



External modulation of Rotimer exudate secretion in monogonant rotifers

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Catechin (PubChem CID: 9064)
Choline chloride (PubChem CID: 6209)
DMSO (PubChem CID: 679)
EDTA (PubChem CID: 6049)
Ethyl nicotinate (PubChem CID: 69188)
Glucose (PubChem CID: 5793)
Glycerol (PubChem CID: 753)
Isoguvacine (PubChem CID: 3765)
KCl (PubChem CID: 4873)
LiCl (PubChem CID: 23681138)
Meclofenoxate hydrochloride (PubChem CID: 19379)
MgCl₂ (PubChem CID: 24644)
NaCl (PubChem CID: 5234)
Selegiline (PubChem CID: 26757)
Spermidine (PubChem CID: 1102)
SrCl₂ (PubChem CID: 6101868)
Taurine (PubChem CID: 1123)

ABSTRACT

The Rotimer, a rotifer-specific biopolymer, is an exogenic bioactive exudate secreted by different monogonant species (e.g. *Euchlanis dilatata* or *Lecane bulla*). The production of this viscoelastic biomolecule is induced by different micro-particles, thereby forming a special Rotimer-Inductor Conglomerate (RIC) in a web format. In this case, the water insoluble Carmine crystals, filtered to size (max. diameter was 50 µm), functioned as an inductor. The RIC production is an adequate empirical indicator to follow up this filamentous biopolymer secretion experientially; moreover, this procedure is very sensitive to the environmental factors (temperature, pH, metals and possible natural pollutant agents). The above mentioned species show completely different reactions to these factors, except to the presence of calcium and to the modulating effects of different drugs. One of the novelties of this work is that the Rotimer secretion and consequently, the RIC-formation is a mutually obligatory and evolutionary calcium-dependent process in the concerned monogonants. This *in vivo* procedure needs calcium, both for the physiology of animals and for fiber formation, particularly in the latter case. The conglomerate covered area (%) and the detection of the longest filament (mm) of the given RIC were the generally and simultaneously applied methods in the current modulating experiments. Exploring the regulatory (e.g. calcium-dependency) and stimulating (e.g. Lucidril effect) possibilities of biopolymer secretion are the basis for optimizing the RIC-production capacities of these micro-metazoans.

Abbreviations: cAMP, cyclic adenosine monophosphate; CCA, conglomerate covered area; DMSO, dimethyl sulfoxide; DW, distilled water; ED, *Euchlanis dilatata*; EDTA, ethylenediaminetetraacetic acid; LB, *Lecane bulla*; LFC, longest filament of conglomerate; NIH, National Institutes of Health; RIC, Rotimer-Inductor Conglomerate; SEM, standard error of the mean.

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1. Introduction

Nowadays, molecular investigations are dominant parts of biological research. Despite of this, the renaissance of supramolecular and experimental biology is manifested in the various applications of their numerous advantages. The metazoan-based ‘micro-in vivo’ systems are inevitable in all types of fundamental researches (e.g. longevity; Maccsai et al., 2018) and for high throughput screening (e.g. viability; Olah et al., 2017; Rico-Martínez et al., 2016).

One of the scientific profiles of the rotifer-type-models is the complex description and characterization of different environmental (temperature, pH and salinity) or biological (toxicity, modulation, abnormalities, biomolecule productions, etc.) effects. Rotifers highly tolerate natural environmental changes; moreover, they are able to survive life-incompatible conditions by halting activities, such as reducing metabolism by suspending active life or egg production (Jönsson and Wojcik, 2017; Kanazawa et al., 2017; Shain et al., 2016). Benefiting from these evolutionally developed anatomic and physiologic abilities (Gilbert, 2017), rotifers respond to external challenges by producing different types of biomolecules (e.g. biopolymers; Datki et al., 2021) applying optimized phenotypic plasticity.

Biopolymers are produced by living organisms from specific monomers; therefore, they can be classified based on their basic chemical structures: polypeptides, polysaccharides, polynucleotides and combined forms (Mohan et al., 2016). The structural formation of polymers always requires cross-linkers, such as metal ions, e.g. calcium (Silva et al., 2009). These biomolecules (e.g. cellulose, chitin, spider silk, casein, collagen, albumin in Plant or Animal kingdoms) have been deeply investigated and their multilevel applications are unquestionable (Niaounakis, 2015). However, their properties are very diverse according to how the producing species adapt to the environment.

As the eco-friendly approach becomes dominant in industrial developments, the relevance of biopolymers gains more importance due to their biodegradable, renewable and environmentally friendly nature (Lalit et al., 2018). These natural agents are applied in environmental protection, agriculture, food-, pharmaco- and energetic industries (Yavuz et al., 2019). The producer, living organisms are in tight connection with their endemic microenvironment, which has crucial regulatory role in the relevant biopolymer production. (Ibrahim et al., 2018).

It is a widely-known evolutionary theory that the rhizomes of life are derived from water; therefore, the first biopolymers were produced by ancient marine species, such as ammonites, sea urchins, snails, etc. (Frenkel-Pinter et al., 2021). These biocomposites were ‘soft’, ‘hard’ or a mix of them, protecting the unicellular organisms or metazoans from thermal, chemical, and ultraviolet stresses (Ehrlich, 2019). As a consequence of diverse natural habitat (e.g. oceans, seas, lakes, rivers, ponds, wetlands, puddles, streams, canals and thermal springs), aquatic organisms have developed different versions of polymers having a wide range of properties such as energy preservatives, structural and metabolic ones, or functions related to cell renewal and water repellence. These complex biomolecules can be found in the extracellular spaces, cell walls, inner tissues and exoskeletons or in exudate form in water and land. Various factors can environmentally influence secretion and formation of biopolymers, including salinity, depth, light intensity, temperature, pressure, density, pH, prey/predator presence, nutrients, dissolved gases, water flow rate, flora and fauna. (Olatunji, 2020).

Water-based ecological niches are extremely sensitive to industrial or chemical pollution; thus, the investigation of concerned aquatic micro-invertebrates is a current scientific issue. The evolutionary ancient rotifers are ideal models for ecotoxicological measurements (Dahms et al., 2011), but they had not been studied in the context of biopolymer secretion. The team of Datki et al. (2021) was first to describe a novel phenomenon in this theme; namely that these animals are capable of secreting filamentous or glue-type exudates following particle-based mechanic irritation. This previously described rotifer-specific bioactive

biopolymer, named Rotimer, is a universally functional molecule of these micro-metazoans. The Rotimer, produced among other things by some monogonant species (e.g. *Euchlanis dilatata* or *Lecane bulla*), is an exogenic and viscoelastic biopolymer having fibrous or glue-like forms. This exudate, in complex formation with particle-type mechanic stimulator (e.g. insoluble Carmine crystals or epoxy-metal beads), forms the Rotimer-Indicator Conglomerate (RIC) in a web structure (Datki et al., 2021). In this RIC formation, the above mentioned biopolymer has a proteinous nature; its exact chemical composition and structure is yet unknown. Based on academic literature it has been proved that numerous species can secrete biopolymers, similarly to rotifers; however, the regulation of this novel Rotimer is yet undescribed.

The aim of this study is to investigate selected environmental factors and possible pollutant agents on Rotimer secretion, experientially monitored by RIC formation. Those chemicals were chosen which might be released to natural waters by certain industries or urban sources. This work intends to shed light on their impacts on the amount and quality of Rotimer, rather than elaborating on their secretion-dependent mechanism of actions.

2. Material and methods

2.1. Materials

Materials applied in this work were the following: yeast (*Saccharomyces cerevisiae*; EU-standard granulated instant form, cat. no.: 2-01-420674/001-Z12180/HU); algae (*Chlorella vulgaris*; BioMenu, Caleido IT-Outsource Kft.; cat. no.: 18255); from Sigma-Aldrich: spermidine (cat. no.: S2626), dimethyl sulfoxide (DMSO; cat. no.: D8418), ethylenediaminetetraacetic acid (EDTA; cat. no.: E9884), ascorbic acid (cat. no.: A1300000), catechin (cat. no.: C-1788), glucose (cat. no.: 47829), taurine (cat. no.: T0625), cyclic adenosine monophosphate (cAMP; cat. no.: A9501), ethyl nicotinate (cat. no.: E40609); from Merck: powdered Carmine crystals (Natural Red 4; cat. no.: 2233), glycerol (cat. no.: 1.04092.1000); distilled water (DW; Millipore SAS, Direct-Q 3 UV, ultrapure; type 1; Molsheim, France); different pH-calibrated media (Mettler Toledo SevenEasy pH meter, Switzerland, InLab 413; cat. no.: Z654272); from Reanal: NaCl (cat. no.: 14064-1-38), KCl (cat. no.: 30080), LiCl (cat. no.: 12033), CaCl₂ (cat. no.: 11024), MgCl₂ (cat. no.: 232-094-6), SrCl₂ (cat. no.: 19066), ethylenediaminetetraacetic acid (EDTA; cat. no.: 9884), choline chloride (cat. no.: 11222); from KRKA Novo Mestro: Lucidril (meclofenoxate hydrochloride; cat. no.: 5812-67/721/1-63); from Other Pharmacy: Selegiline (cat. no.: 903871-2G); from Cambridge Research Biochemicals: isoguvacine (cat. no.: PE7097); from Molecular Probes: Fluo-3 (cell impermeable calcium-specific fluorescent dye; cat. no.: F3715); from Corning or Corning-Costar: 24-well plate (cat. no.: 3524), from Greiner Bio-One GmbH: microplate 96 well plate (clear, half area, cat. no.: 675101); Petri dish (cat. no.: 430167), flasks (cat. no.: 430168); universal plastic web (pore diameter: 50 µm); Acrodisc 13 filter, Low Protein Binding (pore diameter: 0.2 µm; cat. no.: 4454) from Gelman Sciences; standard medium (mg/L): Ca²⁺ 31.05; Mg²⁺ 17.6; Na⁺ 0.9; K⁺ 0.25; Fe²⁺ 0.001; HCO₃⁻ 153.097; SO₄²⁻ 3; Cl⁻ 0.8; F⁻ 0.02; H₂SiO₃ 3.3 (pH = 7.5).

2.2. Animals

The measurements were carried out on invertebrate monogonant rotifers *Euchlanis dilatata* and *Lecane bulla*; thus, according to the current international regulations, no specific ethical permission was needed. The animals were obtained from Red Cross Lake (GPS coordinates: 46° 16' 25" N; 20° 08' 39" E; early summer) in Szeged (Southern Great Plain, Hungary). They have been maintained in a standard laboratory environment for 4 years. The experiments were performed in accordance with globally accepted norms: Animals (Scientific Procedures) Act, 1986, associated guidelines, EU Directive 2010/63/EU for animal experiments, and the National Institutes of Health guide for the care and

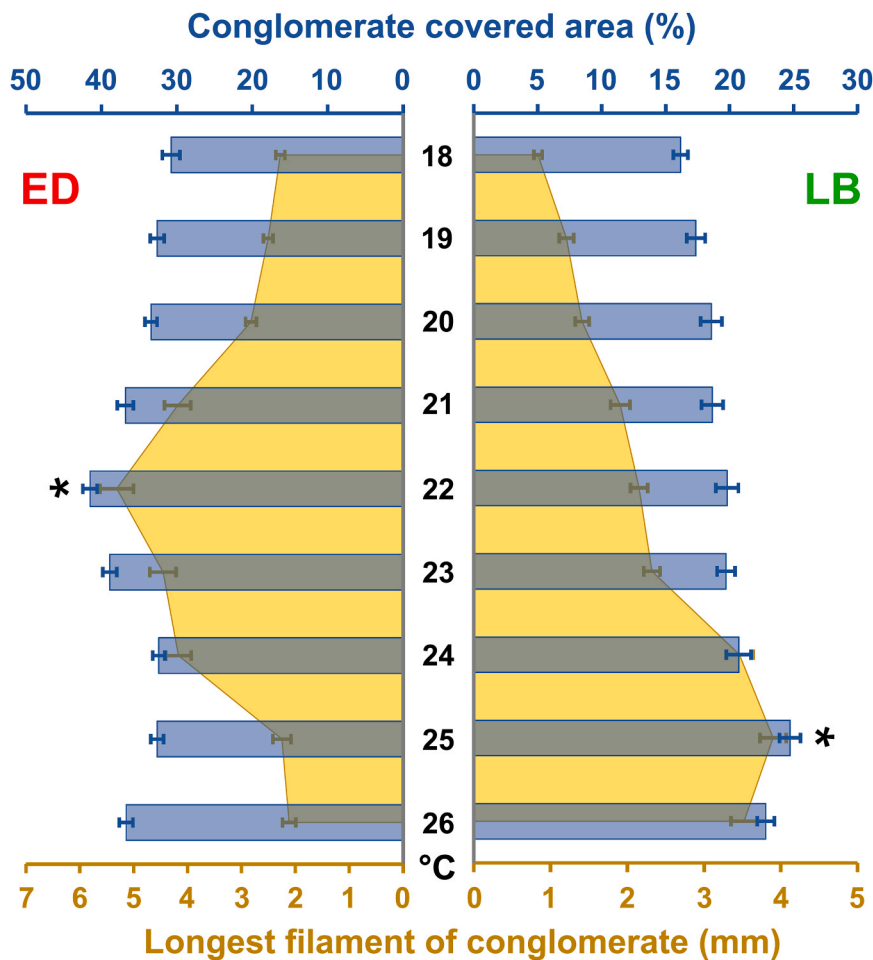


Fig. 1. The effect of temperature on RIC formation. The impacts of temperature on 'Rotimer-Inductor Conglomerate' (RIC) produced by *Euchlanis dilatata* (ED; red) and *Lecane bulla* (LB; green) are presented by the RIC-covered area (%; blue) and the longest filament of conglomerate (mm; yellow). The error bars represent SEM. One-way ANOVA with Bonferroni *post hoc* test was used for statistical analysis, the levels of significance are $p^* \leq 0.05$ (*, significant difference from all the other measured data, indicating the optimum, the significance is higher than or equal to 95% in both measured parameters).

use of Laboratory animals (NIH Publications No. 8023, revised 1978). Animal studies comply with the ARRIVE guidelines. The rotifers were cultured based on the methods previously published by Datki et al. (2021), using flasks (25 cm² area), filled with standard medium. They were fed every second day by heat-inactivated, homogenized and filtered (plastic web; pore diameter: 15 µm) algae-yeast (in 1:3 ratio) suspension (final dose: 600 µg/mL/case). The *E. dilatata* and *L. bulla* are maintained as standard cultures in our *micro-in vivo* laboratory.

2.3. Treatment of rotifers

Every measurement was performed in standard environment (24 °C, pH = 7.5, 40% air humidity, in standard media and 12:12 h dark-light), except for the actual parameter of interest or the optimized experiments (for *E. dilatata*: 22 °C, pH = 7.8; for *L. bulla*: 25 °C, pH = 7.2). The experimentally applied animals were isolated by pouring them into Petri dishes (55 cm² area). In all experiments (n = 24, well; in all groups) related to rotifer-specific biopolymer (named Rotimer), 20–22 mature (with maximal body size or with an egg inside) entities were applied in a 24-well plate (1.8 cm² well-area) with 1 mL working volume per well.

The influence of changing environmental parameters (temperature and pH) and the treatment with various agents (20 µM) lasted for 10 h with a constant number of unfed animals. These RIC-influencing chemicals were the follows: DMSO, ascorbic acid, Lucidril, catechin, glycerol, glucose, taurine, selegiline, cAMP, spermidine, ethyl nicotinate, isoguvacine and choline. The various media with different pH values (from 6.6 to 8.4) were made freshly before all relevant treatments.

The animals were let to rest (washed) in DW for 30 min before being

applied in metal salts (100 mg/L) related experiments. These salts were the follows: NaCl, KCl, LiCl, CaCl₂, MgCl₂ and SrCl₂. These measurements, with constant number of unfed rotifers, were carried out after making 10x dilutions from stock solutions. The RIC induction was initiated with mechanically ultra-powdered water insoluble Carmine crystal particles after 5 min treatment with different metal-salts.

2.4. RIC-analysis

The RIC-analysis is a slightly modified adaptation of the method applied by Datki et al. (2021). The final (working) concentration of the administered inductor (Carmine) of Rotimer-secretion was 50 µg/mL which was diluted from 2 mg/mL stock solution. The Carmine-induced biopolymer formed RIC in a high density web structure after 30 min incubation time. After removing the well solution by pipette, these RIC products were desiccated (dried) at room temperature (24 °C) and at 40% humidity in darkness for 60 min. The inductor particles were applied above the size of 50 µm (filtered with plastic web). All manipulations were performed slowly and carefully to avoid fluid flow destroying the exudate web.

The amount and pattern of RIC was detected by light microscopy (Labovet FS; 63x magnification). The photos (Nikon D5100, 16 MP RAW and ISO 100) were converted in a black and white graphical format (threshold, 2.04 pixel = 1 µm, 408 pixel = 0.2 mm and 8-bit). These images were analyzed with ImageJ program (Wayne Rasband, USA), extracting data related to the conglomerate-covered area (%) of this crystal-biomolecule complex.

Detection of the longest filament of RIC was performed under light microscope with 25x magnification applying Bürker-type square grid in

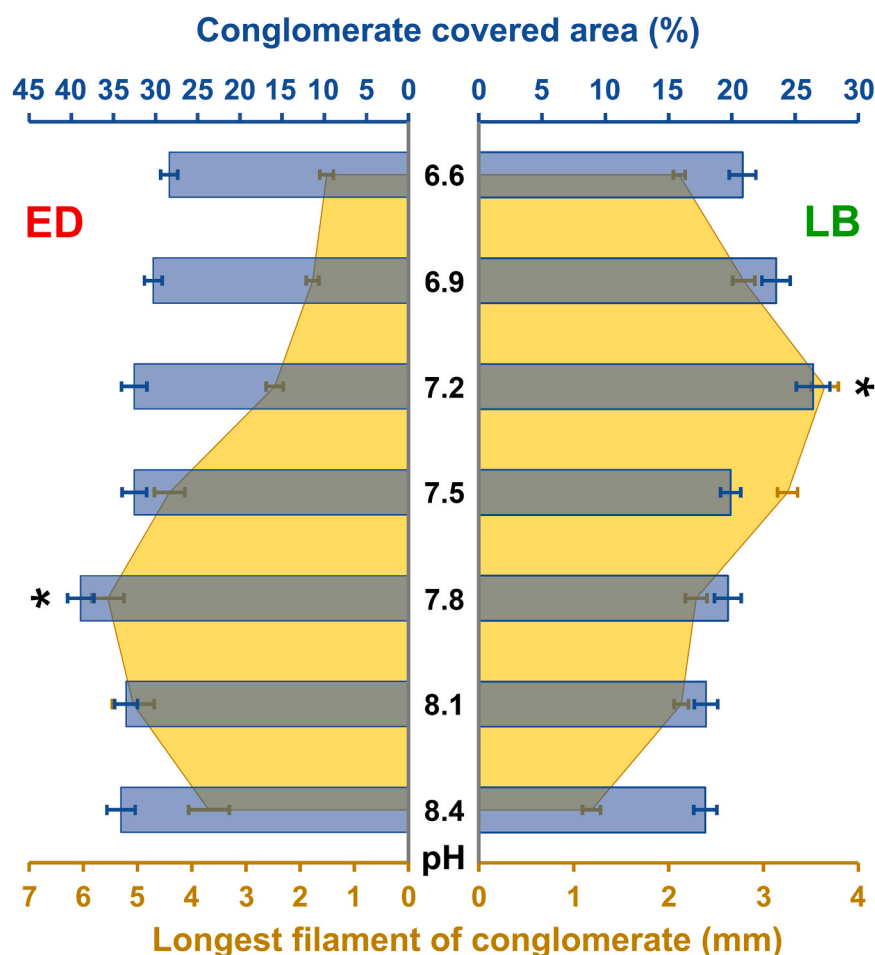


Fig. 2. The effect of pH on RIC formation. The impacts of pH on 'Rotimer-Inductor Conglomerate' (RIC) produced by *Euchlanis dilatata* (ED; red) and *Lecane bulla* (LB; green) are presented by the RIC-covered area (%) and the longest filament of conglomerate (mm; yellow). The error bars represent SEM. One-way ANOVA with Bonferroni *post hoc* test was used for statistical analysis, the levels of significance are $p^* \leq 0.05$ (*, significant difference from all the other measured data, indicating the optimum, the significance is higher than or equal to 95% in both measured parameters).

parallel layering with plate-wells. In the web of RIC (in every relevant well) the longest straight filament with two attachment points was measured (in mm).

2.5. Fluorescent detection of calcium level in rotifer-medium

Detecting the relative changes of calcium ion level in rotifer-media was carried out with a plate-reader-based fluorescent method. The external CaCl_2 concentration in the current media was 5 mg/L (45 μM), supplemented with 205 mg/L NaCl; therefore, the total salt concentration was equivalent with the standard medium. In these experiments, calcium-specific and cell-impermeant Fluo-3 (stock solution: 1 mg/mL; final concentration: 50 μM) was applied. Low CaCl_2 concentration provided 0.9:1 calcium: dye ratio, since without free dye surplus the potential alterations cannot be detected precisely.

The rotifer-free samples (0.15 mL) were filtered with Acrodisc 13 filter (pore diameter 0.2 μm) from the respective well-media (1 mL) of applied 24-well plates. The maximal calcium-specific signal did not decrease in the presence of Carmine in this animal-free setup. The labeling interval with Fluo-3 was 10 min at room temperature in the dark. The readings were carried out in a 96-well (clear) half-area plate, using NOVO star plate-reader (BMG Labtech, Germany). The volume of wells was 95 μL medium supplemented with 5 μL Fluo-3 (from 1 mM stock solution). The extinction/emission was set at 500/530 nm and the number of flash/well/cycle was 30. Before the first turn, orbital shaking was applied where the shaking time was set at 3 s and the plate-rounds were 600/min. The readings and the gain adjustment were normalized to the background of the free-dye. This blank was 650 relative unit, 1% of the maximum fluorescence intensity with dye and without calcium

(210 mg/L NaCl). Fluorescence intensity of the Fluo-3 increased about 14–15-fold after calcium binding (reference samples: 100% relative unit, without animals and Carmine). Optimized conditions (except for the calcium amount) were applied to both rotifer species in photometric experiments.

2.6. Statistics

The error bars represent the standard error of the mean (SEM). For comparative statistical analysis, the one-way ANOVA was used followed by the Bonferroni *post hoc* test with SPSS 23.0 (SPSS Inc, Chicago, IL, USA) software for Windows. The homogeneity and normality of the data were checked, and they were found suitable for ANOVA followed by Bonferroni *post hoc* test. The different levels of significance are indicated as follows: $p^* \leq 0.05$, $p^{***,###} \leq 0.001$ (all marks are defined in the given figure legend).

3. Results and discussion

The rotifers are validated models of toxicity screening related to environmental parameters and chemical agents (Rico-Martínez et al., 2016). The biopolymer producing capacity of these micro-metazoans has just recently been discovered (Datki et al., 2021). The Rotimer is such a biomolecule, which is essential for the survival of the animals (food-trapping, gluing the eggs and water purification) that produce it. Secretion of the above mentioned multifunctional product largely depends on the environmental factors. These influencing factors have various impacts on rotifers according to their degree; however, every species has its own optimum. The *E. dilatata* preferred lower

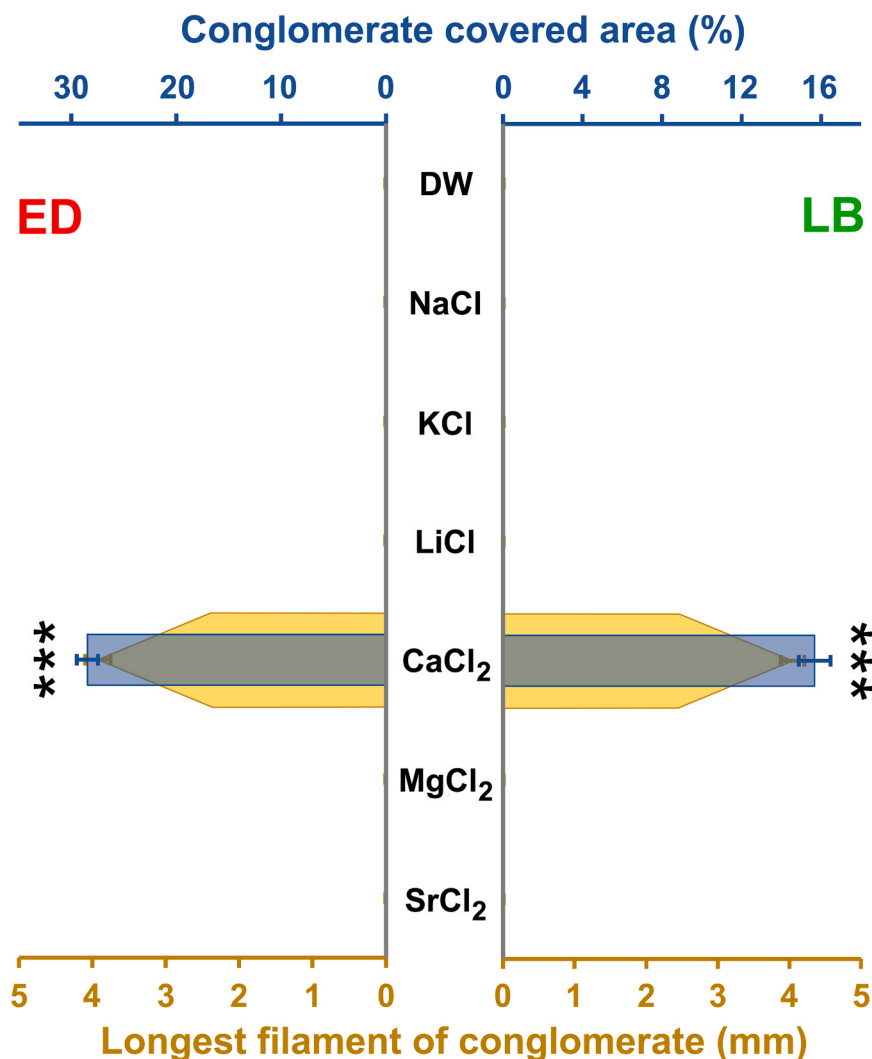


Fig. 3. The effect of different metal ions on RIC formation, The impacts of various metal ions on 'Rotimer-Inductor Conglomerate' (RIC) produced by *Euchlanis dilatata* (ED; red) and *Lecane bulla* (LB; green) are presented by the RIC-covered area (%; blue) and the longest filament of conglomerate (mm; yellow). The error bars represent SEM. One-way ANOVA with Bonferroni *post hoc* test was used for statistical analysis, the levels of significance are $p^{***} \leq 0.001$ (*, significant difference from all the other measured data, indicating the optimum, the significance is higher than or equal to 99.1% in both measured parameters).

temperature (22 °C; Fig. 1) and higher pH (pH=7.8; Fig. 2) than *L. bulla* (25 °C and pH=7.2) did in our laboratory conditions. In case of the species-specific optimum, both RIC markers (conglomerate covered area and longest fiber of conglomerate) showed similarly high values; while distancing from that optimum, parameters decreased, especially in fiber length. In the case of *E. dilatata*, these markers produced approximately 2-fold change in average, compared to *L. bulla*.

Natural waters contain various metal ions which implicitly influence the flora and fauna (Armour, 2016). Applying natural salts (Fig. 3) in artificial conditions revealed that only calcium ion is inevitable for exudate secretion. There is no RIC production in its absence. A similar phenomenon was described related to mucin biopolymers, where the sol-gel transition is pH- and also calcium dependent (Curnutt et al., 2020). It is true to the fact that the freshly harvested rotifers can produce Rotimer in a fully demineralized environment, but the animals washed and let to rest for minimum half an hour in DW cannot secrete the biopolymer (Datki et al., 2021). These micro-metazoans presumably have some amount of stocked metal ions; however, the exudate secretion capacity disappears without natural replacement of the essential minerals. Comparing the ion content of standard culture media (210 mg/L), a lower dose (100 mg/L) was applied, since this concentration level was acceptable in terms of osmolarity, yet neither ion proved to be toxic. The one-component treatment solutions showed significant differences compared to both standard and only calcium-containing media. Surprisingly, neither the magnesium, nor the strontium was able to replace

the calcium, unlike in the case of some physiological process (Saris et al., 2000; Hendrych et al., 2016). These facts prove that the calcium sensors or receptors of rotifers are very specific to this particular ion.

The abiotic factors, such as temperature (Wenjie et al., 2019) and minerals (Hernández-Flores et al., 2020), have significant modulating effects on the global aquatic environment. Rotifers are particularly sensitive to all these factors, thus forming a natural regulating-, viability- and toxicity indicator systems (Pérez-Legaspi and Rico-Martínez, 2001).

To precisely evaluate the calcium-dependency of RIC production, dose-efficacy was measured (Fig. 4). As it turned out, the sodium has no impact on exudate production, the different calcium doses were supplemented with this, equaling the amount of metal ions, similarly to that of the standard media. Assuring constant osmolarity in artificial environment is essential to assess dose-dependency. Regarding biopolymer production, optimums of the two investigated species differ from each other, since 50 mg/L is the most optimal level for the *E. dilatata*, while 100 mg/L is the most favorable for *L. bulla*. Moreover, the first one produces twice as much Rotimer as the latter one. Results suggest that 10–20% of conglomerate formation already starts in the presence of a relatively low dose (0.1 mg/L) of calcium. In the case of both species sudden increase in activity was observed at 5 mg/L dose; moreover, it was found that the production of exudate dramatically reduces in the presence of calcium (50 mg/L) together with EDTA (100 mg/L). The EDTA is a well-known chelator of different metal ions (e.g. calcium,

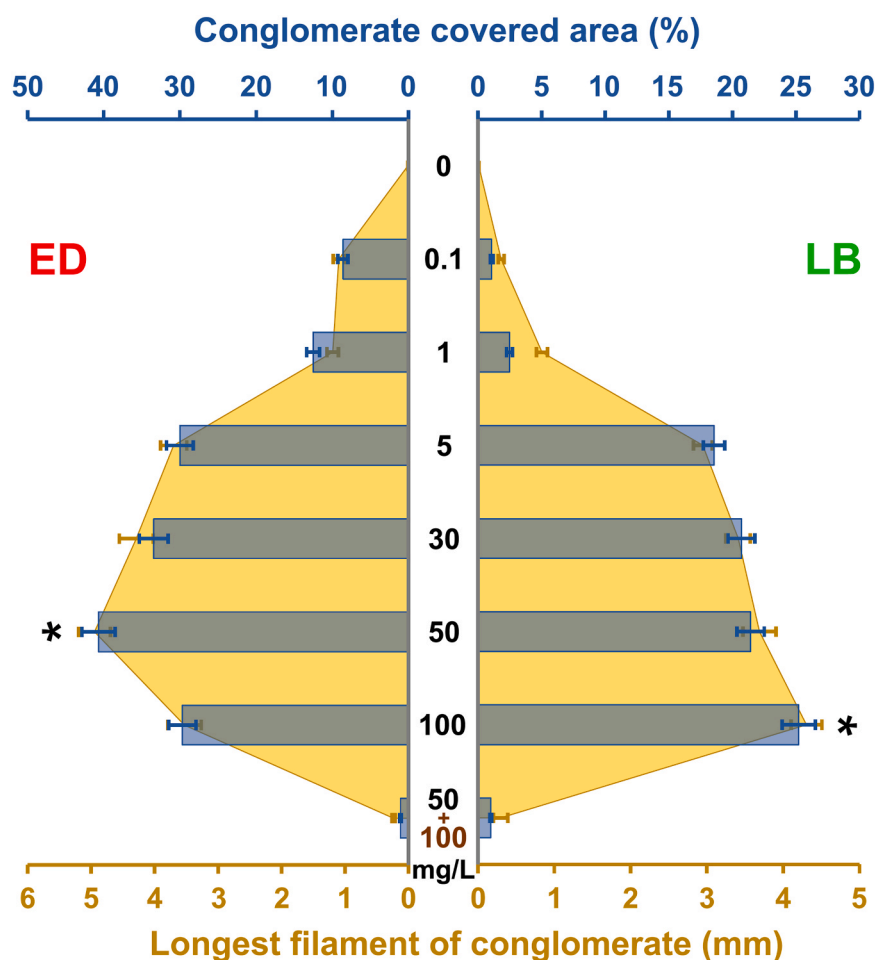


Fig. 4. Dose-dependent effect of calcium on RIC formation. The impacts of various doses of calcium on 'Rotimer-Inductor Conglomerate' (RIC) produced by *Euchlanis dilatata* (ED; red) and *Lecane bulla* (LB; green) are presented by the RIC-covered area (%; blue) and the longest filament of conglomerate (mm; yellow). EDTA (100 mg/L; brown) was used against calcium (50 mg/L; black) in the control measurement (bottom column). The error bars represent SEM. One-way ANOVA with Bonferroni *post hoc* test was used for statistical analysis, the levels of significance are $p^* \leq 0.05$ (*, significant difference from all the other measured data, indicating the optimum, the significance is higher than or equal to 95% in both measured parameters).

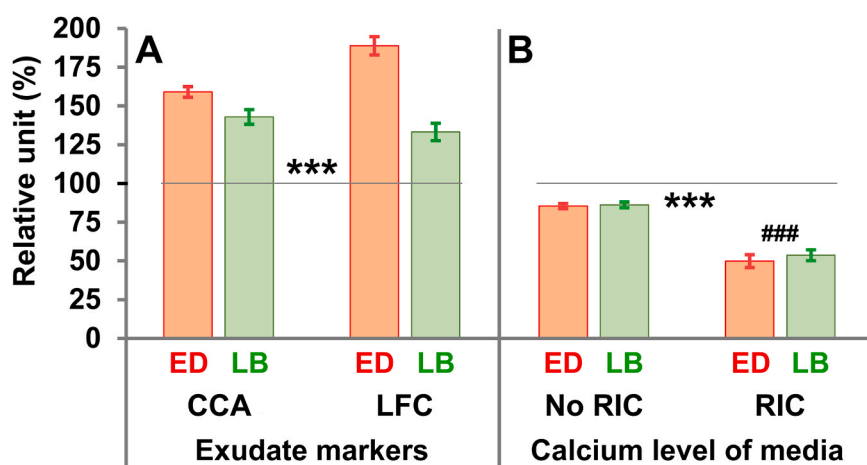


Fig. 5. RIC formation (A) and use of calcium (B) under species-specific optimized conditions. The influencing factors were optimized in species-specific manner in *Euchlanis dilatata* (ED; red) and *Lecane bulla* (LB; green) populations. The percentage of relative units of exudate markers (A) was presented as conglomerate covered area (CCA) and longest filament of conglomerate (LFC). The changes in species-specific calcium uptake from the media (B) were measured in the presence or absence of 'Rotimer-Inductor Conglomerate' (RIC). The error bars represent SEM. One-way ANOVA with Bonferroni *post hoc* test was used for statistical analysis, the levels of significance are $p^{***,###} \leq 0.001$ (*, significant difference from the standard culturing, which was the 100% indicated by the black line; #, significant difference from the 'No RIC' columns).

magnesium, copper or zinc; Zaitoun and Lin, 1997) and it had no negative effect on the viability of the animals up to the level of 200 mg/L dose, which was measured by swimming speed ($\mu\text{m}/\text{sec}$; Suppl. Fig.). The inhibitory effect of non-membrane permeable calcium-specific chelator (George and Brady, 2020) on RIC production proves that this process happens extracellularly, presumably at the inlet of the intestinal tract. The calcium dependency of biopolymer-secretion and formation is not a rare phenomenon in the animal kingdom, especially in aquatic invertebrates (Wang et al., 2003; Ehrlich, 2019).

In the case when the exudate secretion was induced in species-

specific optimal circumstances in modified medium (*E. dilatata*: 22 °C, pH=7.8, calcium 50 mg/L; *L. bulla*: 25 °C, pH=7.2, calcium 100 mg/L), both species showed significantly higher activity (Fig. 5A) compared to their controls (100%; standard environmental factors and media). The RIC amount and the length of filaments show that *E. dilatata* can be better stimulated than *L. bulla*. In another experiment, but also in an optimized environment (except for calcium concentration, which was only 5 mg/L), the rotifers used calcium both for their physiological needs and for RIC production (Fig. 5B). It was shown by non-cell permeable Fluo-3 fluorescent dye (Zhang et al., 2014) that

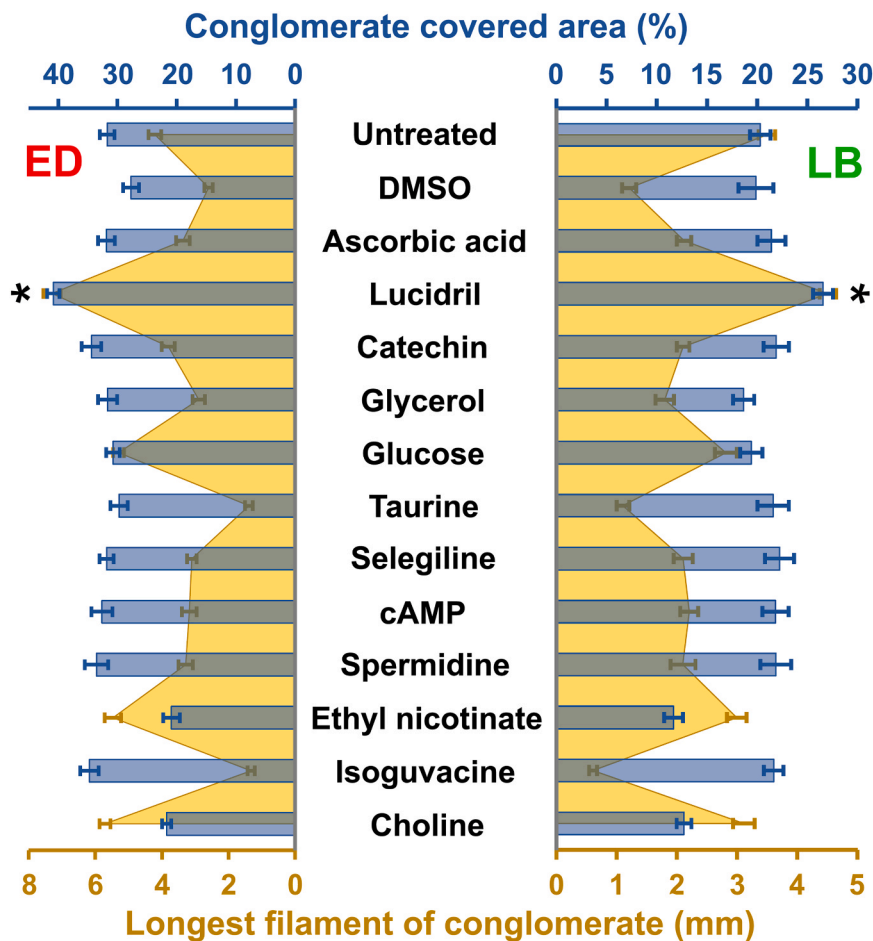


Fig. 6. The effect of different chemical agents on RIC formation. The impacts of various chemicals on 'Rotimer-Inductor Conglomerate' (RIC) produced by *Euchlanis dilatata* (ED; red) and *Lecane bulla* (LB; green) are presented by the RIC-covered area (%) (blue) and the longest filament of conglomerate (mm; yellow). The error bars represent SEM. One-way ANOVA with Bonferroni *post hoc* test was used for statistical analysis, the levels of significance are $p^* \leq 0.05$ (*, significant difference from all the other measured data, indicating the optimum, the significance is higher than or equal to 95% in both measured parameters).

significantly more calcium was used by the animals during RIC production than without its induction. The control measurements were carried out in the same media (with also reduced calcium content) lacking animals and the inductor. The reason for it was to enhance the molarity ratio of the detectable dye. These data suggest that rotifers extract and bind metal ions from their environment. The same process can be observed in the case of marine corals (Mitterer, 1978; Howard and Brown, 1984).

Rotimer as a biomaterial can likely absorb calcium during its formation which may even take part in the global level processes of freshwater and marine sediments. Our present and previous studies show that biopolymer production is essential for the rotifers to live and survive.

Numerous environmental pollutant chemicals may come from industrial sources or inadequately handled communal waste in human habitat (Bieber et al., 2018). The short-term (a few hours) effects of the investigated agents (DMSO, ascorbic acid, Lucidril, catechin, glycerol, glucose, taurine, selegiline, cAMP, spermidine, ethyl nicotinate, isoguvacine and choline) were very different; however, both species reacted with the same tendency (Fig. 6). There were a few cases in which the amount of conglomerate remained average; however, the length of the fibers significantly shortened (glycerol, taurine and isoguvacine) compared to the control. Among the investigated group of agents there were two chemicals (ethyl nicotinate and choline) which effects resulted a decreased amount of RIC with relatively long fibers. In the last variation of the exudate marker related pattern (Lucidril and glucose), high ratio of the covered area was detected with longer fibers than that of the control. Lucidril (Lazarova-Bakarova and Genkova-Papasova, 1989; Verma and Nehru, 2009) may be highlighted for its holistic stimulating effect as a conclusion. Based on the previously described results,

concerning the pollutant agents, it is unequivocal that the activity or sensitivity of *E. dilatata* is higher than that of the *L. bulla* related to the exudate secretion. Here, the goal was not to explore the agents' mechanism of action on the rotifers, but to demonstrate the various and holistic effects of these chemicals on the external secretion of micro-metazoans.

Although both investigated rotifers are monogonants, their numerous phenotypic differences and physiological variations can be explained by their ancient phylogenetic divergence. These species show similarity only in calcium-dependent biopolymer production, suggesting that it is an evolutionarily ancient capability. The fact that rotifers have preserved this ability indicates that this product is a multifunctional bioactive molecule with a highly pronounced bioindicator role. Further investigation of the Rotimer has high potential for future applications. The phenomenon that the natural or artificial molecules influence the microscopic fauna, such as rotifers in the current case, in a relatively quick and different manner, raises the concern of preserving the natural environmental balance.

4. Conclusion

Examination of the conglomerate-covered area and the longest fiber of conglomerate markers revealed that the *E. dilatata* and *L. bulla* rotifer species react differently to all these parameters except for drug effects and calcium dependency of exudate secretion. Identical and opposite trends were also observed in the changes of the two examined RIC markers. The amount of conglomerate rarely correlated with the length of its fibers. The greatest novelty of this work lies in the fact that the production of Rotimer proved to be obligatorily calcium-dependent; moreover, this special exudate is a promising bioindicator of the

natural aquatic environment. Species-specific and simultaneous optimization of natural influencing factors significantly increased the RIC formation. Regulation and stimulation of biopolymer production may yield further investigations and possible industrial production of this substance in the future.

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CRediT authorship contribution statement

Zs.D. and Z.G.O.: Conceptualization. Zs.D., E.B. and Z.G.O.: Methodology. Zs.D., Z.G.O. and F.S.: Validation. Zs.D., Z.G.O., F.S. and E.B.: Formal analysis. Zs.D., E.B. and Z.G.O.: Investigation. Zs.D., J.K.: Resources. Zs.D., Z.G.O., E.B. and B.G.: Data curation. Zs.D. and Z.G.O.: Writing - original draft. Zs.D., Z.G.O., and B.G.: Writing - review & editing. Zs.D.: Visualization. Zs.D., Z.G.O. and J.K.: Supervision. Zs.D., Z.G.O. and E.B.: Project administration. Zs.D. and J.K.: Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.ecoenv.2021.112399](https://doi.org/10.1016/j.ecoenv.2021.112399).

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