeolite A in distilled water has been examined during 24 h. The hydrolysis of zeolite crystals, amorphous aluminosilicate and unreacted gel in water medium and consecutive hydrolytic damage, dissolution and leaching of aluminium and silicon was detected (Fig. 4). These values of aluminium and silicon were considered to be present in experiments with bacterium and yeast. Dissolved amount of aluminium and silicon depends on the chemical and phase composition of commercial zeolite A, such as amount of amorphous phase and unreacted gel. Due to similar chemical composition of tested zeolites, the resulting amount of aluminium and silicon dissolved in water medium had similar profile. The maximum obtained concentrations of aluminium and silicon were 4.94 and 6.29 mg l^{-1}, respectively.

3.4. Mass spectrometry results

During the recording of the ESI mass spectra, sodium from zeolites and chlorine from salt acid formed several intense NaCl-aggregate signals that interfere with the interpretation of the spectra. Thus even the positive spectra of samples with pH 6.0 and 7.0 were almost useless. However, in samples with pH 8.0 the spectra showed clear signal series, which were not originated from sodium adducts. These signals were assigned to stabile, twice charged, three, four or five silicon atoms containing complexes. The change of sample cone voltage or RF-values did not change the shape of these spectra. The spectrum is illustrated in Fig. 5. The spectra of all zeolites A (sample A, M and R) were almost alike at pH 8.0 showing the series with mutual 44u distances. In negative mode only signals assigned to $[(NaCl)_xCl]^-$ could be seen.

Since the mass of these complexes was within 700–1300 Da range and they contained three to five silicon atoms, it could be iterated that the rest of the complex has to be aluminium and/or sodium connected by oxo and hydroxo bridges. The sum formula could be like this: $[Al_mH_nNa_pO_qSi_{3-5}]^{2+}$ or $[(Na(OH))_x\,(AlO(OH))_y\,(Si(OH)_4)_{3-5}]^{2+}$. Unfortunately sodium ^{23}Na and aluminium ^{27}Al are monoisotopes and thus their amount in complexes could not be verified by mass spectrometric methods. So, there are several possible solutions to assign individual signal, but no unambiguous assignation was found to whole series. The regular 44u (88 Da) sift shows some exchange reaction, where the sodium and/or aluminium oxo hydroxides are reacting on the stabile silicate cluster, but no such reaction was unveiled yet.

4. Discussion

The results showed that all three samples of investigated commercial zeolite A had a potential to reduce the survival and activity of microorganisms and cause damage of DNA. Three samples of zeolite A tested in this study acted slightly more toxic towards bacterium *A. junii* (EC_{50} values 0.138–0.328 g l^{-1}) than the reported toxicity of zeolite A to *Pseudomonas putida* isolated from wastewater treatment plant with EC_{50} value of 0.330–0.950 g l^{-1} [2]. The toxicity of tested zeolites A against yeast *S. cerevisiae* was 9–39 times lower when compared to *A. junii*. The presence of yeast cellular wall should be considered in the comparison of toxic effect of zeolites A with respect to bacteria. The cell envelope (consisting of plasma membrane, periplasmic space and cell wall) of yeast is much more ticker than those of bacteria. In *S. cerevisiae* the cell envelope takes about 15% of the total cell volume and the cell wall is remarkably tick (100–200 nm). A slight toxic effect of zeolite A used as food additive to *S. cerevisiae* has been reported [19] where the 70% of survival was obtained at zeolite concentration of 7.5%. The EC_{50} values of 2.88–5.47 g l^{-1} observed for *S. cerevisiae* were much higher than EC_{50} values of 0.425 g l^{-1} reported for freshwater cladoceran *Ceriodaphnia dubia* [20] or EC_{50} values of 1.0–1.8 g l^{-1} for cladoceran *Daphnia* sp. and 0.56–1.00 g l^{-1} for algae reported in

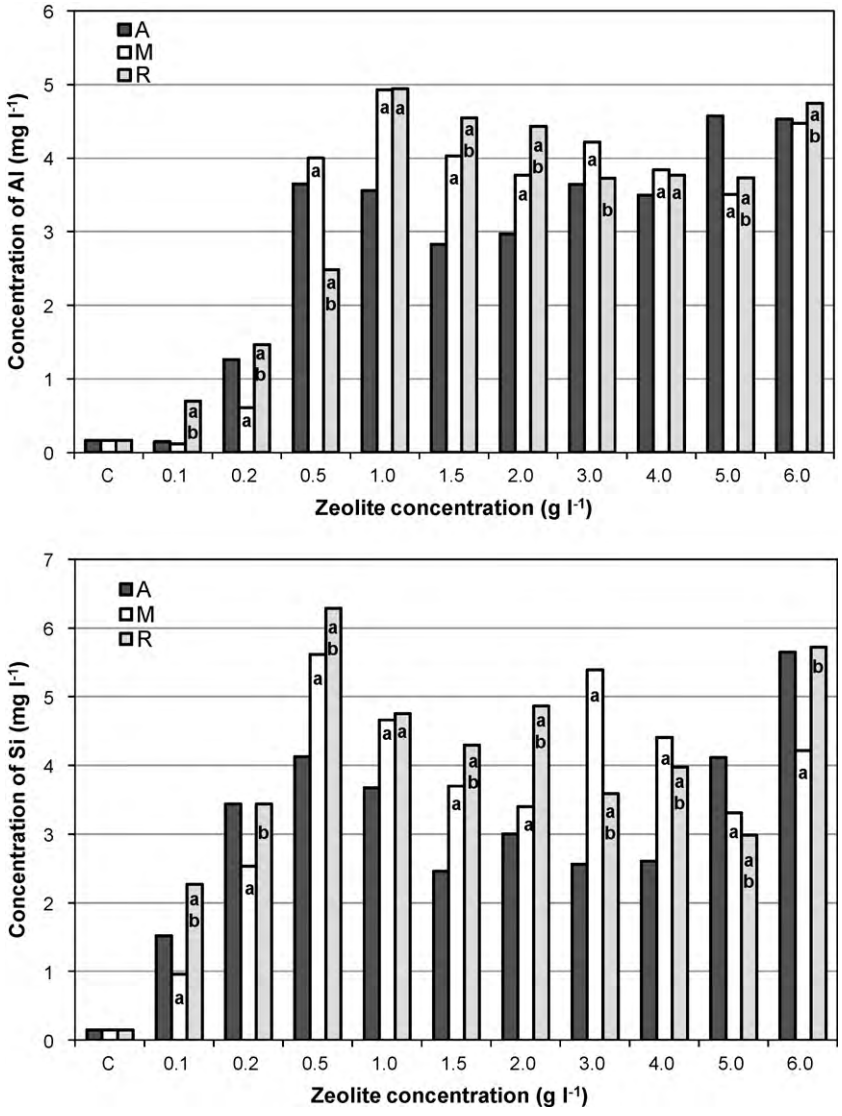

Fig. 4. Concentrations of Al and Si in water released during dissolution and leaching of three samples of zeolite A (A, M and R) in the concentration range of 0.1–6.0 g l^{-1} and corresponding distilled water as negative control (C). Significantly different values: a—compared to zeolite A; b—compared to zeolite M.

material safety data sheet. In the cases of zeolite toxicity to bacterium and yeast, the sample R appeared to be less toxic than the sample A and M, in spite of almost identical chemical composition of materials.

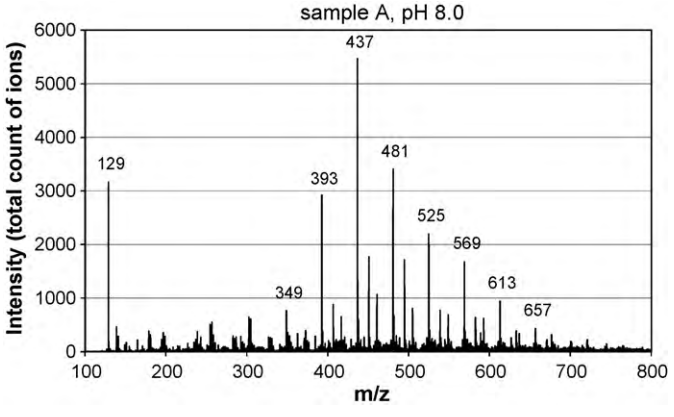

Fig. 5. The ESI MS spectrum of zeolite A (sample A) at pH 8.0. The signal series with mutual 44u distances are clearly seen.

Although the zeolites A are evidently toxic against prokaryotic and eukaryotic cells, little is given about the probable mechanism of its toxicity. The zeolite A hydrolyzes extensively in the water medium and a half life of 1–2 months is typical for waters at a neutral pH [21]. Suspended in water, zeolite A produces hydroxyl ions (OH^-) due to hydrolysis, resulting in the pH of the water slurry above 7:

$$\text{O-(Al, Si)(s)} + H_2O(l) \leftrightarrows [\text{HO-(Al, Si)}]^+(s) + OH^-(aq)$$

Exchange of surface Na^+ ions with H_3O^+ also can raise pH value:

$$Na^+\cdots\text{O-(Al, Si)(s)} + H_2O(l) \leftrightarrows \text{HO-(Al, Si)(s)} + Na^+(aq) + OH^-(aq)$$

In the experiments with *A. junii* the addition of zeolite A resulted in dose-dependent increase of the final pH of medium. Since *A. junii* grows in the pH range from 6.0 to 9.0, the pH can be eliminated as a cause of bacterial decay. In the experiments with *S. cerevisiae* the addition of zeolite A, which was previously equilibrated in distilled water and neutralised, resulted in negligible increase of final pH.

Hydroxyl ions, produced during hydrolysis of zeolite A, enhance the dissolution of aluminium and silicon from the framework and these extra framework aluminium species carry a charge, which

will be again introduced in ternary exchange processes, causing the additionally leaching of aluminium and silicon forms at inert layer of the zeolite surface and this hydrolytic damage produces colloidal material in solution [3,22,23]. The toxic effect of three examined zeolite A can be explained by influence of water soluble species originating from dissolution and leaching of aluminium and silicon in the form of positively charged complexes $[Al_mH_nNa_pO_qSi_{3-5}]^{2+}$ or $[(Na(OH))_x\ (AlO(OH))_y\ (Si(OH)_4)_{3-5}]^{2+}$, which were recorded by ESI mass spectra. The surface of microbial cell wall is predominantly electronegative and major mechanism involved in the interaction on the microbial cell wall/solution interface are electrostatic (e.g., Coulombic, dipol–dipol interaction). On the cell surface with a predominance of reactive electronegative sites (e.g. phosphoryl, carboxyl or hydroxyl), the soluble aluminosilicate polycations can participate in the formation of surface complexes [24].

Aluminium and silicon are toxic to many microorganisms including bacteria and fungi [25]. Aluminium nitrate completely inhibited the growth of *Rhizobium leguminosarum* at concentration of 0.67 mg Al l^{-1} [26]. A 5–10 s exposure of *E. coli* to 6 g l^{-1} of sodium metasilicate resulted in complete bacterial inhibition [27]. The concentrations of aluminium dissolved from examined zeolites A correlated significantly negative with the final log CFU of *A. junii* ($R = -0.649$ for A, -0.681 for M and -0.630 for R sample). The correlation between the dissolved silicon and log CFU of *A. junii* was significantly negative for zeolite samples M ($R = -0.655$) and R ($R = -0.767$), but not significant for sample A ($R = -0.417$). The correlations of the dissolved aluminium with the percentage of dead yeast cells and percentage of inhibition of fermentation were significantly positive for zeolite sample A ($R = 0.886$), but not significant for samples M ($R = 0.600$) and R ($R = 0.600$). The concentrations of dissolved silicon correlated significantly positive with the percentage of dead yeast cells and percentage of inhibition of fermentation for zeolite sample A ($R = 0.829$), but not significant for samples M ($R = 0.314$) and R ($R = 0.486$). We assume that the differences in the levels of primary DNA damage in yeast cells treated with zeolites A, M and R were dependent on degree of dissolution and leaching of zeolite in water medium. The hydrolytic damage of zeolites A definitely influenced the concentration of aluminium in water and amounts of produced reactive oxygen species, which could induce additional indirect DNA damage. Previous studies indicate that Al-stress caused formation of highly toxic oxygen free radicals in cells [28] and indirectly contributed to lipid peroxidation disturbing the protective enzymes as superoxide dismutase, glutathione peroxidase and other [29]. The concentrations of aluminium and silicon dissolved from examined zeolites A were high enough to explain the toxic action of zeolite A to bacterium *A. junii* and yeast *S. cerevisiae*. However, it should be mentioned that the aluminium and silicon dissolved from zeolite A were present in water as a small positively charged aluminosilicate molecules (as determined by mass spectrometry). The toxicity of these molecular species was not tested and their toxicity can be different to the same microorganisms than the toxicity of aluminium and silicon ions. Possible the synergistic effect of aluminium and silicon ions also played a role in the overall toxicity.

It is supposed that the aluminosilicate molecules dissolved from zeolite A in water medium had the main contribution to the toxic effect of zeolite A on tested microorganisms. This can be supported by previous finding [19] that zeolite A did not act toxic or mutagenic to *S. typhimurium* by applying the small zeolite crystals directly on the agar plate, where no dissolution and leaching of zeolite occurred. The accumulation of the zeolite nanoparticles in the cell wall of bacteria [30] and yeast or even inside cells is possible to occur, which can play a role in mechanism of zeolite toxicity. Namely, the 0.5–3.0 μm large particles of zeolite A brought into close contact with cells of Gram-negative bacteria and *S. cerevisiae* caused the ruptures in cell walls, resulting in death of cells [31].

The synthetic zeolite A has a unique architecture completely different from the framework tectosilicates found in nature and in contact with microorganisms is recognised as a strange compound which can be defined as xenobiotic. As in the case with other xenobiotics, microorganisms needs time to adapt to new compound in order to get the energy source for cell. The zeolite A in its structure has no macro- or micro-nutrients which can be interesting for cell. Therefore, the time adaptation of microorganisms to zeolite A cannot be supposed.

5. Conclusions

From ecological point of view, the commercial synthetic zeolite A, if accumulated in environment at concentrations higher than 0.1 g l^{-1}, can result in negative impact on the microbial structure. In the real wastewater containing suspended solids, the interaction of synthetic zeolites with natural microbial population can differ from the ones in laboratory conditions presented in this study. The further interdisciplinary studies on the mechanism of toxicity and antimicrobial activity of synthetic zeolites to microorganisms are needed to elucidate the general impact of synthetic zeolites on the environment. The antimicrobial property of synthetic zeolites can be also used for their possible beneficial application as biocidal material.

Acknowledgements

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