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THE TOXICITY OF ALUMINIUM SALTS TO LECANE INERMIS
ROTIFERS: ARE CHEMICAL AND BIOLOGICAL METHODS
USED TO OVERCOME ACTIVATED SLUDGE BULKING
MUTUALLY EXCLUSIVE?

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Abstract: The aim of this study was to assess the effects of two flocculants that are often used to overcome activated sludge bulking problems – aluminium chloride, $AlCl_3$, and aluminium sulphate, $Al_2(SO_4)_3$ – on *Lecaneinermis* (Rotifera, Monogononta) at three different temperatures: 8, 15 and 20°C. The mean EC50 value (effective concentration, $mg\ dm^{-3}$) calculated for the 24 h mortality test was $0.012\ mg\ Al^{3+}dm^{-3}$. Next, the effects of low concentrations of the Al-salts on the population development from single individuals (parthenogenetic females) were tested in a 21-day experiment. At concentrations as low as EC4.8 and EC0.48, both Al-salts affected rotifer population negatively. However, temperature was the most pronounced factor that modified the toxicity of the Al-salts to the rotifers. On the 12th day of the experiment, there were significant interactions between temperature and the Al-salts, indicating that the chemicals were more toxic to the rotifers at 20°C than at lower temperatures. The weaker rotifers sensitivity to Al-salts (especially to $AlCl_3$) in temperatures below 15°C, when the biggest problems associated with sludge bulking occurs, may mean use both rotifers and chemicals reasonable and effective.

INTRODUCTION

The excessive growth of filamentous bacteria that cause the bulking of activated sludge is a serious problem in many low-loaded wastewater treatment plants. Currently, applied methods are limited to the application of appropriate chemicals, such as aluminium chloride or sulphate. The addition of flocculants improves the settlement characteristics of the sludge. On the other hand, flocculants could also increase the production of excessive sludge [1, 2] or could interfere with nitrification process [2]; the disadvantage of chemical methods is also high cost and aluminium toxicity to non-target organisms [3]. Soddell and

Seviour [4] mentioned some attempts of using different microorganisms, among them ciliates, bacteria or even commercially available bacteria mixture, to control foaming. However, none of those methods proved to be fully successful [4]. Little attention has been paid to the possibility of controlling filamentous bacteria using organisms that occur naturally in the activated sludge. Recently a new, promising biological method was described, having an additional advantage of reducing the excessive sludge production if sufficiently effective consumers are used [5]. Briefly, the idea consists in rotifers mass breeding in chambers submerged in bioreactor volume and letting them out if sludge bulking occurred. The question arises regarding whether Al-salts can be used simultaneously with rotifers, because rotifers may be subjected to toxic effects several days after chemical application since the sludge in wastewater treatment plants circulates in a partially closed system.

Rotifer species differ in their sensitivity to toxicants. McDaniel and Snell [6] compared the toxicity of cadmium and pentachlorophenol to nine rotifers species and noticed that the toxic effect for both chemicals varied considerably between the species tested. They showed that for cadmium, *Brachionuscalyciflorus* had the highest EC50 value at 0.270 mg dm⁻³, which was 27 times higher than the lowest value, observed for *Euchlanisdilatata* at 0.010 mg dm⁻³ with a mean EC50 value for these nine tested species equal to 0.110 mg dm⁻³. Much of these data are available for *Brachionus* spp. rotifers, which are used in standardised toxicological tests, such as Rotokit FTM [7]. However, similar data for *Lecane* spp. are scarce.

McDaniel and Snell [6] tested *Lecanequadridentata* and showed that, compared to other species, *L. quadridentata* was moderately sensitive to cadmium and pentachlorophenol. The most scrupulous study with *Lecane* spp. rotifers and a range of different chemicals was conducted by Pérez-Legaspi and Rico-Martínez [8]. In their study, three rotifer species, *Lecanehamata*, *L. luna* and *L. quadridentata*, were submitted to acute toxicity tests to compare their susceptibility to 11 toxicants, including trace metals such as cadmium, chromium, copper and lead [8]. In acute tests with 48 h exposure of neonates, they showed differences in EC50 values of up to 22-fold in the three species that were susceptible to lead. McDaniel and Snell [6] found an EC50 value of 0.046 mg dm⁻³ for cadmium in an acute test for *L. quadridentata*, while Pérez-Legaspi and Rico-Martínez [8] found a value of 0.23 mg dm⁻³, which is more than six times higher. These data indicate large differences in sensitivity to toxicants, even among members of the same rotifer genus. From another point of view, differences in toxic effects of these substances on the same rotifer species may be greatly influenced by the test conditions. Results for cadmium toxicity for *L. quadridentata* might differ because of differences in exposure duration, rotifer age and the geographical origin of the rotifers. Aluminium toxicity for *Lecane* spp. rotifers was determined recently by Guzmán et al. [9], who conducted acute toxicity tests to determine EC50 values for Al, Fe and Zn using *L. quadridentata*. The authors determined that the EC50 value for Al was 0.1572 mg dm⁻³, which is likely the sole estimation of Al toxicity for *Lecane* rotifers. Thus, there is a serious gap in our knowledge in this matter [10].

The biggest problems associated with bulking and foaming occur when the temperature of the sludge drops below 15°C. In most cases, this is caused by the over proliferation of the most troublesome bacteria, *Microthrixparvicella*, which are able to grow at appreciable rates at temperatures as low as 7°C [11]. Simultaneously, the

population growth rate of rotifers is strongly limited by temperature [12], so in many cases it may be difficult to obtain a sufficient rotifer density in reactors during the winter season, even though *L. inermis* is one of the most fecund species of rotifers [13]. The toxic effect of flocculants may change with temperature, and thus it is particularly important to include this factor when assessing the influence of chemicals on the vitality and reproductive rate of the rotifers.

The aim of presented study was to assess the toxic effects of aluminium chloride, AlCl_3 , and aluminium sulphate, $\text{Al}_2(\text{SO}_4)_3$, flocculants typically used to overcome the bulking problem, on the monogonont rotifer, *Lecaneinermis*. The experiments presented here comprised of testing the toxicity of Al-salts to *L. inermis* at three temperatures (8, 15 and 20°C) that reflect the annual temperature distribution in the majority of municipal wastewater treatment plants in the temperate climate zone. We wanted to assess to what extent temperature factor must be taken into consideration when applying simultaneously the biological and the chemical methods of overcoming the activated sludge bulking problem.

MATERIALS AND METHODS

Two clonal populations of the monogonont rotifer *Lecaneinermis* called Lk1 and Lk3 were used in the experiments. Both strains were isolated from a sludge sample from wastewater treatment plants in southern Poland. The cultures were maintained in darkness at a temperature of 20°C (Sanyo Versatile Environmental Test Chambers). We performed range-finding tests using five concentrations plus controls for each toxicant. Test concentrations represented a logarithmic series and ranged from 0.002 to 20 g dm⁻³ for both of the Al-salts employed here (POCh Poland). The lowest concentration with 100% mortality was chosen as the upper limit and additional intermediate concentrations were included in the final test. Finally, the following concentrations of both Al-salts were applied: 0 (control), 0.0001, 0.0005, 0.001, 0.005, 0.01, 0.05 and 0.1 mg dm⁻³. Rotifers from strains Lk1 and Lk3 (ca 10 individuals, age <1 day) were picked up with the micropipette and transferred into separate wells (24 wells Cell Wells™, Corning). Then, 1 ml of aluminium chloride, AlCl_3 , or aluminium sulphate, $\text{Al}_2(\text{SO}_4)_3$, solution was added. The Al-salt solutions were enriched with molasses (Greenland Technology, Poland) as a medium [14]. The culture plates were incubated at 8, 15 and 20°C, and the number of alive and dead rotifers was checked after 24 h of exposure. Tests in which the control mortality was 10% or higher were considered to be invalid. Four replicates of each treatment were then applied. The EC50 (the effective concentration caused 50% mortality in mg dm⁻³) was calculated from a log-linear model [13].

After the EC50 was determined, we measured effects of low concentrations of AlCl_3 and $\text{Al}_2(\text{SO}_4)_3$ on the rotifer reproduction and population growth rate. Single individuals of Lk1 or Lk3 strains (age <1 day) were transferred into separate wells, and 1 ml of 0.0005 or 0.005 mg dm⁻³ of Al-salt solutions with molasses were added. The culture plates were then incubated at 8, 15 and 20°C. Ten replicates were used for controls, and six replicates each were used for Al-salts, both divided in two experimental series. The number of alive and dead rotifers, as well as the number of eggs, was counted directly in each well after 4, 7, 12 and 21 days of the experiment under magnification. Wells containing only dead rotifers were excluded from further observations and analyses. For each well and

treatment mortality rate and internal growth rate (r) were calculated [12]. Mortality rate was determined by dividing all dead rotifers in an experimental well by the number of active rotifers, while the internal growth rate (r) was calculated as $r = (\ln N_{t+1} - \ln N_t) / t$.

A multifactor ANOVA was used to compare differences between the treatments and the interactive effects between the tested factors. When significant differences between groups were found, the means were compared using a Tukey's HSD test. Non significant interactions were removed from the model. First, EC50 values were evaluated to identify any significant differences between Al-salt treatments, strains and tested temperatures, as well as the interactive effects between them. Next, the effects of the experimental treatments on particular variables measured in low-concentration experiment were tested. These variables included the following separately for AlCl_3 and $\text{Al}_2(\text{SO}_4)_3$ on each day of the experiment: the number of alive rotifers, the number of eggs, the mortality rate and the internal growth rate (r). All statistical analyses were conducted using Statgraphics Centurion XVI.

RESULTS

EC50 values did not differ between Al-salts and temperature treatments or between rotifer strains and the mean value was $0.0519 \text{ mg dm}^{-3}$ Al-salts, which corresponded to $0.012 \text{ mg Al}^{3+} \text{ dm}^{-3}$. Thus, Al-salts of 0.005 mg dm^{-3} that were tested in a next step corresponded to an EC value of 4.8 (i.e., the concentration caused a mortality rate of ca. 5%), whereas $0.0005 \text{ mg dm}^{-3}$ resulted in an EC value of 0.48 (i.e., the concentration caused a mortality rate of ca 0.5%). Even such low concentrations affected population characteristics negatively during the 21-day experiment.

The number of live rotifers reached up to 255 individuals per well (Lk3 cultured 12 days at 20°C and $0.0005 \text{ mg dm}^{-3}$ of $\text{Al}_2(\text{SO}_4)_3$). Temperature was the most important factor affecting the number of live rotifers ($F > 102$, $p < 0.0001$). On the fourth and seventh days of the experiment, the number of live rotifers was similar at the lower temperatures (8°C and 15°C) and was significantly higher at 20°C . After the fourth day of the experiment, the differences in the number of live rotifers number between particular temperatures were intensified and were the highest on the 12th day of the experiment, when numbers of live rotifers ranged from lowest to highest at the following temperatures: $8^\circ\text{C} < 15^\circ\text{C} < 20^\circ\text{C}$. After the 12th day of the experiment, significant increases in the number of live rotifers were observed at 15°C . Conversely, at 20°C , the increase was not observed, and on the 21st day of the experiment, the number of rotifers at 20°C was similar to that at 15°C .

The Lk3 strain reached a higher population density than the Lk1 strain, and there were significant interactions between the strain and temperature effects ($p < 0.005$). On the 4th, 7th and 12th days of the experiment, this interaction indicated that at 20°C , the Lk3 strain reached a higher population density than the Lk1 strain. In turn, on the 21st day of the experiment, the interaction indicated that at 15°C , the Lk3 strain reached a higher population density than the Lk1 strain ($p < 0.004$).

The effect of negative Al-salts on the number of live rotifers was not observed until the 12th day of the experiment ($p < 0.0001$ for AlCl_3 and $p < 0.0002$ for $\text{Al}_2(\text{SO}_4)_3$ contamination). A negative effect of $\text{Al}_2(\text{SO}_4)_3$ continued to be observed on the 21st day of the experiment ($p < 0.004$). The interaction between the strain and the Al-salt concentration

was nonsignificant, indicating that both strains were similarly sensitive to Al-salts. The most interesting result was the significant interaction between temperature and the concentration of Al-salts on the 12th day of experiment. At 20°C, the chemicals were more toxic to the rotifers than at lower temperatures, and the effect was more distinct for AlCl_3 (Fig. 1A, B).

The mortality rate was as high as 9.07 (Lk1 after 21 days at 20°C in a control treatment). The mortality rate of rotifers during the low-concentration experiment was affected mainly by temperature. On fourth day of the experiment, the Lk1 strain was characterised by a higher mortality rate than the Lk3. However, a significant interaction

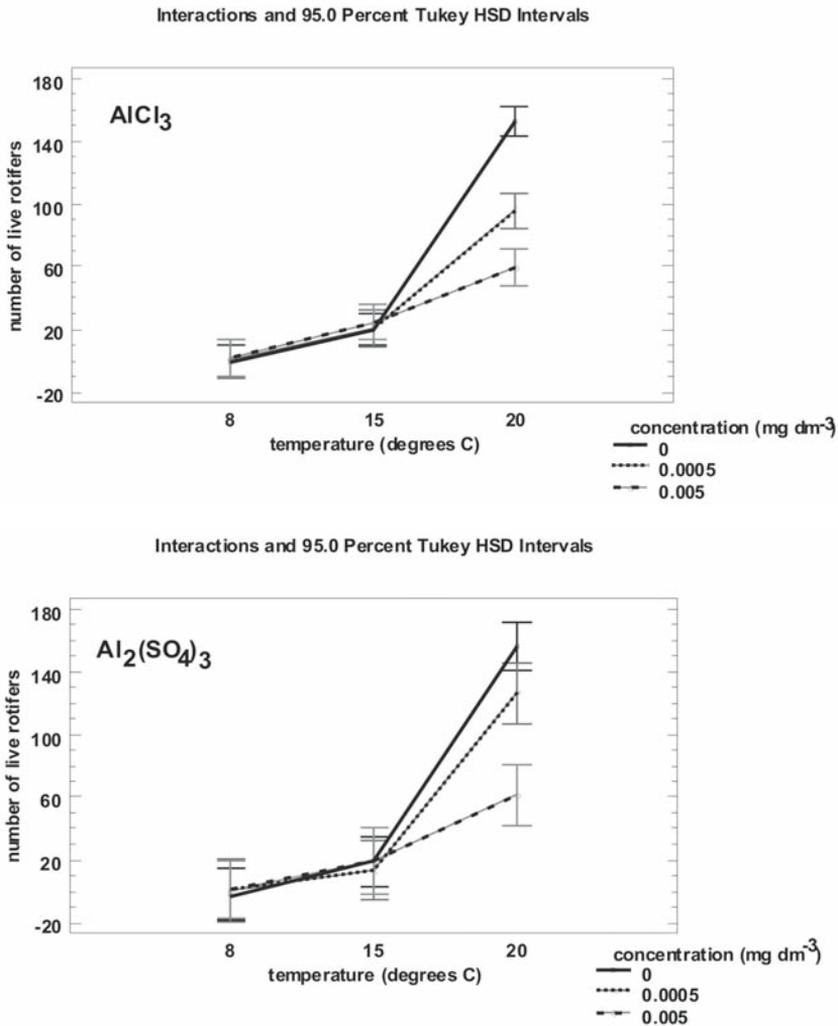


Fig. 1. Effects of the interaction between temperature and (A) AlCl_3 and (B) $\text{Al}_2(\text{SO}_4)_3$ on the number of live rotifers per well on the 12th day of the experiment. Central points indicate the sample means and error bars indicate 95% Tukey HSD intervals.

between temperature and strain ($p < 0.0001$ for both AlCl_3 and $\text{Al}_2(\text{SO}_4)_3$ contamination) indicated that the mortality rate of the Lk1 strain was only higher at 8°C . The opposite effect was observed at subsequent measurements, as the Lk3 strain exhibited a higher mortality rate than the Lk1 on the 12th day ($p < 0.002$ for AlCl_3 and $p < 0.04$ for $\text{Al}_2(\text{SO}_4)_3$ contamination) and on the 21st day ($p < 0.04$ for AlCl_3). The effect of toxicants depended on temperature. On the 12th day, both AlCl_3 and $\text{Al}_2(\text{SO}_4)_3$ contamination caused a higher mortality rate than that observed in the controls ($p < 0.0005$ and $p < 0.004$, respectively), and there was a significant interaction between temperature and Al-salt concentration ($p < 0.02$ and $p < 0.0001$, respectively). This indicated that, at 20°C , the chemicals caused a higher mortality rate than that at lower temperatures (Fig. 2 A, B).

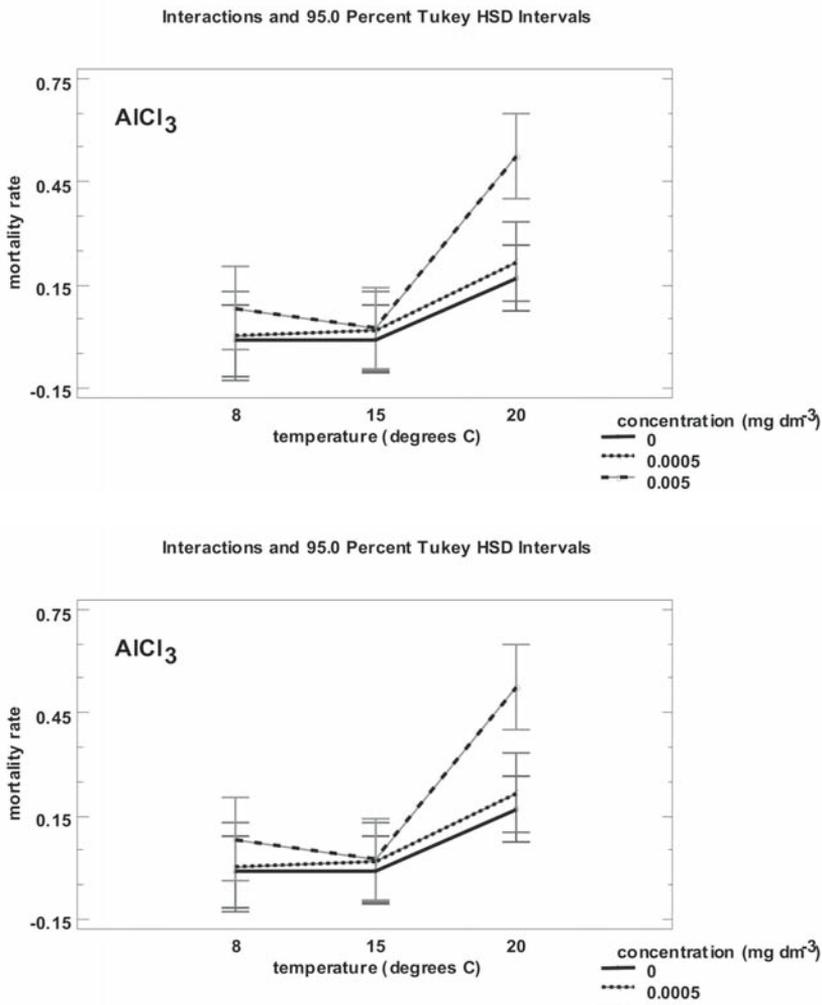


Fig. 2. Effects of the interaction between temperature and (A) AlCl_3 and (B) $\text{Al}_2(\text{SO}_4)_3$ on rotifer mortality rates on the 12th day of experiment Central points indicate the sample means and error bars indicate 95% Tukey HSD intervals

The number of eggs was as high as 255 per well (found in Lk3 cultured for seven days at 20°C and 0.0005 mg dm⁻³ of Al₂(SO₄)₃). The number of eggs per well increased with the experiment duration up to the 12th day and was slightly lower on the 21st day. Temperature positively affected the number of eggs laid. A negative effect of both AlCl₃ and Al₂(SO₄)₃ on eggs number was observed on the 12th and the 21st day of the experiment. On the 12th day, there was a significant interaction between temperature and the concentration of Al-salts ($p < 0.0001$ for both AlCl₃ and Al₂(SO₄)₃ contamination), indicating that, at 20°C, the negative influence of chemicals on the number of laid eggs was stronger than at lower temperatures (Fig. 3 A, B). The Lk3 rotifer strain produced more eggs than the Lk1 strain, especially at 20°C, but there was no difference between the strains on the 21st day of the experiment.

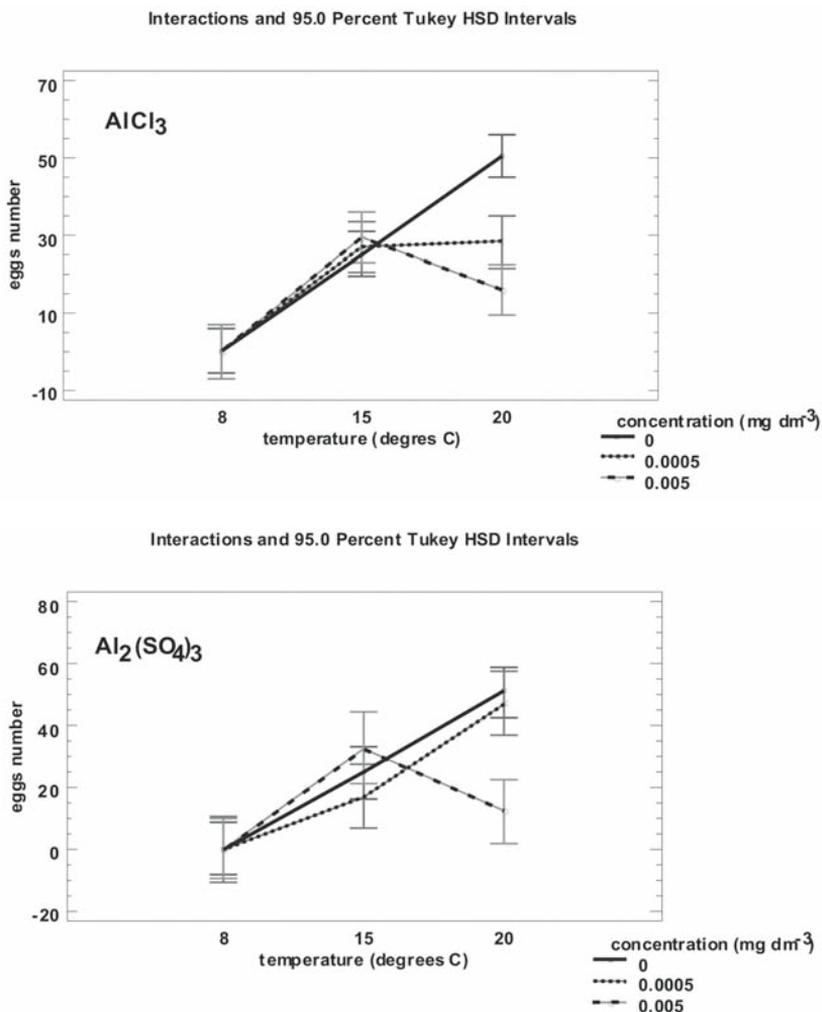


Fig. 3. Effects of interaction between temperature and (A) AlCl₃ and (B) Al₂(SO₄)₃ on the number of eggs laid per well on the 12th day of the experiment. Central points indicate the sample means and error bars indicate 95% Tukey HSD intervals.

Population growth rate (r) depended strongly on temperature. The temperature effect was highly significant ($p < 0.0001$) for each experimental period (0–4, 4–7, 7–12 and 12–21 days). Initially (between days 0 and 4), the r value was the highest at 20°C, but at the end of the experiment (between the 12th and 21st day), the r value reached a maximum at 15°C, as did the number of eggs per well. There was a significant reduction of the r value because of the Al-salt contamination, though the effect was significant only between the 7th and 12th days of the experiment ($F = 10.60$, $p < 0.0001$ for $AlCl_3$ and $F = 9.62$, $p < 0.0003$ for $Al_2(SO_4)_3$) and between the 12th and the 21st day of the experiment for $Al_2(SO_4)_3$ ($F = 3.81$, $p < 0.03$). In each case, there was a significant interaction between Al-salt contamination and temperature (Fig. 4 A, B), indicating that, at 20°C, the chemicals reduced the r value to a greater extent than at lower temperatures.

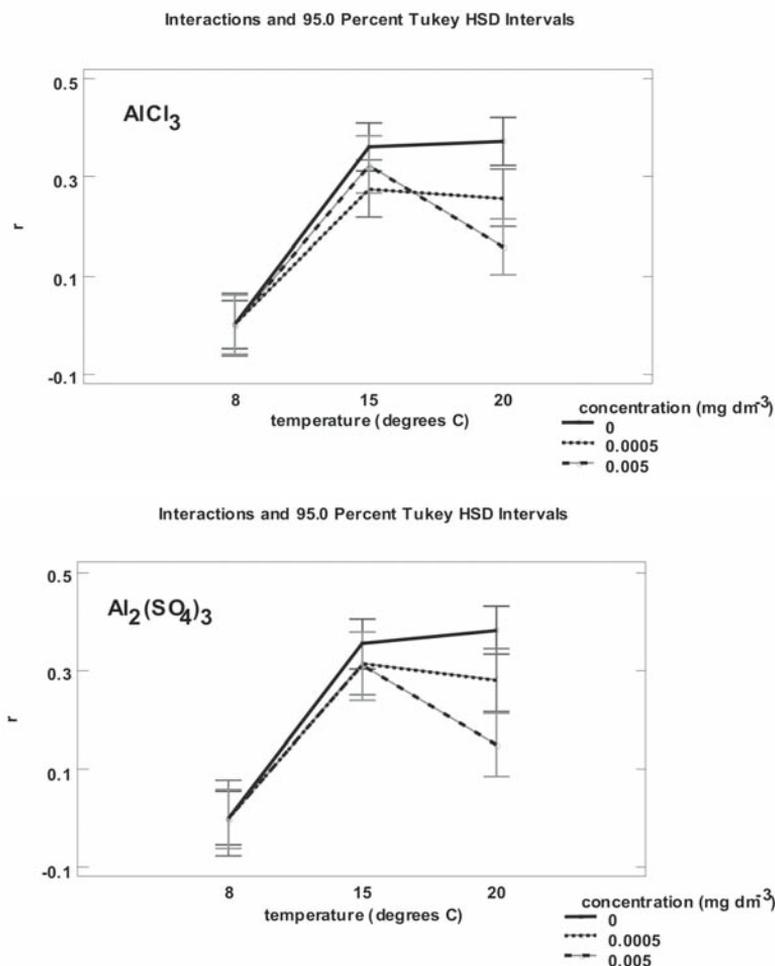


Fig. 4. Effects of the interaction between temperature and (A) $AlCl_3$ and (B) $Al_2(SO_4)_3$ on the internal growth rate (r) of rotifers on the 12th day of the experiment. Central points indicate the sample means and error bars indicate 95% Tukey HSD intervals.

DISCUSSION

Even though the biology of *L. inermis* is relatively well described [13], up to now there was no ecotoxicological data available. The EC50 value for aluminium ions applied as $AlCl_3$ estimated for other *Lecane* spp. rotifers, i.e., *L. Quadridentata* was determined on $0.1572 \text{ mg Al}^{3+} \text{ dm}^{-3}$ [9]. In our study, the mean EC50 values calculated for both Al-salts employed in experiments was $0.012 \text{ mg Al}^{3+} \text{ dm}^{-3}$. As mentioned earlier, considerable differences exist between the sensitivity of *Lecane* species to toxicants. In our study, the differences in EC50 values may have been due to the rotifers originating from wastewater treatment plants. Bioreactors with activated sludge constitute substantially different environments in comparison to natural water bodies or rivers, and some differences in rotifer biology may occur despite prolonged laboratory cultivation.

It is unclear whether Al-salts can be used simultaneously with rotifers to overcome activated sludge bulking. The recommended concentration of Al^{3+} for controlling activated sludge bulking resulting from the intensive growth of *Microthrixparvicella* ranged from 2.0 to 2.5 g kg^{-1} of dry mass of sludge [15]. Laboratory trials have shown that the dosage of Al^{3+} should be less than $7 \text{ g Al}^{3+} \text{ kg}^{-1}$ of dry mass of sludge. With an increased dosage, an increase in the free bacteria and a decrease of the protozoa activity are observed [16]. Usually, dosing the appropriate chemicals is given as grams of Al^{3+} per kilogram of dry mass of sludge, but there are some difficulties in relating this value to the concentration of aluminium ions in experimental solutions, especially since the pollutants in wastewater are associated with particles to such a large extent [1]. Determining the lowest effective concentration of flocculants that is sufficient to eliminate sludge bulking is important, as it reduces both costs and the chemicals concentration in excessive sludge. Moreover, the filamentous bacteria may develop resistance to flocculants [17]. Rotifers may experience toxic effects several days after the application of chemicals, as the sludge in wastewater treatment plants circulate in a partially closed system. The age of the sludge is an important parameter describing a treatment plant functioning [18]. Thus, it was important to assess the prolonged effects of the low concentration of flocculants. We tested the effects of Al-salts on rotifer populations during a 21-day experiment. In our experiment, the rotifer population was the biggest on the 12th day of the experiment, and at the same time, the toxic effects of the Al-salt were most noticeable. After the 12th day of the experiment, there were symptoms of decay in the rotifers population, both in experimental treatments and controls. This was likely caused by the accumulation of rotifer metabolic products in the medium. Generally, rotifer populations cultured in a confined environment, such as in closed batch systems, tend to decrease significantly towards the end of the culture period [19] and such effect is not expected when rotifers are cultured in flow systems.

The aim of this study was to compare the toxic effects of Al-salts to *Lecane* rotifers in three different temperatures. The temperature in bioreactors in wastewater treatment plant depends on air temperature, and in a temperate climate zone may fall below 10°C in the winter season. Temperature affects the duration of the developmental period in poikilothermic animals, which increases with lower temperatures and decreases with higher temperatures [20]. At lower temperatures, there is a delay in the onset of reproduction, embryonic egg development and hatching time. The effect of temperature on rotifers is also the result of the plasticity of indirect effects of the changes in body

size and egg volume, which was demonstrated to be significantly correlated with life expectancy at hatching, generation time and net reproductive rate [21].

From a theoretical perspective, chemical toxicity increases with temperature in aquatic environments [22]. However, detoxification mechanisms and excretory processes increase with temperature, and these may serve to cancel out the temperature effect. In other words, a temperature change may make a given chemical more or less toxic to an organism. Experimental results regarding trace metal toxicity to invertebrates appear to confirm that extreme temperatures increase the toxicity of metals [23]. Such results were obtained by Rathore and Khangarot [24] in an experiment with the sludge worm *Tubifex tubifex*. They showed that the acute toxicity of the trace metals increases with temperature. Also, Gupta et al. [25] showed similar relationships for copper toxicity in the freshwater pond snail, *Viviparus bengalensis*. Small aquatic animals maybe exposed to greater amounts of heavy metals because of increased diffusion or activity that induces increases in the rate of metal movement from water to the cell membranes. With a greater metabolic rate at higher temperatures, the heavy metals would act more rapidly on the cells, and death would occur sooner [24].

Generally, temperature was the most pronounced factor affecting the rotifer population characteristics in our experiment and strongly modifying the toxic effects of the Al-salts. The interactions between temperature and the Al-salts indicated that the effect of both chemicals was more toxic at higher than at lower temperatures. These interactions were significant for both of the Al-salts tested, though during the 21 days of the low-concentration experiment, $AlCl_3$ seemed to be more toxic to the rotifers than $Al_2(SO_4)_3$. Interactions between temperature and the concentration of the Al-salts caused the number of live rotifers, the number of eggs laid and the r coefficient to decrease, especially at $20^\circ C$, compared to the respective control treatments. A simultaneous increase in the mortality rate was also observed. It must be noticed, however, that we determined temporary mortality rate in our experiment, and as dead individuals decay during a few days, so our mortality rate assessments were not absolute values.

The negative effect of Al-salts, especially $AlCl_3$ on the rotifers population size of *L. inermis* rotifers was almost two times bigger in $20^\circ C$ than in $15^\circ C$ or $8^\circ C$. The significantly smaller rotifers sensitivity to Al-salts in lower temperatures is, however, good news, as the biggest problems with sludge bulking occur in lower temperatures. As *L. inermis* reproduction rate and their effectiveness in filaments removal is much higher in higher temperatures the most reasonable may be preventive introduction of rotifers to the bioreactors before winter season and then chemicals addition when temperature decreases. Detecting the toxic effects may depend on population parameters, which were considered in an experiment [26]. Our study indicates that seasonal temperature changes are important in determining the amount of flocculants that may be used in wastewater treatment plants. However, temperature is still a major factor affecting the rotifer population size and influencing the possibilities for using rotifers as a tool to overcome activated sludge bulking.

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TOKSYCZNOŚĆ SOLI GLINU DLA WROTKÓW *LECANE INERMIS*:
CZY CHEMICZNE I BIOLOGICZNE METODY ZWALCZANIA PUCHNIĘCIA OSADU CZYNNEGO
WYKLUCZAJĄ SIĘ WZAJEMNIE?

Sole glinu są powszechnie stosowanym flokulantem, służącym zwalczaniu puchnięcia osadu czynnego w biologicznych oczyszczalniach ścieków. Nowa idea biologicznego zwalczania tego niekorzystnego dla prawidłowej pracy oczyszczalni zjawiska polega na zastosowaniu wrotków z gatunku z *Lecaneinermis*. Wrotki te naturalnie występują w osadzie czynnym i są w stanie zjadać bakterie nitkowate, jak *Microthrixparvicella*, sprawiające najwięcej problemów w eksploatacji oczyszczalni podczas miesięcy zimowych. Celem badań było porównanie toksyczności chlorku glinu $AlCl_3$ oraz siarczanu glinu $Al_2(SO_4)_3$ dla wrotków *Lecaneinermis* w trzech temperaturach: 8, 15 and 20°C. Średnią wartość EC_{50} (stężenie powodujące 50% efekt, $mg\ dm^{-3}$) dla śmiertelności wrotków na podstawie 24-godzinnego testu ustalono na poziomie $0.012\ mg\ Al^{3+}\ dm^{-3}$. Następnie, badano wpływ niskich stężeń soli glinu na tempo wzrostu populacji z pojedynczego osobnika (partenogenetyczna samica) w 21-dniowym eksperymencie. Ustalono, że stężenia na poziomie odpowiadającym $EC_{4.8}$, a nawet $EC_{0.48}$ wpływają negatywnie na tempo wzrostu populacji. Temperatura silnie wpływała na toksyczność glinu. W 12-tym dniu eksperymentu stwierdzono, że zachodzi istotna interakcja pomiędzy toksycznością glinu i temperaturą, wskazująca, że w 20°C glin jest bardziej toksyczny dla wrotków niż w niższych badanych temperaturach. Mniejsza wrażliwość wrotków na glin w temperaturze poniżej 15°C może oznaczać, że łączenie tych dwóch metod zwalczania puchnięcia osadu czynnego w miesiącach zimowych może być racjonalnym i efektywnym rozwiązaniem.