

**Szkoła Doktorska Nauk Ścisłych
i Przyrodniczych Uniwersytetu Łódzkiego**

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**Adaptacje zooplanktonu do wybranych
zanieczyszczeń w miejskich zbiornikach
wodnych**

Adaptations of zooplankton to selected
pollutants in urban water ponds

Praca doktorska

wykonana w Katedrze UNESCO
Ekohydrologii i Ekologii Stosowanej,
Instytut Ekologii i Ochrony Środowiska
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4. Wprowadzenie

4.1. Uzasadnienie podjętej tematyki badawczej

Miasta są ostoją człowieka od czasów starożytnych i mają one niezaprzeczalny wpływ na środowisko naturalne. Szacuje się, że w regionach rozwiniętych 79% populacji ludzkiej zamieszkuje tereny zurbanizowane, a odsetek ten wzrośnie do 87% do 2050 roku (World Cities Report 2022). Wraz z postępującym rozwojem transportu, drogi w miastach ulegały przekształceniom, począwszy od dróg gruntowych, poprzez pierwsze drogi utwardzane kamieniem, aż po znane nam dziś jezdnie i autostrady.

W rejonach półkuli północnej, gdzie w sezonie zimowym występują temperatury w okolicach 0°C, drogi takie wymagają odpowiedniego zabezpieczenia przed śniegiem i lodem. Sól drogowa, czyli najczęściej wykorzystywany w tej roli chlorek sodu (NaCl), jest najpowszechniejszą substancją używaną w celu odladzania powierzchni. Początki stosowania chlorku sodu jako substancji odladzającej sięgają lat 40. ubiegłego wieku i od tego czasu obserwuje się wzrost jego popularności (Hintz i in., 2022). Inne sole nieorganiczne, takie jak chlorek magnezu ($MgCl_2$) czy chlorek wapnia ($CaCl_2$), jak również zamienniki niezawierające chlorków takie jak octany czy mrówczany mogą być używane w celu zimowego zabezpieczania dróg, jednak chlorek sodu, w porównaniu do innych substancji, jest najbardziej ekonomiczny w użytkowaniu (Hintz i in., 2022).

Warto jednak zwrócić uwagę na to, że pomimo ważnej roli jaką pełni NaCl w zwiększaniu bezpieczeństwa na drogach w warunkach zimowych, ma on niekorzystny wpływ na środowisko. Sól drogowa wraz ze spływem powierzchniowym oraz poprzez infiltrację do gleby przedostaje się do wód powierzchniowych i podziemnych, gdzie powoduje podwyższone stężenia jonów chlorkowych oraz sodu. Ponadto, jon Cl^- jest konserwatywny – nie ulega on w łatwym stopniu biotransformacji ani rozpadowi, co powoduje, że może się utrzymywać w środowisku przez dłuższy czas (Hintz i Relyea, 2019).

Problem zanieczyszczenia spowodowanego stosowaniem soli drogowej jest często bagatelizowany o czym świadczy fakt, że w wielu krajach brak jest konkretnych wytycznych dotyczących stężeń granicznych, jeżeli chodzi o oddziaływanie na biologię dla jonów chlorkowych i sodu w wodach słodkich. Granice takich stężeń zostały wyznaczone w Stanach Zjednoczonych (230 mg Cl^-/L – stężenia chroniczne, 860 mg Cl^-/L stężenia ostre) (US EPA, 1988) oraz w Kanadzie (120 mg Cl^-/L – stężenia chroniczne i 640 mg Cl^-/L – stężenia

ostre) (CEQG, 2011). Podobnych limitów brak w krajach Unii Europejskiej, z kilkoma wyjątkami. Na przykład, w Niemczech za wody lekko zanieczyszczone solą uważa się wody o stężeniu od 50 do ≤ 200 mg Cl⁻/L, natomiast te charakteryzujące się stężeniem od 200 do 400 mg Cl⁻/L klasyfikowane są jako wody średnio zanieczyszczone solą (Hintz i in., 2022). W Austrii wartość graniczna dla jezior i rzek to 150 mg Cl⁻/L (średnia roczna), na Węgrzech od 20 do 60 mg Cl⁻/L dla rzek oraz przewodność od 20 do 1500 μ S/cm wyznaczona dla jezior (średnie roczne), w Holandii od 40 do 300 mg Cl⁻/L dla rzek i od 40 do 200 mg Cl⁻/L dla jezior (średnie sezonowe), natomiast w Polsce jest to przewodność od 300 do 850 μ S/cm dla rzek i 100 do 600 μ S/cm dla jezior (średnie roczne) (Kelly i in., 2023). Ponadto, jak wskazuje Schuler i współautorzy (2019), obecne przepisy są niewystarczające, aby skutecznie chronić słodkowodne ekosystemy przed zasoleniem, ponieważ nie wiążą się one z konsekwencjami prawnymi. Dodatkowo, nie zapewniają one skutecznych rozwiązań w przypadkach, gdy stężenia jonów odpowiedzialnych za podwyższone zasolenie są przekraczane.

Niestety, jak wskazują przykłady z literatury, w wodach słodkich narażonych na zanieczyszczenie NaCl, zarówno stężenia chroniczne jak i ostre wyznaczone przez kanadyjskie i amerykańskie limity, są często wielokrotnie przekraczane. Podwyższone stężenia jonów chlorkowych obserwuje się w przydrożnych zbiornikach burzowych (Marsalek i in. 2003; Gallagher i in. 2011; Tixier i in. 2011), jeziorach (Mueller i Gaechter, 2012, Rogora i in. 2015; Dugan i in. 2017), a także w strumieniach i rzekach (Lax i in. 2017; Hubbart i in. 2017). Chlorek sodu może również przedostawać się do wód podziemnych i wraz z nimi migrować do wód powierzchniowych, powodując podwyższone stężenia jonów chlorkowych również w miesiącach ciepłych (Lax i Peterson, 2009), a także przyczyniać się do podwyższonego stężenia chlorków w studniach z wodą pitną (Bester i in. 2006).

Podwyższone stężenia jonów chlorkowych w wodzie niosą za sobą skutki zarówno abiotyczne jak i biotyczne. Do tych pierwszych zaliczyć można m.in. wpływ na zmianę struktury gleb (Robinson i in. 2017) czy wzrost stężenia metali ciężkich w ekosystemach słodkowodnych (Schuler i Relyea, 2018a). Zaobserwowano również szereg konsekwencji podwyższonego zasolenia na organizmy żywe, w tym mikroorganizmy (Cochero i in., 2017), glony (Hintz i in., 2017), bezkręgowce (Clements i Kotalik, 2016) i kręgowce (Karraker i Gibbs, 2011; Tollefsen i in., 2015).

Zooplankton stanowi kluczowe ogniwo w słodkowodnych sieciach troficznych,

zapewniając przepływ energii od producentów do konsumentów wyższych rzędów (Sinclair i Arnott, 2018). Zooplankton, a w szczególności wioślarki z rodzaju *Daphnia*, dzięki odżywianiu się poprzez filtrację, kontrolują obfitość fitoplanktonu, przyczyniając się tym samym do polepszania jakości wody w ekosystemach słodkowodnych (Lampert i Sommer, 2001). Jednakże, badania wskazują, że podwyższone stężenia chlorków ($600 \text{ mg Cl}^-/\text{L}$) mogą wpływać na zwiększenie śmiertelności wioślarek (Searle i in., 2016), a także wywoływać szereg skutków sub-letalnych, takich jak spadek wielkości lęgu, spadek całkowitej liczby potomstwa, spadek średniej długości życia, spadek współczynnika reprodukcji netto (R_0) oraz spadek wewnętrznego przyrostu naturalnego (r) (Martínez-Jerónimo i Martínez-Jerónimo, 2007). Ponadto, zaobserwowano, że zwiększone zasolenie może powodować spadek szybkości pionowych wędrówek wioślarek, przy czym spadek ten jest tym większy im dłuższa jest ekspozycja na zasolenie (Huber i in. 2023). Zmiany spowodowane podwyższonym stężeniem chlorków obserwuje się również na poziomie słodkowodnych sieci troficznych, wskutek zachodzących interakcji top-down i bottom-up. Badania prowadzone przy użyciu zbiorników, których zadaniem jest imitowanie warunków naturalnych (tzw. *mesocosm studies*) wykazały, że w warunkach podwyższonego zasolenia ($1000 \text{ mg Cl}^-/\text{L}$) nastąpił wzrost zagęszczenia fitoplanktonu oraz peryfitonu, natomiast spadek zagęszczenia widłonogów oraz wioślarek (Lind i in., 2018), czy zdominowanie społeczności zooplanktonu przez wrotki, w tym gatunek charakterystyczny dla wód słonych – *Brachionus plicatilis* (Van Meter i Swan, 2014). Zaobserwowano również synergistyczny efekt pomiędzy wysokim zasoleniem a obecnością drapieżników (ryb), który doprowadził do spadku bogactwa gatunkowego oraz zagęszczenia zooplanktonu, czego skutkiem był wzrost zagęszczenia fitoplanktonu (Hintz i in. 2017).

Badania wskazują jednak na możliwość wykształcenia przez wioślarki tolerancji na podwyższone zasolenie. Coldsnow i współautorzy (2017) wykazali, że *Daphnia pulex* po 2,5 miesięcznej ekspozycji na podwyższone stężenia chlorków (100 , 250 i $1000 \text{ mg Cl}^-/\text{L}$), wykazywała wyższą przeżywalność w wysokich stężeniach chlorków ($1300 \text{ mg Cl}^-/\text{L}$) w porównaniu do organizmów kontrolnych. Autorzy zwracają jednak uwagę na koszty takiej tolerancji, np. mniejsze rozmiary ciała czy spadek reprodukcji, w porównaniu do populacji *Daphnia* żyjących w niezasolonych wodach (Coldsnow i in. 2017). Natomiast Hintz i współpracownicy (2019) wskazują na możliwość wytworzenia przez *D. pulex* tolerancji krzyżowej, tzn. *D. pulex* wykazujące tolerancję w stosunku do podwyższonych stężeń NaCl , mogą również być bardziej odporne

na podwyższone stężenia $MgCl_2$. Jednakże, autorzy również podkreślają koszty związane z wytworzeniem tolerancji na zasolenie, tj. wolniejszy przyrost populacji w początkowych etapach eksperymentu w porównaniu do kontroli.

Stosowanie chlorku sodu jako substancji odladzającej niesie za sobą poważne konsekwencje dla środowiska naturalnego, jednak jak dotąd brak jest skutecznych zamienników lub innych rozwiązań, które mogłyby pozwolić na zaprzestanie jego stosowania w przyszłości. Na rynku dostępne są zamienniki $NaCl$ w postaci innych soli nieorganicznych, głównie $MgCl_2$ oraz $CaCl_2$. Jednak stosowanie tych soli nie rozwiązuje problemu przedostawania się jonów chlorkowych do środowiska. Dodatkowo, niektóre badania wskazują na wyższą toksyczność $MgCl_2$ dla zooplanktonu w porównaniu do $NaCl$ (Coldsnow i Relyea, 2021). Inne substancje, często reklamowane jako „przyjazne dla środowiska,” takie jak octany, substancje oparte o mocznik czy wzbogacone ekstraktem z soku z buraka w badaniach toksyczności wykazywały podobną do $NaCl$ lub nawet większą toksyczność dla zooplanktonu (Natile i Solan, 2019). Z tego względu konieczne jest dokładne poznanie możliwych efektów, jakie mogą wywoływać zamienniki soli drogowej w ekosystemach słodkowodnych.

Alternatywą dla zminimalizowania szkodliwego wpływu zasolenia na słodkowodne ekosystemy mogą być rozwiązania oparte na naturze (ang. *nature-based solutions*), które opierałyby się o wykorzystanie odpowiednio dobranych gatunków roślin znoszących zasolenie. Doniesienia z literatury wskazują na zdolność do akumulacji w tkankach jonów Na^+ oraz Cl^- przez wybrane gatunki łobody (Suairé i in., 2016), pałki oraz situ (Guesdon i in., 2016). Jednakże jest to temat, który wciąż wymaga dalszych badań.

W niniejszej rozprawie doktorskiej starano się podejść do problemu zanieczyszczenia solą drogową wieloaspektowo. Po pierwsze, zbadano, jak zanieczyszczenie solą drogową wpływa na zooplankton zamieszkujący miejskie zbiorniki wodne. W tym celu wybrano cztery stawy zlokalizowane na terenie miasta Łodzi: Arturówek Górny, Julianów Górny, Zbiornik Wasiaka oraz Zbiornik Zgierska. Aby ocenić stopień zanieczyszczenia tych ekosystemów solą drogową oraz zbadać wpływ zasolenia na strukturę zooplanktonu w tych zbiornikach, prowadzono trzyletni monitoring w latach 2019-2021 różniących się pod względem zimowej pogody i tym samym ilości stosowanej soli drogowej. Po drugie, aby ocenić sukces wylęgu zooplanktonu w warunkach zanieczyszczenia $NaCl$ przeprowadzono serię badań laboratoryjnych, a także oceniono

wpływ ekspozycji NaCl na strukturę jaj przetrwalnikowych z użyciem mikroskopu skaningowego. Po trzecie, zbadano toksyczność pięciu różnych zamienników tradycyjnej soli drogowej dostępnych na polskim rynku dla czterech gatunków zooplanktonu: *Brachionus calyciflorus*, *Thamnocephalus platyurus*, *Ceriodaphnia dubia* i *Daphnia magna*. Wreszcie, w czwartym etapie badań, oceniono wpływ zasolenia na mechanizmy antyoksydacyjne dwóch gatunków polskiej flory: situ rozpierzchłego (*Juncus effusus*) oraz oczeretu jeziornego (*Schoenoplectus lacustris*), a także zbadano zdolność tych roślin do akumulacji jonów sodu, w kontekście wykorzystania ich do ograniczenia zanieczyszczenia solą drogową w ekosystemach słodkowodnych.

4.2. Cele i hipotezy badawcze

Publikacja I - „*The effects of road salt on freshwater ecosystems and solutions for mitigating chloride pollution – A review*”

Celem publikacji przeglądowej było podsumowanie aktualnej wiedzy na temat wpływu soli oładzających na środowisko słodkowodne oraz wskazanie potencjalnych kierunków ograniczenia degradacji ekosystemów, spowodowanej stosowaniem soli drogowej. Cel realizowano w następujących etapach: 1) **analiza zagrożeń** – ocena, na podstawie danych literaturowych, w jakim stopniu naturalne ekosystemy słodkowodne narażone są na zanieczyszczenie jonami Cl⁻; 2) **analiza przyczynowo-skutkowa**, mająca na celu wskazanie konsekwencji wynikających ze stosowania soli drogowej dla jakości i biologicznej aktywności gleby, wód podziemnych i ekosystemów słodkowodnych, a także ocenę wpływu zasolenia na złożone zależności między parametrami abiotycznymi i biotycznymi; 3) **rozwijanie i optymalizacja metod** - przedstawienie możliwych alternatyw dla tradycyjnych metod zimowego utrzymania dróg.

Publikacja II - „*Does winter application of road salt affect zooplankton communities in urban ponds?*”

Hipoteza: 1) Zagęszczenie zooplanktonu jest związane ze stężeniami jonów chlorkowych w wodzie powierzchniowej, a podwyższone stężenia chlorków w wodzie obserwowane podczas surowej zimy wpłyną na spadek zagęszczenia zooplanktonu w zbiornikach miejskich; 2) Warunki pogodowe oraz charakterystyka badanych zbiorników będą miały istotny wpływ na stężenia badanych jonów w wodzie oraz zagęszczenie zooplanktonu.

Wiele badań skupiających się na poznaniu wpływu zasolenia na organizmy zooplanktonowe to badania przeprowadzane w kontrolowanych warunkach (tzw. *mesocosm studies*) lub badania toksyczności z wykorzystaniem biotestów. Znacznie mniej doniesień literaturowych omawia wpływ zasolenia na zooplankton w warunkach naturalnych. Z tego względu, w publikacji II starano się ocenić zależność między stężeniami jonów chlorkowych oraz zagęszczeniem zooplanktonu w naturalnym środowisku, tj., w zbiornikach miejskich. Głównym celem publikacji II było zbadanie efektu wpływu stężenia jonów chlorkowych na całkowite zagęszczenie zooplanktonu oraz zagęszczenie poszczególnych grup zooplanktonu. Wybrano cztery miejskie zbiorniki zlokalizowane

w Łodzi, różniące się stopniem narażenia na zanieczyszczenie solą drogową. Badania przeprowadzono w trakcie trzech lat hydrologicznych (2019-2021), różniących się warunkami pogodowymi w trakcie zimy. Zbadano stężenia jonów w toni wodnej oraz w wodzie międzyosadowej, jak również przeprowadzono analizy jakościowe i ilościowe organizmów zooplanktonowych zamieszkujących zbiorniki.

Publikacja III - „*Acute toxicity of seven de-icing salts on four zooplankton species – is there an “eco-friendly” alternative?*”

Hipoteza: Dostępne na rynku zamienniki soli drogowej, w tym te reklamowane jako „przyjazne dla środowiska”, charakteryzują się podobną toksycznością dla zooplanktonu co tradycyjna sól drogowa NaCl.

Zamienniki tradycyjnej soli drogowej oraz efekty jakie mogą wywoływać na słodkowodnych ekosystemach są mniej poznane niż efekty działania NaCl, jednak niektóre badania wskazują na zwiększoną toksyczność takich soli jak CaCl₂ czy MgCl₂. Ponadto, niewiele wiadomo na temat efektów zamienników promowanych jako „przyjazne dla środowiska,” które często nie przechodzą wystarczająco dokładnych badań przed dopuszczeniem na rynek. W badaniach toksykologicznych często wykorzystywane są substancje chemicznie czyste, a nie te dostępne komercyjnie, które mogą zawierać w swoim składzie dodatkowe komponenty. Badania dotyczące toksyczności wybranych substancji na zooplankton najczęściej skupiają się na wybranych gatunkach wioślarek, natomiast znacznie mniej badań wykorzystuje przedstawicieli pozostałych grup zooplanktonu. Z tego względu, w publikacji III przeprowadzono testy ekotoksyczności mające na celu zbadanie wpływu tradycyjnej soli drogowej NaCl oraz jej sześciu zamienników dostępnych na polskim rynku, na czterech przedstawicieli zooplanktonu pochodzących z czystych linii laboratoryjnych, tj. *Daphnia magna*, *Ceriodaphnia dubia*, *Thamnocephalus platyurus* oraz *Brachionus calyciflorus*.

Publikacja IV - „*The effect of road salt (NaCl) treatment on the hatching success of Daphnia magna and Thamnocephalus platyurus*”

Hipoteza: 1) Ekspozycja jaj przetrwalnikowych *Daphnia magna* oraz *Thamnocephalus platyurus* na podwyższone stężenia NaCl wpłynie na zmniejszenie sukcesu wylęgu tych organizmów, a sukces wylęgu będzie odwrotnie proporcjonalny do stężeń NaCl;

2) Wcześniejsza inkubacja jaj w podwyższonych stężeniach NaCl przyczyni się do tego, że sukces wylęgu organizmów będzie większy niż z jaj przetrwalnikowych nie poddanych wcześniejszej inkubacji.

Konsekwencje jakie zasolenie może mieć na rozród słodkowodnych skorupiaków są wciąż słabo poznane. Z tego względu celem publikacji IV było zbadanie wpływu soli drogowej (NaCl) na sukces wylęgu dwóch organizmów zooplanktonowych, tj. *Daphnia magna* oraz *Thamnocephalus platyurus*, które wykazały wysoką wrażliwość na zasolenie w badaniach przedstawionych w publikacji III. Przeprowadzono badania polegające na ocenie sukcesu wylęgu organizmów z jaj przetrwalnikowych po ich ekspozycji na zwiększające się stężenia NaCl: a) bez wcześniejszej inkubacji, b) po wcześniejszej inkubacji jaj przetrwalnikowych w różnych stężeniach NaCl przez trzy, siedem i 28 dni.

Publikacja V (manuskrypt) - „Odpowiedź antyoksydacyjna situ rozpięzchłego (*Juncus effesus*) i oczeretu jeziornego (*Schoenoplectus lacustris*) na zanieczyszczenie solą drogową”

Hipoteza: 1) Systemy antyoksydacyjne situ rozpięzchłego i oczeretu jeziornego skutecznie chronią rośliny przed stresem spowodowanym ekspozycją na sól drogową; 2) Rośliny akumulują jony Na^+ w swoich tkankach, a ich stężenie w tkankach roślin będzie wzrastało wraz z gradientem stężeń NaCl zastosowanych w czasie ekspozycji.

Zastosowanie roślin do fitoekstrakcji jonów sodowych z wody zanieczyszczonej solą drogową jest obiecującym kierunkiem badań. Jednak, aby skutecznie zastosować rozwiązania oparte o fitotechnologie, należy dobrać gatunki roślin, które charakteryzować się będą tolerancją na zasolenie, jak również zdolnością do akumulacji jonów Na^+

w swoich tkankach. W tym celu wybrano dwa gatunki roślin rodzimych dla flory Polskiej – sit rozpięzchły i oczeret jeziorny i przeanalizowano odpowiedź systemów antyutleniających tych roślin na zasolenie, jak również ich zdolność do akumulacji jonów Na^+ w nadziemnych tkankach. W tym celu przeprowadzono 60 dniową ekspozycję roślin na wzrastające stężenia NaCl, zbadano wybrane parametry stresu oksydacyjnego oraz określono zawartość jonów Na^+ zakumulowanych w roślinach w trakcie ekspozycji na zasolenie.

4.3. Materiały i metody badawcze

Badania terenowe - teren badań, materiał i metody poboru prób

Teren badań stanowiły cztery stawy miejskie zlokalizowane na terenie miasta Łodzi (51.7500000° N, 19.4666700° E):

1. Arturówek Górny (Ryc. 1a) położony na rzece Bzurze, znajdujący się na terenie kompleksu wypoczynkowo-rekreacyjnego „Arturówek.” Stanowił on zbiornik referencyjny ze względu na zakaz stosowania soli drogowej w zlewni zbiornika (znajdującej się w obszarze miejskiego Lasu Łągiwnickiego). Ograniczony dopływ zanieczyszczeń wynika również z zastosowanych w tym ekosystemie rozwiązań ekohydrologicznych (Jurczak i in., 2018).
2. Zbiornik Wasiaka (Ryc. 1c), położony na rzece Sokołówce. Pierwszy od źródła zbiornik na Sokołówce stale wypełniony wodą. Zlewnia zdominowana zabudową jednorodzinną, gęstą siecią dróg osiedlowych oraz kilkoma większymi drogami międzyosiedłowymi, z których wody opadowo-roztopowe odprowadzane są siecią kanalizacyjną do rzeki.
3. Zbiornik Julianów Górny (Ryc. 1b), położony na rzece Sokołówce (drugi stale wypełniony wodą), leżący na terenie Parku im. Adama Mickiewicza. Zagospodarowanie zlewni pomiędzy zbiornikami Wasiaka i Julianów Górny takie samo jak w przypadku zlewni zbiornika Wasiaka, dodatkowo z dwujezdniową drogą.
4. Zbiornik Zgierska (Ryc. 1d), położony na rzece Sokołówce (trzeci zbiornik od źródeł – po Wasiaku i kompleksie zbiorników Julianów). Położony jest poniżej dwukierunkowej jezdni i torowiska, otoczony nieutwardzonymi ścieżkami dla pieszych. W sąsiedztwie zbiornika znajduje się osiedle domów wielorodzinnych. Zasilany przelewem ze zbiornika Julianów Dolny, brak zidentyfikowanych dopływów z kanalizacji deszczowej.

Wcześniejsze badania prowadzone w zlewni rzeki Sokołówki wykazały stężenia chlorków w spływie porztopowym sięgające od 1 243 mg Cl⁻/L do nawet 12 086 mg Cl⁻/L – wyloty z kanalizacji deszczowej pomiędzy zbiornikami Wasiaka i Julianów Górny (Szkłarek i in. 2015).



Ryc. 1. Badane zbiorniki wodne. a: Arturówek Górny, b: Staw Wasiaka, c: Julianów Górny, d: Zbiornik Zgierska. Autorzy fotografii Sebastian Szklarek i Rafał Witczak.

Materiał stanowiły trzy grupy zooplanktonu: wrotki, wioślarki i widłonogi pobrane z w/w stawów miejskich. Próby zooplanktonu pobierane były co dwa tygodnie, od rozpoczęcia kalendarzowej wiosny aż do zakończenia kalendarzowej jesieni, w latach 2019, 2020, 2021. Próby pobierano w strefie limnetycznej, przy wlocie do zbiorników z warstwy 1 metr od powierzchni wody. Próba wody o objętości 20 L była filtrowana za pomocą siatki zooplanktonowej o średnicy oczek 50 μm . Wydajność sieci o tym rozmiarze oczek jest wysoka, nawet przy pobieraniu próbek z mniejszych objętości wody, co zostało zweryfikowane podczas wstępnych prac terenowych. Przy objętości próbki 20 litrów i gęstej siatce, w każdej próbce uzyskano od kilkuset do kilku tysięcy osobników nie tylko wrotków, ale także skorupiaków planktonowych. Następnie próby zagęszczano do 10 ml, a w celu utrwalenia prób zastosowano płyn Lugola. Zooplankton analizowano jakościowo i ilościowo przy użyciu szklanej komory Sedgewick-Rafter o pojemności 1 ml i mikroskopu Nikon (powiększenie 40-100x). Gdy liczebność osobników danej grupy taksonomicznej była wysoka (> 150 osobników na podpróbę), wszystkie zwierzęta zooplanktonowe w komorze były liczone standardowo (liczono zawartość jednej komory). Gdy liczebność zooplanktonu w jednej podpróbce była niższa, pobierano drugą podpróbę i liczono osobniki z kolejnej komory. Procedura ta jest modyfikacją metody opisaną przez

Mack i in., 2012 jako "metoda 150/próbka." Analizy morfologiczne przeprowadzono w oparciu o publikacje Amoros (1984), Benzie (2005) oraz Ejsmont-Karabin i in. (2004).

Pobór wody oraz osadów do analiz chemicznych odbywał się dwa razy w miesiącu, a w okresie roztopów co kilka dni. Woda z toni wodnej była pobierana 20 cm pod powierzchnią, pobór osadów został przeprowadzony przy pomocy czerpacza osadów, z górnej 20 cm warstwy osadu. Wodę międzyosadową uzyskano poprzez wirowanie prób osadów (3000 rpm, 10 minut).

Materiał do badań eksperymentalnych

1. Organizmy zooplanktonowe:

- a) pochodzące z naturalnych ekosystemów, pobierane w terenie zgodnie z opisem powyższej;
- b) pochodzące z linii laboratoryjnych (Microbiotest Inc.), wykorzystane do badań z biotestami oraz w eksperymentach dotyczących sukcesu wylęgu. Wykorzystane gatunki to *Daphnia magna*, *Ceriodaphnia dubia*, *Thamnocephalus platyurus*, *Brachionus calyciflorus*.

2. Materiał roślinny

Sadzonki situ rozpięzchłego (*Juncus effusus*) oraz oczeretu jeziornego (*Schoenoplectus lacustris*) nabyte w firmie oferującej rośliny wodne (Perfekt Klik).

Metody badań

W celu realizacji badań terenowych i eksperymentalnych wykorzystane zostały poniższe techniki badawcze:

- 1. Analizy jakościowe i ilościowe prób zooplanktonu (metodyka opisana w publikacji II),
- 2. Analiza wody z toni wodnej oraz wody międzyosadowej przeprowadzona za pomocą chromatografii jonowej (chromatograf jonowy Dionex®) (metodyka opisana w publikacji II),
- 3. Analizy z wykorzystaniem biotestów Rotoxkit F, Thamnotoxkit F, Daphtoxkit F magna oraz Daphtoxkit F pulex (Microbiotests Inc.) (metodyka opisana w publikacji III),
- 4. Analiza struktury jaj przetrwalnikowych *Daphnia magna* za pomocą skaningowego mikroskopu elektronowego (Phenom ProX) (metodyka opisana w publikacji IV),

5. Analizy dotyczące systemów antyoksydacyjnych u roślin (metodyka opisana w manuskrypcie V),
 - a) stężenie białka oznaczono przy użyciu odczynnika Folina-Ciocalteu metodą opisaną przez Lowry'ego i in. (Lowry i in., 1951),
 - b) stopień utlenienia lipidów oznaczono przy użyciu kwasu tiobarbiturowego (TBA) opisanego przez Stocks i Dormandy (Stocks i Dormandy, 1971) z modyfikacją Rice-Evans i in. (Rice-Evans i in., 1991),
 - c) stężenie zredukowanego glutationu (GSH) oznaczono przy użyciu odczynnika Ellmana (kwas 5,5'-ditiobis(2-nitrobenzoesowy); DTNB) (Ellman, 1959),
 - d) aktywność katalazy została oznaczona spektrofotometrycznie w oparciu o metodę Aebi (1984),
 - e) do oznaczenia całkowitego nieenzymatycznego potencjału przeciwutleniającego zastosowano metodę opisaną przez Benzie i Strain (1996).
6. Badania dotyczące zawartości jonów sodu w tkankach roślin prowadzono w oparciu o metodykę opisaną przez Iseki i in., 2017 (metodyka opisana w manuskrypcie V).
7. Analizy statystyczne otrzymanych danych terenowych i eksperymentalnych.

5. Omówienie prac wchodzących w skład rozprawy doktorskiej

5.1. Publikacja I - *The effects of road salt on freshwater ecosystems and solutions for mitigating chloride pollution – A review*

Problem zanieczyszczenia solą drogową został poruszony w publikacji przeglądowej [publikacja nr I]. W publikacji ujęto problem zasolenia opierając się na podejściu ekohydrologicznym, tzn. skupiając się na trzech krokach: 1. monitoringu zagrożeń, 2. analizie przyczynowo skutkowej, 3. tworzeniu i optymalizacji rozwiązań (Zalewski, 2014). W sekcji pierwszej artykułu zwrócono uwagę na podwyższone stężenia jonów chlorkowych, które obserwowane są w wodach słodkich, począwszy od zbiorników wód burzowych, poprzez stawy i jeziora, a także źródła, strumienie i rzeki. Dodatkowo, opisano problem przenikania soli drogowej do wód gruntowych oraz ich migracji wraz z wodami podziemnymi do wód powierzchniowych. Sekcja druga publikacji skupia się na wpływie zanieczyszczenia solą drogową na aspekty abiotyczne oraz biotyczne. Do tych pierwszych zaliczyć można zmiany w strukturze gleb oraz wypłukiwanie z niej metali ciężkich, zwiększone wydzielanie nieorganicznego i organicznego węgla z sedimentów rzecznych czy zaburzenia w miksi jezior. Zwrócono również uwagę na szeroki aspekt oddziaływania zasolenia na organizmy żywe – mikroorganizmy, fitoplankton, zooplankton, a także bezkręgowce bentosowe, ryby i makrofity. W ostatniej sekcji publikacji opisano możliwe metody przeciwdziałania i minimalizacji zanieczyszczenia solą drogową. Zaliczyć do nich można dobre praktyki przechowywania i stosowania soli drogowej, możliwe zamienniki chlorku sodu, metody mechaniczne, takie jak systemy grzewcze instalowane w drogach oraz obiecujący kierunek, jakim są rozwiązania bliskie naturze, w tym wykorzystanie roślin halofilnych zdolnych do akumulacji jonów sodu i chlorkowych w tkankach.

Przegląd danych literaturowych zaprezentowany w publikacji wskazał na szerokie konsekwencje, jakie ma stosowanie soli drogowej na słodkowodne ekosystemy oraz pozwolił zidentyfikować luki w badaniach nad wpływem soli drogowej na zooplankton. Większość badań dotyczących zooplanktonu to badania toksyczności, głównie skierowane na pojedynczy gatunek zooplanktonu lub badania z wykorzystaniem sztucznych zbiorników (tzw. *mesocosm*), w celu obserwacji zmian w słodkowodnych sieciach troficznych. Wyniki te niewątpliwie rzucają światło na złożoność zależności pomiędzy podwyższonymi stężeniami jonów Cl^- a organizmami zooplanktonowymi, jednak bardzo mało publikacji porusza problem stosowania soli drogowej w oparciu o badania

prowadzone w naturalnych ekosystemach.

5.2. Publikacja II - *Does winter application of road salt affect zooplankton communities in urban ponds?*

W celu lepszego zrozumienia jak sól drogowa wpływa na organizmy zooplanktonowe w naturze, przeprowadzono badania na wybranych miejskich zbiornikach wodnych, w różnym stopniu narażonych na zasolenie [publikacja nr II]. Teren badań stanowiły cztery zbiorniki zlokalizowane na terenie miasta Łodzi: Arturówek Górny (zbiornik referencyjny), Zbiornik Wasiaka, Zbiornik Julianów Górny oraz Zbiornik Zgierska. Teren w pobliżu zbiorników oraz ich szczegółową charakterystykę przedstawiono na **Rycinie 1 w publikacji nr II** oraz w **Tabeli S1 w Suplemencie do publikacji nr II**. Badania prowadzono na przestrzeni trzech lat hydrologicznych, różniących się od siebie warunkami pogodowymi w czasie zimy. Zgodnie z definicją Instytutu Meteorologii i Gospodarki Wodnej rok hydrologiczny w Polsce rozpoczyna się pierwszego listopada i trwa do 31 października. Rok 2019 charakteryzował się przeciętną zimą, rok 2020 łagodną, bezśnieżną zimą, natomiast rok 2021 surową zimą i opadami śniegu. Przeprowadzono monitoring jakości wody z toni oraz wody międzyosadowej, a także analizy jakościowe i ilościowe organizmów zooplanktonowych zamieszkujących zbiorniki.

Zaobserwowano, że w stawach narażonych na dopływ soli drogowej, tj. w Zbiorniku Wasiaka, w Zbiorniku Julianów Górny oraz Zbiorniku Zgierska, stężenia chlorków w wodzie międzyosadowej utrzymywały się na poziomie powyżej limitu chronicznego (120 mg Cl⁻/L) przez większą część roku 2020. Natomiast stężenia chlorków w wodzie międzyosadowej w roku 2021 przekraczały stężenia ostre (640 mg Cl⁻/L) również w ciągu miesięcy wiosennych. Stężenia chlorków przekraczające limit dla stężeń ostrych utrzymywały się również w wodzie z toni do marca [Rycina 2 i 3 w publikacji II]. Świadczy to o tym, że problem zanieczyszczenia solą drogową nie ogranicza się wyłącznie do miesięcy zimowych – podwyższone stężenia chlorków mogą utrzymywać się w zbiornikach na długo po zakończeniu zimy i wpływać na organizmy słodkowodne także w ciągu pozostałych miesięcy. Potwierdzono rolę stawu Arturówek Górny jako zbiornika referencyjnego. W tym ekosystemie stężenia chlorków i sodu utrzymywały się na najniższym poziomie we wszystkich badanych latach [Rycina 4 i 5 w publikacji II].

Struktury zooplanktonu we wszystkich zbiornikach charakteryzowały się średnim bogactwem gatunkowym. Największa liczba gatunków (27) została zaobserwowana w Arturówku Górnym w roku 2019. Do najczęściej obserwowanych gatunków należały

Bosmina longirostris oraz *Chydorus sphaericus*, a także *Keratella cochlearis*, *Keratella quadrata* oraz wrotki z rodzaju *Polyarthra*. Zbiornik Zgierska, w którym zagęszczenie całkowitego zooplanktonu było najwyższe, charakteryzował się dominacją wrotków w strukturze zooplanktonu. Czynnikiem wyjaśniającym wysokie zagęszczenia wrotków w tym stawie może być duża ilość materii organicznej w zbiorniku, która mogła stanowić dobre źródło pożywienia dla wrotków. Wioślarki z rodzaju *Daphnia* we wszystkich badanych zbiornikach obserwowane były rzadko i pozostawały nieliczne.

Przeprowadzone analizy RDA (ang. *redundancy analysis*) wskazały, że zarówno czynniki środowiskowe, jak i charakterystyka poszczególnych stawów w znaczący sposób wpłynęły na zagęszczenie zooplanktonu w zbiornikach. Jednak czynniki środowiskowe, w tym stężenia chlorków, miały mniejszy wpływ na zagęszczenie zooplanktonu (11%), w porównaniu do charakterystyki stawów (62%) [rycina 6 w publikacji nr II]. W zbiorniku referencyjnym Arturówek Górny, stężenia jonów Cl^- oraz Na^+ nie miały wpływu na zagęszczenia zooplanktonu obserwowane w zbiorniku. W przypadku tego stawu czynnikiem, który w największym stopniu decydował o zagęszczeniu zooplanktonu były warunki pogodowe. Jeżeli chodzi o pozostałe trzy stawy, które narażone były na zanieczyszczenie solą drogową, całkowite zagęszczenie zooplanktonu oraz zagęszczenie poszczególnych grup (wioślarki, widłonogi i wrotki) było negatywnie skorelowane ze stężeniem jonów Cl^- w wodzie powierzchniowej. Jednak co ważne, czynnikiem najbardziej wpływającym na zagęszczenie organizmów zooplanktonowych była charakterystyka zbiorników.

Wyniki otrzymane w tym etapie badań wskazały, że jony chlorkowe były ważnym, ale niedecydującym czynnikiem wpływającym na strukturę zooplanktonu w badanych stawach miejskich.

5.3. Publikacja III - *Acute toxicity of seven de-icing salts on four zooplankton species – is there an “eco-friendly” alternative?*

Zdecydowana większość doniesień literaturowych dotyczących wpływu stosowania soli drogowej na słodkowodne ekosystemy skupia się na wykorzystaniu chlorku sodu lub innych soli nieorganicznych tj. CaCl_2 lub MgCl_2 . Znacznie mniej informacji dotyczy efektów stosowania innych zamienników, w tym tych reklamowanych jako „przyjazne dla środowiska” (Schuler i Relyae, 2018b). Z tego względu, w drugim etapie badań skupiono się na zbadaniu toksyczności sześciu zamienników tradycyjnej soli drogowej dostępnych

w Polsce **[publikacja nr III]**. Substancjami badanymi były: tradycyjna sól drogowa NaCl (z dodatkiem substancji przeciwbrylającej), produkt DONSOL (CaCl_2), produkt DONSOL Drogowy ($\text{NaCl} + \text{CaCl}_2$, z dodatkiem inhibitora korozji), produkt DONSOL Magnez ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$), posiadający eko-etykieta produkt Lodołamacz (CaCl_2 , z dodatkiem inhibitora korozji oraz $(\text{NH}_4)_2\text{HPO}_4$) oraz produkt IceGo w postaci granulek oraz płynu (CH_3COOK). Organizmami badanymi były cztery gatunki zooplanktonu: wioślarki *Daphnia magna* i *Ceriodaphnia dubia*, widłonóg *Thamnocephalus platyurus* oraz wrotek *Brachionus calyciflorus*. W badaniach wykorzystano cztery rodzaje mikrobiotestów firmy MicroBioTests Inc. z wymienionymi organizmami, w celu oszacowania skutecznych stężeń EC_{10} i EC_{50} . Zastosowany w badaniach szereg stężeń chlorków wynosił: 15 200 mg/L, 7 600 mg/L, 3 800 mg/L, 1 900 mg/L oraz 950 mg/L. W przypadku CH_3COOK w formie granulek konieczne było zwiększenie stężenia wyjściowego dwukrotnie, ze względu na brak efektu przy założonym szeregu rozcieńczeń. Dla CH_3COOK stężenia wynosiły 30 400 mg/L, 24 320 mg/L, 21 280 mg/L oraz 18 240 mg/L. Organizmy wystawiono na działanie substancji przez okres 24 godzin. Po ekspozycji zliczano organizmy aktywnie pływające oraz te nieruchome.

Dawki EC_{50} różniły się pomiędzy organizmami oraz poszczególnymi substancjami **[Rycina 1 w publikacji nr III]**. Tradycyjna sól drogowa NaCl okazała się najbardziej toksyczna dla *T. platyurus* ($\text{EC}_{50} = 3\,289$ mg/L) oraz *B. calyciflorus* ($\text{EC}_{50} = 3\,318$ mg/L). Dawki EC_{50} NaCl dla wioślarek wynosiły natomiast 4 328 mg/L dla *C. dubia* oraz 5 244 mg/L dla *D. magna*. Spośród pozostałych substancji, jedynie CH_3COOK w formie granulek okazał się mniej toksyczny dla wszystkich badanych organizmów i wywoływał efekt przy najwyższych stężeniach (EC_{50} w zakresie od 19 274 do 22 786 mg/L). Substancja DONSOL (CaCl_2), DONSOL Magnez ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$) oraz CH_3COOK w postaci płynu posiadały istotnie statystycznie niższe dawki EC_{50} w porównaniu do soli drogowej NaCl. Natomiast dawki EC_{50} dla Lodołamacza (CaCl_2) oraz DONSOL Drogowy ($\text{NaCl} + \text{CaCl}_2$) nie różniły się statycznie od EC_{50} dla tradycyjnej soli drogowej **[Rycina 2 w publikacji nr III]**. Otrzymane wyniki wskazały również na różną wrażliwość badanych gatunków. Spośród badanych organizmów dawki EC_{50} dla *D. magna* były istotnie statystycznie wyższe niż dawki dla pozostałych trzech organizmów. Natomiast dawki obliczone dla *T. platyurus* były istotnie statycznie niższe od wszystkich pozostałych organizmów. Dawki EC_{50} dla *B. calyciflorus* oraz *C. dubia* nie różniły się istotnie pomiędzy sobą **[Rycina 3 w publikacji nr III]**.

Uzyskane wyniki pozwoliły na wyciągnięcie dwóch istotnych wniosków.

Po pierwsze, organizmy zooplanktonowe różnią się w swojej odporności na zasolenie. Niniejsze badania wskazały, że *T. platyurus* wykazał się najmniejszą odpornością na podwyższone zasolenie, a *D. magna* największą. Po drugie, wiele substancji odładowujących, mimo obietnic ze strony producentów, nie wykazuje mniejszej toksyczności w porównaniu do tradycyjnych soli drogowej NaCl. Z tego względu istnieje konieczność dokładnego badania wszelkich zamienników soli drogowej oraz ich wpływu na ekosystemu słodkowodne.

5.4. Publikacja IV - *The effect of road salt (NaCl) treatment on the hatching success of Daphnia magna and Thamnocephalus platyurus*

Biorąc pod uwagę wyniki otrzymane w drugim etapie badań, etap trzeci skupił się na dwóch przedstawicielach zooplanktonu – *D. magna* oraz *T. platyurus*, w celu oceny sukcesu wylęgu organizmów z jaj przetrwalnikowych: 1. przy różnych stężeniach NaCl bez wcześniejszego okresu inkubacji oraz 2. po okresie inkubacji jaj w różnych stężeniach NaCl przez trzy, siedem i 28 dni **[publikacja nr IV]**. Jaja przetrwalnikowe pochodziły ze standaryzowanych testów Daphtoxkit i Thamnotoxkit (MicroBioTests Inc). Dokładna metodyka eksperymentu z wylęgami została przedstawiona na **Rycinie 1 w publikacji nr IV**. Dodatkowo, w celu sprawdzenia jak ekspozycja na różne stężenia NaCl wpływa na jaja przetrwalnikowe i zarodki *D. magna*, ehippia wcześniej wystawione na różne stężenia NaCl przez trzy i siedem dni zostały zanalizowane pod elektronowym mikroskopem skaningowym. Ehippia zostały otworzone a obserwacje podzielono na następujące kategorie: 1. puste ehippium, 2. pojedyncze jajo, 3. dwa jaja, 4. pojedynczy zarodek, 5. pojedynczy zarodek z widocznym okiem, 6. dwa zarodki, 7. dwa zarodki z widocznym okiem, 8. jajo i zarodek.

W pierwszej kolejności sprawdzono sukces wylęgu prób kontrolnych, tzn. jaj przebywających w pożywce (EPA dla *T. platyurus* i ISO dla *D. magna*), bez dodatku NaCl. Nie zaobserwowano istotnie statystycznych różnic w pierwszym i drugim wylęgu pomiędzy jajami inkubowanymi przez zero (T0), trzy (T3) i siedem dni (T7). Jednakże, statystycznie niższy wylęg zaobserwowano po inkubacji przez 28 dni (T28), dlatego w dalszych badaniach nie brano pod uwagę inkubacji 28-dniowej **[Rycina 2 w publikacji nr IV]**.

Następnie przetestowano sukces wylęgu jaj, które nie były wystawione na inkubację (T0) w warunkach podwyższonego stężenia NaCl. Podczas pierwszego wylęgu

zaobserwowano, że sukces wylęgu spadał wraz ze zwiększającymi się stężeniami NaCl. Dla *T. platyurus* liczba wykłutych osobników w pożywce EPA i w najniższym stężeniu NaCl-5 (0,95 g/L) nie różniła się istotnie statystycznie. Liczba osobników w stężeniu NaCl-4 (1,90 g/L), NaCl-3 (3,80 g/L) i NaCl-2 (7,60 g/L) różniła się statystycznie istotnie od kontroli, od stężenia najniższego NaCl-5 oraz między sobą. Dla *D. magna* liczba osobników wykłutych w pożywce ISO, w stężeniu NaCl-5 (0,95 g/L), NaCl-4 (1,90 g/L) oraz NaCl-3 (3,80 g/L) nie różniła się istotnie statystycznie między sobą. Statystycznie istotnie mniej osobników wykłuło się natomiast w stężeniu NaCl-2 (7,60 g/L). Przy najwyższym stężeniu NaCl-1 (15,20 g/L) nie wykłuły się żadne osobniki, zarówno *T. platyurus* jak i *D. magna* **[Rycina 3 w publikacji nr IV]**. Podczas drugiego wylęgu, podobna liczba organizmów *T. platyurus* wykłuła się w stężeniu NaCl-5 (0,95 g/L) oraz NaCl-4 (1,90 g/L). W stężeniu NaCl-3 (3,80 g/L) suma z dwóch wylęgów nie różniła się statystycznie od sumy wylęgów zaobserwowanych w kontroli, stężeniu NaCl-5 oraz NaCl-4. Suma wylęgów w stężeniu NaCl-1 nie różniła się statystycznie od sumy wylęgów w stężeniach NaCl-3 i NaCl-4 **[Rycina 3 w publikacji nr VI]**. Dla *D. magna* zaobserwowano nagły spadek wykłutych osobników przy stężeniu NaCl-2 (7,60 g/L). Osobniki wykłuły się również w stężeniu najwyższym NaCl-1 i było ich statystycznie mniej niż w kontroli oraz stężeniach NaCl-5, NaCl-4 i NaCl-3 **[Rycina 3 w publikacji IV]**.

Wyniki analiz jaj przetrwalnikowych, które wcześniej wystawione były na trzydniową oraz siedmiodniową ekspozycję NaCl, nie wykazały istotnej zależności pomiędzy czasem inkubacji a stężeniami NaCl, która wpływałaby na sukces wylęgu. Wykazano jednak istotne zmiany w sukcesie wylęgu pomiędzy stężeniami **[Rycina S1 i S2 w Suplemencie do publikacji nr IV]**. Ostatnim etapem badań było sprawdzenie czy przepłukanie jaj po trzydniowej i siedmiodniowej inkubacji wpłynie na sukces wylęgu. Nie wykazano statystycznie istotnych zależności pomiędzy stężeniami NaCl i czasem inkubacji, które wpływałyby na sukces wylęgu. Jednakże, zaobserwowano różnice w zsumowanej liczbie osobników wykłutych z obu wylęgów u *T. platyurus* po przepłukaniu jaj przetrwalnikowych wodą **[Rycina S3 i S4 w Suplemencie do publikacji nr IV]**.

Analizy z użyciem elektronowego mikroskopu skaningowego zostały wykorzystane, aby sprawdzić czy różne stężenia NaCl mają bezpośredni wpływ na ehippia oraz znajdujące się w nich jaja i zarodki. Do tych badań wykorzystano ehippia, które wcześniej inkubowane były przez trzy dni w stężeniach NaCl-5, NaCl-2, NaCl-2 (przepłukane wodą) i NaCl-1 oraz NaCl-1 (przepłukane wodą). Do drugiej grupy należały ehippia inkubowane przez siedem dni w stężeniach NaCl-3, NaCl-2 i NaCl-1. Dla grupy inkubowanej przez trzy

dni wykazano statystycznie istotne różnice pomiędzy kontrolą a stężeniem NaCl-2 oraz kontrolą i stężeniem NaCl-1, a także kontrolą i stężeniem NaCl-1 (przepłukane). W przypadku siedmiodniowej inkubacji, wszystkie stężenia różniły się statystycznie od kontroli [**Tabela 1 w publikacji nr IV**]. Ephippia kontrolne najczęściej były puste po otwarciu, natomiast te wystawione na inkubację w NaCl częściej zawierały w środku jajo lub zarodek na różnym etapie rozwoju [**Rycina 4 w publikacji nr IV**]. Mogłoby to sugerować, że inkubacja w NaCl w jakimś stopniu uniemożliwiła zarodkom opuszczenie ehippium wpływając na sukces wylęgu, jednak jak wykazano we wcześniejszych eksperymentach, nie znaleziono potwierdzenia na istotną interakcję pomiędzy czasem inkubacji a sukcesem wylęgu.

Trzeci etap badań pozwolił na wyciągnięcie wniosków, że stężenia NaCl miały kluczowe znaczenie na sukces wylęgu *T. platyurus* oraz *D. magna*. Podobnie jak wykazano w publikacji nr III, również na tym etapie badań *T. platyurus* charakteryzował się większą wrażliwością na podwyższone zasolenie w porównaniu do *D. magna*.

5.5. Publikacja V – Odpowiedź antyoksydacyjna situ rozpięzchłego (*Juncus effesus*) i oczeretu jeziornego (*Schoenoplectus lacustris*) na zanieczyszczenie solą drogową (MANUSKRYPT)

Wyniki przedstawione w Publikacji II pokazały, że problem zanieczyszczenia solą drogową nie ogranicza się wyłącznie do miesięcy zimowych – wysokie stężenia jonów chlorkowych i sodu obserwowane były również w miesiącach wiosennych. W związku z tym konieczne staje się poszukiwanie rozwiązań, które mogłyby niwelować stopień zasolenia w celu ochrony słodkowodnych ekosystemów. W dobie postępujących zmian klimatu kluczowe staje się wykorzystywanie rozwiązań opartych na naturze (ang. *Nature Based Solutions*), których działanie oparte jest o naturalne procesy występujące w przyrodzie. Przykładem takich rozwiązań są sztuczne mokradła (ang. *constructed wetlands*), które dzięki doborowi odpowiednich gatunków roślin pozwalają na remediację zanieczyszczonej wody czy gleby.

Celem ostatniego etapu badań była ocena tolerancji na zasolenie oraz potencjału fitodesalinizacji dwóch gatunków roślin flory Polski: situ rozpięzchłego (*Juncus effesus*) oraz oczeretu jeziornego (*Schoenoplectus lacustris*). Aby to osiągnąć przeprowadzono dwumiesięczną ekspozycję roślin na dwa stężenia NaCl: stężenie niskie (200 mg/L) oraz wysokie (1000 mg/L). W trakcie pierwszego miesiąca rośliny podlewane były wodą

destylowaną wzbogaconą pożywką Hoagland'a w rozcieńczeniu 1/4, natomiast podczas drugiego miesiąca pożywkę zastąpiono wodą kranową, w celu sprawdzenia czy wspomaganie mineralne miało znaczenie w warunkach stresu. Schemat przebiegu ekspozycji na zasolenie przedstawiono na **Rycinie 1 w publikacji V**. Po okresie ekspozycji, zbadano wybrane parametry stresu oksydacyjnego w komórkach roślin: stopień peroksydacji lipidów, stężenie zredukowanego glutationu (GSH), aktywność katalazy, całkowity nieenzymatyczny potencjał antyoksydacyjny. Ponadto, określono zawartość jonów Na^+ w nadziemnych tkankach roślin. Zastosowano następujące nazwy wariantów badanych: K – kontrola w czasie aklimatyzacji podlewana wodą kranową, K1 – kontrola podlewana wodą destylowaną z pożywką, C11 – woda destylowana + pożywka + niskie stężenie NaCl (200 mg NaCl/L), C12 – woda destylowana + pożywka + wysokie stężenie NaCl (1000 mg NaCl/L), K2 - kontrola podlewana wodą kranową, C21 – woda kranowa + niskie stężenie NaCl (200 mg NaCl/L), C22 – woda kranowa + wysokie stężenie NaCl (1000 mg NaCl/L).

Badane gatunki różniły się pod względem zbadanych parametrów stresu oksydacyjnego. Dla sita rozpięzchłego poziom peroksydacji lipidów w roślinach z kontroli z pożywką (K1) był istotnie mniejszy niż w roślinach traktowanych wodą kranową i wysokim stężeniem NaCl (1000 mg NaCl/L) (C22). Dla oczeretu jeziornego nie zaobserwowano żadnych różnic pomiędzy badanymi wariantami [**Ryc. 2a w publikacji V**]. W opozycji do tej obserwacji, stężenie zredukowanego glutationu w sitie rozpięzchłym utrzymywało się na takim samym poziomie we wszystkich badanych wariantach. U tego gatunku stężenie GSH było wyższe niż u oczeretu jeziornego we wszystkich wariantach, z wyjątkiem roślin w czasie aklimatyzacji (K). Natomiast, dla oczeretu jeziornego zaobserwowano mniejsze stężenie GSH w roślinach traktowanych wodą z pożywką i niskim stężeniem NaCl (200 mg NaCl/L) niż w roślinach traktowanych wodą kranową i niskim stężeniem NaCl (200 mg NaCl/L) [**Ryc. 2b w publikacji V**]. Wyraźne różnice w odpowiedzi antyoksydacyjnej wybranych gatunków zaznaczyły się w aktywności katalazy. W sitie rozpięzchłym aktywność katalazy była znacząco wyższa niż w oczerecie jeziornym. Dodatkowo, w sitie aktywność katalazy w kontroli z pożywką (K1) była wyższa niż w roślinach traktowanych wysokim stężeniem soli, zarówno tych podlewanych pożywką (C12) jak i wodą kranową (C22). Dla oczeretu jeziornego nie wykazano żadnych istotnych różnic między badanymi wariantami [**Ryc. 2c w publikacji V**]. Oczeret jeziorny wykazał znacząco wyższy całkowity nieenzymatyczny potencjał antyoksydacyjny niż sit rozpięzchłe. Dodatkowo, u oczeretu jeziornego potencjał ten był znacząco wyższy

w roślinach w trakcie aklimatyzacji (K) niż w kontroli z wodą kranową oraz w roślinach traktowanych NaCl [**Ryc. 2d w publikacji V**].

Zarówno sit rozpierzchły jak i oczeret jeziorny zakumulowały znaczące stężenia jonów sodu w swoich tkankach [**Ryc. 3 w publikacji V**]. W przypadku situ rozpierzchłego zawartość Na^+ w roślinach podlewanych wodą kranową z wysokim stężeniem NaCl (C22; średnia 119,3 mg Na^+ /g suchej masy) była wyższa niż w roślinach z kontroli podlewanej wodą kranową (K2; średnia 13,3 mg Na^+ /g suchej masy). Dla oczeretu jeziornego zawartość jonów Na^+ była znacząco wyższa w roślinach podlewanych wodą kranową z wysokim stężeniem NaCl (C22; średnia 77,7 mg Na^+ /g suchej masy) niż w roślinach czasie aklimatyzacji (K; średnia 14,1 mg Na^+ /g suchej masy), z kontroli podlewanej wodą z pożywką (K1; średnia 24,3 mg Na^+ /g suchej masy), podlewanych wodą z pożywką i NaCl (stężenie 200 mg/L) (C11; średnia 18,3 mg Na^+ /g suchej masy) oraz z kontroli podlewanej wodą kranową (K2; średnia 22,4 mg Na^+ /g suchej masy). Sit rozpierzchły zakumulował w swoich tkankach więcej Na^+ , jednak różnica ta nie była istotna statystycznie.

Wyniki otrzymane w trakcie ostatniego etapu badań wskazały, że odpowiedź antyoksydacyjna situ rozpierzchłego i oczeretu jeziornego różniła się od siebie – oczeret jeziorny wykazał mniejszą zmienność pod względem przeanalizowanych parametrów stresu oksydacyjnego, co może wskazywać na jego większą tolerancję na zasolenie. Pomimo różnic, systemy antyoksydacyjne obu gatunków były w stanie ochronić rośliny przed stresem spowodowanym zasoleniem. Oba zbadane gatunki roślin wykazały zdolność do akumulacji jonów Na^+ w swoich nadziemnych tkankach, przy czym sit rozpierzchły zakumulował ich więcej niż oczeret jeziorny, co mogło spowodować większy stopień peroksydacji lipidów zaobserwowany u tego gatunku.

6. Wnioski

1. Problem zanieczyszczenia słodkowodnych ekosystemów solą drogową nie ogranicza się wyłącznie do okresu zimowego. Uzyskane w **publikacji II** wyniki wskazują, że podwyższone stężenia jonów chlorkowych utrzymywały się w wodzie powierzchniowej i międzyosadowej również w miesiącach wiosennych. Może to wpływać negatywnie na organizmy zooplanktonowe podczas sezonu wylęgowego.
2. Zaobserwowano negatywną korelację pomiędzy zagęszczeniem zooplanktonu a stężeniami jonów chlorkowych w wodzie przekraczającymi stężenia chroniczne. Jednakże, charakterystyka stawów była głównym czynnikiem decydującym o zagęszczeniu zooplanktonu w badanych zbiornikach **[publikacja II]**.
3. Zamienniki soli drogowej dostępne na polskim rynku, również te posiadające „eko-etykietę” były równie lub bardziej toksyczne dla organizmów zooplanktonowych w porównaniu z tradycyjną solą drogową **[publikacja III]**. Konieczne jest dokładne badanie zamienników soli drogowej zanim trafią one do środowiska naturalnego, zważywszy, że skutki ich działania oraz możliwe interakcje z innymi substancjami nie są jak dotąd wystarczająco poznane. Dodatkowo, wyniki otrzymane w publikacji III wskazują na konieczność poszukiwania alternatyw dla soli nieorganicznych zawierających chlorki.
4. Organizmy zooplanktonowe różnią się w swojej wrażliwości na sól drogową. Wyniki uzyskane w **publikacji III i IV** wskazały na wyższą odporność *Daphnia magna* w porównaniu do *Thamnocephalus platyurus*. Podwyższone stężenia NaCl były decydującym czynnikiem wpływającym na sukces wylęgu tych dwóch gatunków z jaj przetrwalnikowych.
5. Wstępne badania zaprezentowane w **publikacji V** wskazują, że zarówno sit rozpięzchły jak i oczeret jeziorny wykazują potencjał do fitodesalinizacji terenów zanieczyszczonych solą drogową. Oba gatunki zakumulowały w swoich tkankach znaczące ilości jonów Na^+ , a ich systemy antyoksydacyjne były w stanie skutecznie ochronić je przed stresem spowodowanym zasoleniem.

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7. Streszczenie w języku polskim

Stosowanie soli drogowej (głównie chlorku sodu) do zimowego utrzymania dróg jest rutynowym zabiegiem w Polsce oraz innych krajach półkuli północnej. Jednakże, stosowanie soli drogowej jest jedną z przyczyn wtórnego zasolenia słodkowodnych ekosystemów. Podwyższone zasolenie ma toksyczny wpływ na słodkowodne organizmy, w tym na kluczowe ogniwo słodkowodnych sieci troficznych – wioślarki z rodzaju *Daphnia*. Pomimo swojego szkodliwego działania, wciąż brak jest skutecznych rozwiązań, które pozwoliłyby na redukcję użycia soli drogowej. Z tego względu konieczne staje się po pierwsze, dogłębne poznanie zagrożeń jakie sól drogowa oraz jej zamienniki stwarzają dla ekosystemów słodkowodnych, a po drugie poszukiwanie nowatorskich rozwiązań, które pozwoliłyby na redukcję zanieczyszczenia solą drogową.

W niniejszej rozprawie doktorskiej przedstawiono wyniki badań terenowych oraz laboratoryjnych, które rzucają światło na problem zanieczyszczenia solą drogową. Badania terenowe przeprowadzone na czterech zbiornikach wodnych w Łodzi pokazały, że problem zanieczyszczenia słodkowodnych ekosystemów solą drogową nie ogranicza się wyłącznie do okresu zimowego – podwyższone stężenia jonów chlorkowych utrzymywały się w wodzie również w miesiącach wiosennych. Ponadto, w oparciu o naturalne ekosystemy, udało się zaobserwować negatywną korelację pomiędzy zagęszczeniem zooplanktonu a stężeniami jonów chlorkowych w wodzie przekraczającymi stężenia chroniczne. Jednakże, to charakterystyka stawów była głównym czynnikiem decydującym o zagęszczeniu zooplanktonu w badanych zbiornikach.

Przeprowadzone badania laboratoryjne pokazały, że podwyższone stężenia NaCl były decydującym czynnikiem wpływającym na sukces wylęgu *Daphnia magna* i *Thamnocephalus platyurus* z jaj przetrwalnikowych. Dodatkowo wykazano, że zamienniki soli drogowej, również te posiadające „eko-etykietę,” są równie lub nawet bardziej toksyczne dla zooplanktonu w porównaniu do tradycyjnej soli drogowej.

Badania laboratoryjne z użyciem situ rozpięzchłego (*Juncus effesus*) oraz oczeretu jeziornego (*Schoenoplectus lacustris*) wskazały na potencjał tych gatunków do fitodesalinizacji terenów zanieczyszczonych solą drogową, co stwarza możliwość na przyszłe wykorzystanie ich w rozwiązaniach bliskich naturze, takich jak sztuczne mokradła.

8. Streszczenie w języku angielskim (Summary)

Application of road salt (mainly sodium chloride) during winter is a routine measure in Poland and other countries of the northern hemisphere. However, the use of road salt is one of the causes that leads to secondary salinization of freshwater ecosystems. Elevated salinity has toxic effects on freshwater organisms, including a key link in freshwater foodwebs – cladocerans from the *Daphnia* genus. Despite its harmful effects, there is still no effective solution for reducing the use of road salt. Therefore, it becomes necessary, firstly, to understand the threats that road salt and its substitutes pose to freshwater ecosystems, and secondly, to search for innovative solutions to reduce road salt pollution.

This dissertation presents the results of field and laboratory studies that shed light on the problem of road salt pollution. Field studies conducted on four urban ponds located in Lodz showed that the problem of road salt pollution was not limited to the winter period - elevated concentrations of chloride ions persisted in the water during the spring months as well. Moreover, based on natural ecosystems, it was possible to observe a negative correlation between zooplankton density and chloride ion concentrations in water exceeding chronic concentrations. However, it was the characteristics of the ponds that were the main factor determining the density of zooplankton in the studied ponds.

Laboratory studies showed that elevated NaCl concentrations were a main factor determining in the hatching success of *Daphnia magna* and *Thamnocephalus platyurus* from spore eggs. In addition, road salt substitutes, including those with an "eco-label," were shown to be equally or even more toxic to zooplankton compared to traditional road salt.

Laboratory studies using *Juncus effesus* and *Schoenoplectus lacustris* have indicated the phytodesalinization potential of these species, which creates a possibility for future use of these plants in nature-based solutions, such as constructed wetlands, that could mitigate the road salt pollution.

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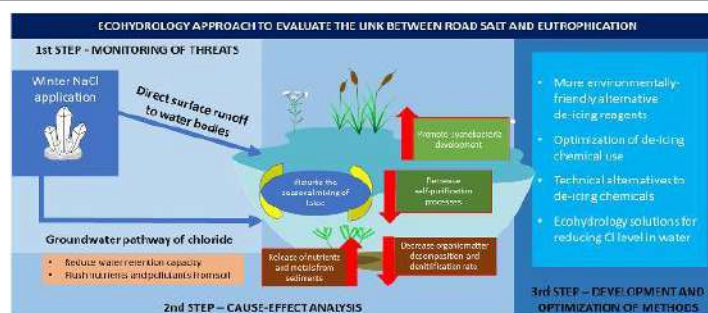
The effects of road salt on freshwater ecosystems and solutions for mitigating chloride pollution – A review

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HIGHLIGHTS

- NaCl application causes short- and long-term negative impacts on water and soil.
- NaCl releases pollutants from soil and sediments.
- Increasing Cl concentration decreases the self-purification processes of water.
- Alternative de-icing agents had similar effects to NaCl, or more negative ones.
- System solutions preventing water pollution by road salt are necessary.

GRAPHICAL ABSTRACT



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ABSTRACT

Road salt (mainly NaCl) is commonly used during the winter to ensure road and pavement safety; however, the long-term application of NaCl has negative consequences on soil and the water environment. The aims of the present review were to evaluate the impact of road salt on catchment processes which accelerate the eutrophication of waters, and to identify a possible approach for reducing the impact of winter salt treatments of roads and sidewalks, on water body quality. The objectives were implemented in accordance with the ecohydrological approach, which recommends using hierarchical steps to solve problems. The first step was the monitoring of threats, in which the causes of high chloride (Cl) concentrations in groundwater and surface water were identified. The results indicate that long-term winter application of road salt increases the annual mean concentrations of Cl in rivers and lakes, due to Cl entering groundwater. The second step was a cause-effect analysis of the impact of NaCl on the abiotic processes in soil and water, and on the biotic response to chloride exposure. Chlorides appear to decrease the biodiversity of aquatic animals and plants but favour the growth of phytoplankton, especially cyanobacteria. Moreover, Cl reduces the self-purification processes of water by decreasing nutrient accumulation in macrophytes, decreasing the denitrification rate and reducing organic matter decomposition. The third step was to evaluate possible solutions for reducing the negative impact of NaCl on the environment, and to improve the effectiveness of alternative de-icing agents. An analysis of available literature indicates that a system-based approach integrating engineering knowledge with an understanding of biological and hydrological processes is necessary to indicate solutions for reducing environmental risks from road salt use.

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1. Introduction

The development of motorization has changed road construction technology, with roads evolving from stone, gravel or sand paths to smooth asphalt and concrete surfaces. This change has been accompanied by an increased risk of accidents due to sliding during the winter period. To improve safety, sodium chloride (NaCl) is commonly used, mainly due to its widespread availability and low cost. In countries located at higher latitudes, where winter temperatures drop below 0 °C and snow is often present, very large amounts of NaCl are applied to roads each year. For example, in 2016, 20.3 million tons of salt were used for ice control and road stabilization in the U.S. (Bolen, 2020); in 2009, more than 6 million tons were applied in Canada (Prosser et al., 2017); in addition, 10 million tons are applied each year in France (Barbier et al., 2018) and according to the General Director for National Roads and Motorways in Poland, approximately 490,000 tons of road salts were applied for the winter season 2019/2020. It is worth noting that those values often do not consider the usage of road salts by people on their private properties or on other commercial, industrial and urban areas. Despite the necessity for ensuring road safety, such widespread usage of road salts is one of the factors associated with the increased salinization of surface water bodies.

Although road salt application may ensure safe movement in the short term, it also has long-term effects on the environment. The first visible consequences of road salt application are car and infrastructure corrosion, deteriorating vegetation near the roads (Zítková et al., 2018) and the corrosion of asphalt (Xiong et al., 2019). Road salt has also been found to influence air quality through the aerosolization of Cl^- and Na^+ at $\text{PM}_{2.5}$ concentrations (particulate matter 2.5 μm or less in size) (Kolesar et al., 2018). In addition, road salt application exerts a much wider impact by influencing nearby soil, groundwater and freshwater ecosystems. Road salts change the physicochemical properties of soil structure and water, which in turn influence their biocenoses. Although organisms have varying degrees of sensitivity to chloride pollution, freshwater ecosystems have been found to demonstrate limit values of 120 mg/L for chronic toxicity and 640 mg/L for acute toxicity in Canada (CEQC, 2011), and 230 mg/L and 860 mg/L (respectively) in the USA (US EPA, 1988).

Increased urbanization and transport, are associated with greater emission of pollutants such as road salt; these enter the water ecosystems with surface runoff, degrading the quality of water and soil. Water is the most important and limited resource necessary for the functioning of the environment and the development of society, and as such, there is a strong need to protect water supplies (Lal, 2013). Road salt pollution has not been considered in studies of water resource quality, but recent years have seen a growing number of publications reporting the negative impact of long-term use of NaCl. The aim of this review is to analyse the threat posed by road salt, especially the most commonly-used chloride salts, on the soil, groundwater and freshwater environment, which could lead to their degradation, accelerate the process of surface water eutrophication and increase the risk of algal blooms.

The study discusses the problem of salt pollution from the perspective of its effect on the mutual interactions between the hydrological cycle and the biocenoses that has developed over the course of evolution. To ensure sustainable development, it is necessary to reduce the negative impact of anthropogenic pollution on the environment, and this can best be achieved by linking ecohydrological processes with system solutions (Zalewski, 2014). To achieve this, this paper takes an Ecohydrology approach comprising the following steps:

- **Monitoring of threats** (Section 2) – identify the pathways of chloride migration after winter salt application by performing a qualitative and quantitative evaluation of processes.
- **Cause and effect analyses** (Section 3) – determine the total impact of chlorides on physicochemical processes and determine the toxicity threat for biota compounds in the environment and biological processes, which together could accelerate the process of eutrophication.
- **Development and optimization of methods** (Section 4. Prevent chloride pollution) – review potential solutions which could decrease the threat from chlorides, remove them from the environment or identify alternative de-icing methods. Such optimization is aimed at improving purification efficiency, but as methods of chloride pollution removal are still being developed, those parts do not include references about optimization.

- **System solution** (Section 5. Conclusion) – summarise current knowledge and indicate potential directions to reduce ecosystem degradation caused by road salt application.

2. Monitoring of threats

2.1. Surface water pollution

In most areas, the primary source of Cl pollution is winter road salt application, most of which reaches surface water via stormwater sewer systems, which has been well documented in a range of studies. Water analyses of highway runoff showed Cl levels to range from 124 to 25,500 mg/L of Cl in Southern Ontario, Canada (Mayer et al., 2011; Prosser et al., 2017); Cl values were found to rise from 183 mg/L to 3568 mg/L during salting period near Azerailles, France, close to the German border (Barbier et al., 2018); finally, Cl concentrations ranged between 1950 and 2740 mg/L in January in Västerås, Sweden, 100 km west of Stockholm (Waara and Färm, 2008). High Cl concentrations were also noted in runoff from urban catchments during melting periods in Lodz city, central Poland, where Cl levels ranged from 1243 mg/L to 12,086 mg/L (Szklarek et al., 2015).

The amount of chlorides that reach the rivers or ponds is correlated with the degree of cover by impermeable surfaces. In Highland Creek (Ontario, Canada) 316 mg/L of Cl (with 1390 mg/L maximum in January/February) was observed in a location with an urbanised area greater than 80% of the catchment. Monitored urbanised sub-watersheds in Illinois creeks (Upper Kickapoo Creek and Upper Little Kickapoo, USA) noted Cl in the range from 36 to 1344 mg/L (Lax et al., 2017). Hubbart et al. (2017) describe a study of chloride concentrations carried out in Hinkson Creek Watershed, Missouri, USA in the years 2010–2014. Samples were collected four times per week from five sampling sites, which were chosen to divide the catchment into five consecutive sub-basins located along the river continuum. They noted that the number of days with a chronic Cl level of 230 mg/L increased from eight days at site #2 to 106 at site #3 during the study period (October 2009–May 2014). The sub-catchment between those sites was highly urbanised: the area of the catchment changed from 102.8 km² to 116.2 km² and urban land cover doubled from 6.1% to 12.7% (Hubbart et al., 2017).

These findings indicate that the part of the river connected with a highly-urbanised area was subject to the greatest threat of acute toxicity for the aquatic ecosystem. While the risk of acute toxicity decreased in the lower part of the river, the risk of chronic toxicity increased. For example, long-term research in Canada (nine lakes in the Halifax Regional Municipality of Nova Scotia) indicated that in most lakes, after an initial period of accumulation, Cl concentrations tended to stabilise after urbanization stopped; however, all lakes demonstrated a higher annual Cl concentration range than noted before urbanization (Scott et al., 2019).

Similar observations were made in a long-term study at 30 sites in 19 streams in the United States – even just a few percent of urban land cover resulted in a higher Cl level (Corsi et al., 2015); at these sites, Cl increased by 2.9 mg/L per % urban land cover for the period 1990–1994 and by roughly twice as much, i.e. 5.8 mg/L per % urban land cover, during 2006–2010 (Corsi et al., 2015). Other long-term studies in 12 watersheds in Maryland's Upper Piedmont, USA found that a 1% increase in impervious surface resulted in increased conductivity by 15 µS/cm over a 25-year period (1986–2010) (Baker et al., 2019).

High annual Cl concentrations in runoff and in rivers influence the level of salinity observed in ponds and lakes. For example, in the Kingston Stormwater Pond (Canada) Cl concentrations reached nearly 1000 mg/L in the pond water and 1290 mg/L in the interstitial water (Marsalek et al., 2003). Also in the 68 stormwater management ponds located in the Red Run watershed (Maryland, USA) mean chloride concentrations were found to range from 7.9 to 3730.8 mg/L during spring 2007 and 2008, with a maximum Cl concentration over 22,000 mg/L

(Gallagher et al., 2011). Tixier et al. (2012) found the Cl concentration in stormwater pond pore water to be in the range of 214–4740 mg/L.

A continuous supply of chloride results in increasing amounts in lakes and ponds. For example, in Lake Simcoe (Canada), the Cl concentration in the outflow increased from 0.7 mg/L/year in 1971 to 39 mg/L in 2007 (Winter et al., 2011). In Lakes Garda, Maggiore, and Iseo (Italy), the Cl concentration increased from 21 to 39% for Cl in the period 1988–2012; in addition, the Cl load analyses for Lake Maggiore showed that 40% of the load come from road salt application (Rogora et al., 2015). In 38 urban lakes in the Minneapolis–St. Paul Twin Cities Metropolitan Area (USA), the annual mean Cl concentration increased by 0.6–3% (depending on the lake) per year in the period 1984–2005 (Novotny et al., 2008). An increasing trend in Cl level was also noted in 125 of 371 lakes during long-term studies in North America in the period 1985–2010 (Dugan et al., 2017).

Long-term trends in surface waters are early warning indicators of significant local, regional and global changes. Hydrological, catchment and weather conditions affect the number of chronic and acute days of Cl concentration observed in rivers or lakes. Corsi et al. (2015) noted that the number of chronic acute days (230 mg Cl/L - US EPA, 1988) increased from 5 to 14 per year for watersheds with a 25% urban area and from 95 to 231 days per year for watersheds with a 90% urban area. A pond receiving runoff from a 14 ha commercial lot noted acute toxicity, i.e. a Cl level of 640 mg/L (CEQG, 2011), until June in surface water (in the range 1500–2500 mg/L from March until halfway through May) and until September in over-sediment water (above 2000 mg/L from March until June, with a maximum 5500 mg/L in March); in addition, in both cases, chronic Cl levels did not decrease below 130 mg/L (CEQG, 2011) for the whole year of the study (Lembcke et al., 2017).

2.2. Groundwater migration to surface water

Although road salt application typically results in pollution of surface water in the winter-spring period, it can also influence the chloride concentration in baseflow, possibly due to the subsurface flow of chloride. Road salt stored in an unsaturated zone was found to be diluted and slowly released into groundwater during a non-winter period: 84 days of NaCl application resulted in a Cl concentration of up to 1900 mg/L in surface soil, which decreased to 100 mg/L at a depth 2.5 m below the surface (Lax and Peterson, 2009); even so, some Cl remained in the unsaturated zone by the next winter. The model made by Lax and Peterson (2009) indicated that 84-day NaCl application each winter for 11 years resulted in the soil systems reaching an equilibrium between Cl input and output: the unsaturated zone was characterised by permanently elevated concentrations of Cl, and this served as a constant source of groundwater and surface water contamination. Robinson et al. (2017) report that the storage capacity for salts in sandy soil increases with the amount of organic matter and that salt release from soil can take 2.5–5 months, depending on field conditions such as longer dry periods and deeper soils. Perera et al. (2013) noted that approximately 40% of applied chloride enters a shallow aquifer in the Highland Creek watershed (the City of Toronto, Canada).

Such groundwater migration poses a threat not only to the quality of surface water, but also to that of underground drinking water resources. For example, in the period 1970–2000, the water pumped from Greenbrook wellfield, the primary source of water for the City of Kitchener (southwestern Ontario, Canada), had been showing steadily increasing chloride concentrations, with some wells demonstrating Cl concentrations exceeding the 250 mg/L drinking water limit (Bester et al., 2006). Similar observations were reported in Connecticut (USA), where the Cl concentration was found to increase from base range 0–2.5 ppm in 1894–1902 to above 250 ppm in 2002–2007 (with maximum 1500 ppm) at hot spots near major highway corridors (Cassanelli and Robbins, 2013).

Chloride contamination could infiltrate the underlying aquifer and increase the Cl levels above base-line concentration in water supply wells by 21% and 54% (during mild and sever winter, respectively) (Rivett et al., 2016).

This soil-groundwater path of Cl migration is also responsible for increases in annual Cl concentrations in surface water, including springs. Chloride concentrations were observed to correlate with the degree of urbanization in a 16-spring site in Ontario, Canada, with the highest chloride concentration at the spring site being 1345 mg/L (mean 1092 mg/L) (Williams et al., 2000). A monitoring study of 52 forest springs in lower mountain ranges of Central Germany showed that the effect of road salt application was still detectable 830 m from the road (Schweiger et al., 2015). Chloride also enters rivers through streambanks. Cl concentrations not exceeding 100 mg/L in samples taken from the streambed interface (0-cm depth) (Halifax, NS, Canada), but those taken from groundwater in a layer 5 to 10 cm below the sediment surface could be as high as 3200 mg/L, and up to 3000 mg/L in a layer 25 to 50 cm below the sediment surface (Roy et al., 2015). Such long-term migration of Cl through groundwater resulted in increased salinity in a nearby urban stream (Kelly et al., 2008; Cooper et al., 2014). Complex studies of chloride migration have been conducted in Urmia city (Iran). During winter 2005, Cl concentrations as high as 1910 mg/L were recorded in snowmelt runoff (mean 445 mg/L), as well as concentrations up to 720 mg/L in January in surface water outflowing from the city area (the lowest level being 30 mg/L, noted in November) and up to 415 mg/L in groundwater samples (mean 131.3 mg/L) (Aghazadeh et al., 2012).

To summarise, road salt application results in Cl concentration exceeding limit values of 120 mg/L for chronic toxicity and 640 mg/L for acute toxicity in Canada (CEQC, 2011), and 230 mg/L and 860 mg/L (respectively) in the USA (US EPA, 1988) in runoff, surface and groundwater (Fig. 1). Moreover, long-term usage of road salt increases annual concentration of Cl in water bodies which presents a threat for abiotic and biotic processes.

3. Cause-effect analysis

3.1. Abiotic processes

The increase of chloride ions in the environment following road salt application not only occurs directly as a consequence of the application per se, they can also migrate indirectly through the soil and groundwater pathways (Fig. 2). Regular use of NaCl as road salt influences the structure of nearby soils, with application resulting in the replacement of Ca, Mg and K with Na, and the depression of porewater pH (Robinson et al., 2017). Such decreases in pH result in the ammonium and dissolved organic matter being flushed from the soils, and the mineralization of organic-N and nitrification being decreased, even at low winter temperatures (Green et al., 2009, 2008; Green and Cresser, 2008). Garakani et al. (2018) found loessial soil pore diameter to be reduced after road salt application (NaCl, KCl, CaCl₂), resulting in a decrease in the retention capacity of soil – further research is needed to examine the impact of NaCl on the hydro-mechanical behavior of unsaturated collapsible soils.

Road salt application can increase the concentrations of heavy metals in the freshwater environment, by flushing from soils (Schuler and Relyea, 2018a) and by increasing fluvial erosion from streambanks (Hoomehr et al., 2018). In addition, greater salinization in rivers significantly increases the release of dissolved inorganic (DIC) and organic (DOC) carbon from sediment; such release was found to be statistically significant in seven of eight cases ($r^2 = 0.64-0.99$, $p < 0.05$) (Duan and Kaushal, 2015). The release of DOC was 7.8 ± 1.9 times higher at a Cl concentration of 4000 mg/L than in controls with 0 mg/L. Salinization also influences the release of total dissolved Kjeldahl nitrogen (TKN) (ammonium + ammonia + dissolved organic nitrogen) from sediment ($r^2 = 0.72-0.95$, $p < 0.05$); this was 13.3 ± 5.1 times higher at 4000 mg/L than at 0 mg/L. The negative impact of salinization was compensated for by an increase in nitrate retention; however, retention was only 1.6 ± 0.4 times higher at 4000 mg/L than at 0 mg/L (Duan and

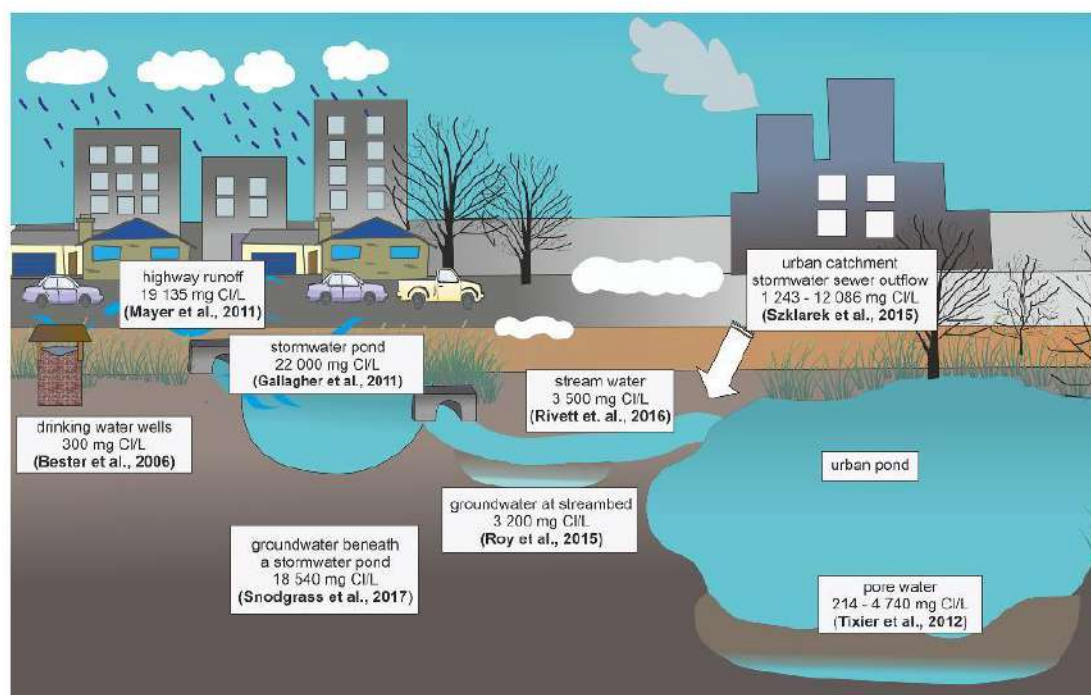


Fig. 1. Monitoring of highest chloride threats in surface water and groundwater.

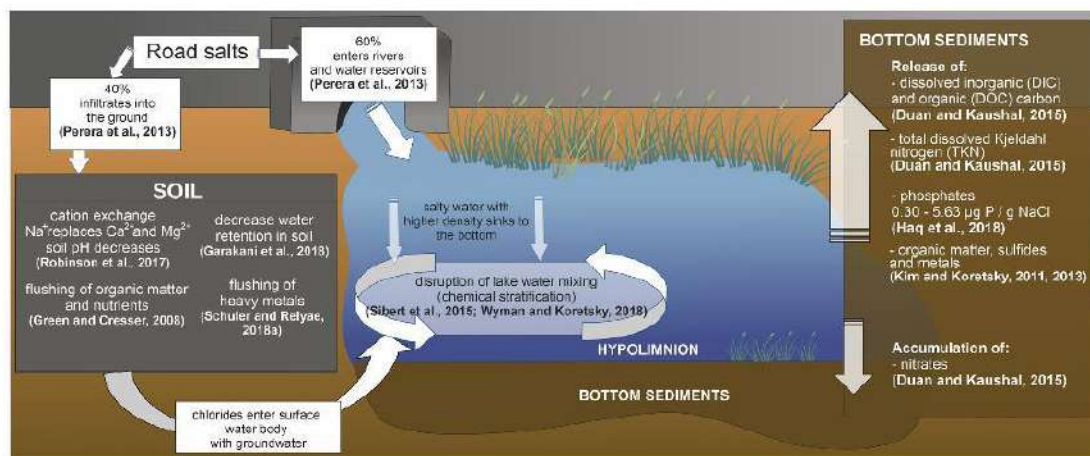


Fig. 2. Impact of chlorides on abiotic processes correlated with eutrophication in the water and soil environment.

Kaushal, 2015). Haq et al. (2018) noted significant phosphorus release from sediments — 0.30–5.63 μg of P per 1 g NaCl. McNaboe et al. (2017) noted that urban runoff with NaCl near the parking lot on the University of Connecticut campus in Storrs (CT, USA) mobilized radium (Ra) and radon (Rn) in the groundwater. In some sampling wells, ion exchange between Ra and Na in the soil particle resulted in a high concentration of Ra, which exceed drinking water standards.

The hydrology and physical characteristics of the catchment area influence the reaction by a lake to chloride pollution. In some cases, the concentration of chlorides in bottom sediments may be several times higher than in surface waters. In Lake Brownie (Minneapolis-St. Paul Twin Cities Metropolitan Area, USA) about 800 mg/L of chlorides were recorded in the bottom water (0.5 m above the sediments) in the June/July period, while it did not exceed 200 mg/L in the surface water, i.e. 0.5 m below the surface (Novotny et al., 2008). In Tanners Lake (Minneapolis-St. Paul Twin Cities Metropolitan Area, USA), a 7 m-thick saline layer was identified at the bottom of the lake, in which chloride concentrations reached 400 mg/L (Novotny et al., 2008). In some lakes, such layering causes chemical stratification at the deepest part, which could prevent or delay the full or partial mixing of lake water during spring or fall, thus generating anoxic conditions in the bottom (Sibert et al., 2015; Wyman and Koretsky, 2018); this would lead to the accumulation of Mn^{2+} , H_2S , and NH_4^+ in the hypolimnion and would impact the aquatic ecosystem (Kim and Koretsky, 2013). While higher concentrations of Cl in sediment porewater has been found to increase the concentrations of chloride ligands capable of metal complexation in sediment, such as Fe, Cu, Pb, Zn and Mn, but mainly Cd(II) (Mayer et al., 2008), application of NaCl and CaCl_2 could also stimulate the release of nutrients from sediments and stimulate primary production by the reduction of Mn(IV) and Fe(III). It was found that Mn(III) and Fe(II) levels increased to 35 ppm and 110 ppm, respectively, during 100 days of incubation of wetland sediments collected along the shoreline of Asylum Lake, Kalamazoo, MI. The greatest increase was noted for 5000 mg CaCl_2 (Kim and Koretsky, 2011).

The examples given above illustrate a number of pollution sources and disorders in abiotic processes which arise as a results of winter application of road salts and which might increase eutrophication processes. The most significant examples are summarized in Fig. 2; however, further research is needed to understand the wide impact of chlorides on abiotic element of soil and water environment.

3.2. Impact on biota

The pathways of Cl migration in the water and soil environment described above present a risk for the biotic component of freshwater

ecosystems, both directly as a toxic response and indirectly by changes in abiotic processes (Fig. 3). Many studies have examined the impact of road salts on freshwater macrophytes, phytoplankton, zooplankton and amphibians (Denoël et al., 2010; Hopkins et al., 2013), fish (Meland et al., 2010), benthic communities (Kotalik et al., 2017) and the freshwater mesocosm (Hintz and Relyea, 2019). The effect of chlorides on trophic pyramid might accelerate the eutrophication of surface waters. It is worth noting that both plants and aquatic animals vary greatly in their preferences and tolerances for salinity, mainly due to differences in their osmoregulatory abilities.

3.2.1. Microorganisms

In addition to flushing nutrients from the catchment and realising them from sediments, Cl might also impact on the efficiency of denitrification process in wastewater treatment plants (WWTP), with the specific denitrifying activity approaching zero in sewage with high salinity (above 1500 mg/L–1.5 wt%) (Ji et al., 2018). NaCl concentrations above 12,000 mg/L significantly reduced the respiration activity of the microorganisms in the activated sludge taken from Brno WWTP (513 000 PE, Czech Republic) (Vítezová et al., 2016). Microorganisms in activated sludge are typically inhibited or killed after exposure to high salinity, and they need time to adapt, during which purification processes are slowed (He et al., 2017). This adaptation time could be prolonged from about three hours at 0.2 M NaCl (11,688 mg/L of Cl) to 30 h at 1 M NaCl (58,440 mg/L of Cl) (Vítezová et al., 2016). The length of this lower efficiency period depends on the salinity of the sewage, the proprieties of the species composition in sludge and the employed treatment method. Interestingly, Ji et al. (2018) report that after high salinity exposure, the microbial community structure changes from one populated with *Gracilibacteria*, *Bacteroidetes*, *Parcubacteria*, *Chloroflexi*, *Ignavibacteriae* with dominant *Proteobacteria* (58.42% community abundance) to one almost fully dominated by *Proteobacteria* (93.39%). Such a monoculture is susceptible to other forms of occasional pollution, thus decreasing the efficiency of the WWTP. Furthermore, the combination of low temperature with high NaCl has a negative effect on the metabolism of the microorganisms in activated sludge: the acclimation phase of the activated sludge was prolonged for 50 h when 0.2 M NaCl (11,688 mg/L of Cl) was applied at temperature of 6 °C; however, no effect was observed at 13 °C and 20 °C (Vítezová et al., 2018).

Denitrification processes were also found to be decreased by Cl treatment in environmental studies. Similar denitrification ratios were observed in forested and urban wetlands when Cl was below 1000 mg/L compared to controls (0 mg/L); although the findings were similar to those observed in freshwater sediments (Lancaster et al.,

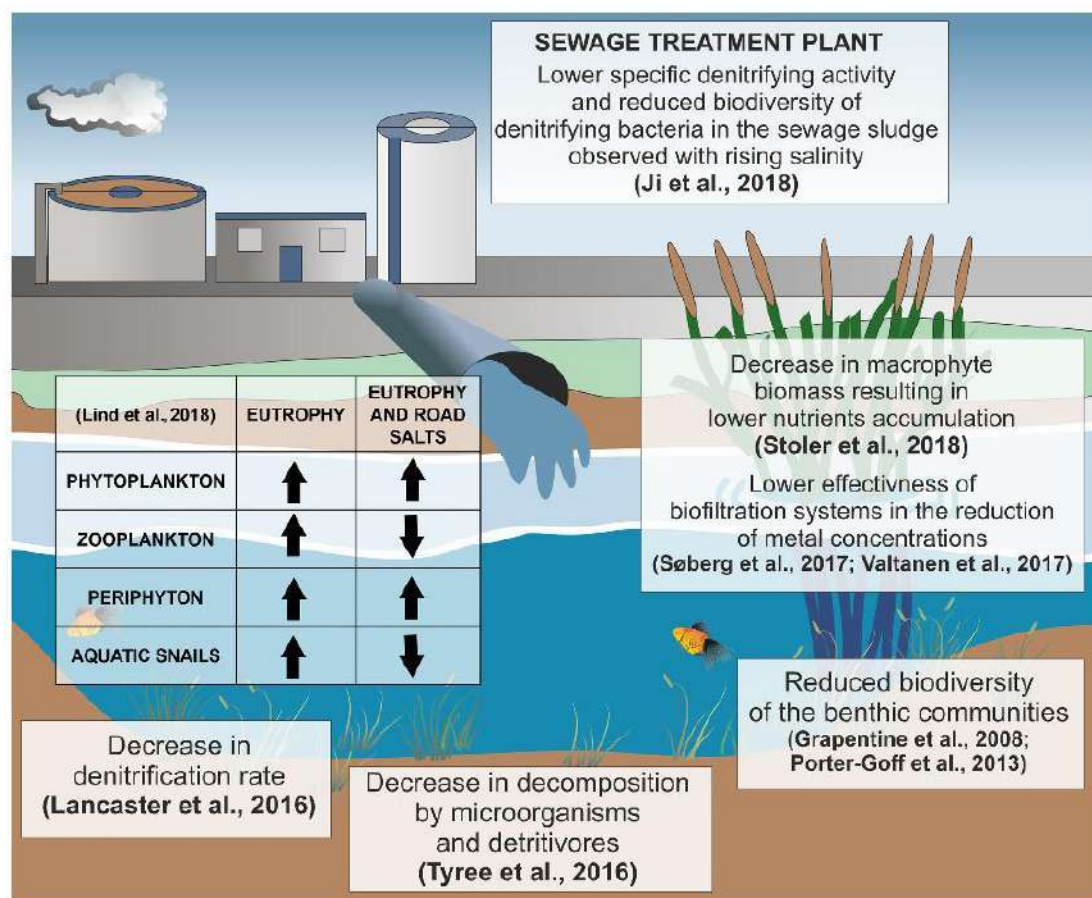


Fig. 3. Impact of chlorides on biota correlated with eutrophication in surface water and sewage treatment plant.

2016), the forested wetland denitrification ratio decreased by 99% at 2500 mg/L, and urban wetland by 90%. This significant difference ($p < 0.05$) might have resulted from the distinct differentiation in microbial community due to previous exposure and adaptation to Cl in the urban site (Lancaster et al., 2016); nevertheless, the decrease was still significant in both cases.

3.2.2. Benthic communities

Tyree et al. (2016) note that even a small addition of NaCl (214 mg/L of Cl and 140 mg/L of Na) decreases the growth of *Lirceus* spp. detritivores, by 4% for body length and 12% for telson width, compared to low NaCl levels (3.9 mg/L of Cl and 3.0 mg/L of Na); *Lirceus* also plays an important role in the decomposition of organic matter.

A study of an urban pond in Terraview-Willowfield Stormwater Management Park (Toronto, Canada) found the densities and taxon richness of benthic macroinvertebrate communities were lowest in the deeper part, where conductivity ranged from 1000 to 5000 $\mu\text{S}/\text{cm}$ (chloride concentrations > 1000 mg/L), particularly compared to two other ponds (Grapentine et al., 2008). In addition, the endobenthic communities were also exposed to chlorides that had migrated with groundwater. Half of the 15 groundwater-discharging sites along urban rivers demonstrated Cl concentrations above long-term chronic levels (120 mg/L) and as much as 70% of all samples in one site exceeded chronic toxicity levels of 640 mg/L (Roy, 2019) during summer through autumn. Among the macroinvertebrates, the most salt-sensitive stream insects are mayflies, stoneflies, and caddis flies (Pond, 2010), while dragonflies, crustaceans, beetles, and true flies tolerate

higher salt levels (Cañedo-Argüelles et al., 2013). Even so, the most sensitive stream insects were affected by road salt at concentrations of approximately 240 mg Cl/L, and many were not affected until Cl concentrations exceeded 800 mg/L chloride (Blasius and Merritt, 2002).

In some organisms, increased salinity induces a biologically-relevant reaction, allowing them to be used as biomarkers for field research. Freshwater bivalves, particularly their larvae (glochidia) and juvenile stages, are particularly sensitive to salinity. In southern Ontario (Canada) significant increases in concentrations were observed in 23 of 24 long-term (1975–2009) monitoring sites. Cause and effect analysis showed that long-term road salt use and retention appears to contribute to a gradual increase in baseline chloride concentrations in at-risk mussel habitats, which may affect their reproduction (Todd and Kaltenecker, 2012). In studies on the effect of salinity on freshwater mussel, *Lampsilis fasciola*, significantly reduced glochidia viability (56%) was observed after exposure to 1000 mg/L of NaCl. The results suggest that the loss of larvae vitality observed after exposure to high concentrations of NaCl (1000 mg/L) may be caused by ion regulatory disorders associated mainly with an increased inflow of Cl (Nogueira et al., 2015).

Benthic diatom community structure was also affected by chloride concentrations. Out of 41 sites in streams in southern Ontario, the communities with low Cl concentrations (< 35 mg/L) had more predictable assemblages, containing species such as *Meridion circulare*, *Diatoma vulgaris*, *Encyonema caespitosum* and *Pinnularia microstauron*, while those with higher Cl concentrations were less predictable, and usually included *Nitzschia hungarica*, *Hippodonta capitata* and *Caloneis*

amphisbaena; this difference might be caused by the higher chloride concentrations associated with multiple co-varying stressors, with elevated Cl being a marker of human impact on the watershed (Porter-Goff et al., 2013).

3.2.3. Macrophytes

Macrophytes play an important role in natural purification processes and in nature-based solutions, such as constructed wetlands and sequential sediment-biofiltration systems (Jurczak et al., 2018; Szklarek et al., 2018); however, they are also exposed to Cl from stormwater and meltwater or by increases in Cl concentration in surface waters. Søberg et al. (2017) conducted a pilot-scale study in 24 bioretention columns under controlled laboratory conditions for a total of 18 weeks. Their findings indicate that high concentrations of chlorides (>2000 mg/L) have a negative impact on the removal of dissolved Cu and Pb in bioretention cells of *Juncus conglomeratus*, *Phalaris arundinacea* and *Carex panacea*, with reductions being 20–40 times lower compared to controls with 50 mg/L Cl.

A combination of physical (turbidity) and chemical (road salt) stressors could have positive and negative effects on the productivity of macrophytes. Stoler et al. (2018) in an experiment conducted at the Rensselaer Aquatic Facility in Troy, observed that *Ceratophyllum demersum* and *Elodea canadensis* exhibited increased net and gross primary productivity during turbid conditions and elevated NaCl (3000 mg/L); however, no significant or negative effect was observed for another five investigated species (*Potamogeton robbinsii*, *Stuckenia pectinata*, *Myriophyllum spicatum*, *Elodea nuttallii* and *Najas flexilis*). In another experiment, carried out in April, July and October 2010 in Brittany (France), increasing freshwater salinity was found to promote the invasion of *Myriophyllum aquaticum* and decrease the success of *Ludwigia grandiflora* (Thouvenot et al., 2012). These results suggest there is a need to properly select macrophytes to support the work of stormwater treatment systems.

3.2.4. Phytoplankton

The most important group of primary producers in most water bodies is phytoplankton. Different groups of phytoplankton communities are adapted to withstand a certain range of salinity; however, species diversity is usually considerably reduced at high salinities. When frequent changes occur in salinity level, phytoplankton need to increase their respiratory activity to maintain the osmotic balance. Studies confirm that increasing NaCl concentration increases respiratory rate and reduces the photosynthetic evolution of O₂ of a non-axenic unialgal culture of *Scenedesmus protuberans* (Flameling and Kromkamp, 1994). These studies were conducted under laboratory conditions using the culture collection of the laboratory of Microbiology of the University of Amsterdam. Tonk et al. (2007) conducted two types of experiments: with gradually increasing salt concentrations and with salt shock. The results of these studies indicate that *Microcystis* spp., freshwater cyanobacteria, have high salt tolerance: their growth rate and microcystin production rate collapsed only when cells were exposed to salinities exceeding 10,000 mg/L for several weeks. Experiments with salt shock show that *Microcystis aeruginosa* can temporarily withstand high salinity; in addition, cell number and production at salinity levels above 10,000 mg/L corresponded to the values found in the experiments with gradually-increasing salinity. When *M. aeruginosa* was exposed to very high salinity levels (15,000 and 17,500 mg/L), cell growth rate and microcystin production decreased, but *M. aeruginosa* was able to grow and produce microcystin for at least one week. During this period, the mean cell size did not decrease, which indicates that the surviving cells were still able to withstand high turgidity pressure. However, the concentration of extracellular microcystin increased to 30% of the total concentration of microcystin in cultures exposed to salinity of 17,500 mg/L, which may indicate that some of the cells had disintegrated. Tonk et al. (2007) indicate that the high tolerance of *Microcystis* spp. to salinity can result in blooms in freshwater

ecosystems, even those with increasing salt concentrations, especially as diatoms and green algae had lower tolerance for salinity.

3.2.5. Zooplankton

Through the top-down effect, zooplankton plays an important role in controlling phytoplankton biomass. Therefore, any negative effect on this group may result in an increase of phytoplankton biomass and a greater risk of water blooms. A laboratory experiment by Jones et al. (2017) found that cultures with medium and high NaCl concentrations (480 and 780 mg/L, respectively) demonstrated significantly lower levels of zooplankton than the controls and those with low NaCl concentrations (20 and 150 mg/L, respectively), after nine and 22 days of incubation. Similarly, Van Meter and Swan (2014) found their mesocosm experiment to be dominated by the rotifer family, *Brachionidae* (93%), at a high Cl concentration (1067 mg/L), while it was composed of 27% of *Brachionidae*, about 30% of copepods and about 25% of cladocerans, with the remainder being made up of other rotifers and ostracods, at low Cl concentrations (177.2 mg/L).

However, long exposure to Cl could promote resistance in the zooplankton community. Coldsnow et al. (2017) found that *Daphnia* were able to adapt to higher salt concentration. During phase I of a 2.5-month experiment, individual *Daphnia pulex* were first exposed to chloride concentrations from 100 to 1000 mg/L over the course of 5–10 generations. In phase II, the populations were then exposed to Cl concentrations ranging from 1300 to 1900 mg/L. At 1300 mg Cl/L, the *Daphnia* populations exposed to Cl in phase I demonstrated a higher survival ratio than the control population. However, at Cl concentrations above 1500 mg/L, mortality increased and was not found to be significantly different to controls.

In addition, Hintz et al. (2019) suggest the possibility of *Daphnia pulex* developing cross-tolerance to different kind of salts after evolving tolerance to NaCl. Three populations of *Daphnia pulex* were exposed to low, moderate and high concentrations of NaCl, MgCl₂ and CaCl₂. The three tested populations consisted of a native population with no previous exposure to elevated NaCl concentrations, as well as two populations with acquired tolerance to 250 mg/L and 1000 mg/L of Cl respectively. After 60 days of mesocosm study, *Daphnia pulex* from the populations with evolved tolerance showed signs of cross-tolerance: they were less affected by moderate and high CaCl₂ concentrations or by moderate MgCl₂ concentrations compared to the native population. In addition, this cross-tolerance also mitigated salt-induced cascades: no differences in phytoplankton abundances were observed in variants with salt-tolerant *Daphnia* populations compared to low-salt controls, while the phytoplankton abundances in native populations were higher; however, all of the results depended on salt type and concentration and varied through the course of the experiment. The authors suggest that, instead of cross-tolerance, the results could indicate that *Daphnia* populations had evolved a new tolerance to the particular salt types, but this possibility was described as unlikely due to the short span of the study. However, it is worth noting that the evolutionary tolerance of *Daphnia* to the road salt NaCl came at the expense of a slower population growth rate. After 21 days of the experiment, the population density of daphnids with evolved salt tolerance was three times lower than the native population. This was probably due to smaller size or number of offspring, reduced offspring survival or increased time to reproduction due to the cost of maintaining the evolved tolerance (Hintz et al., 2019). Long Lake, Ontario, Canada is located 1.4 km from the nearest road, with summer Cl concentrations of only 0.4 mg/L. In this lake, with a low risk of exposure to Cl, it was found that the abundance of zooplankton communities was significantly altered by experimental treatments of 250 mg/L of Cl, while their biomass was altered but not significantly. In contrast, no such changes were observed in a community taken from Loughborough Lake, which is intersected by a two-lane highway, and has higher summer Cl concentrations of 19 mg/L (Sinclair and Amott, 2018).

Increasing salinization is usually connected with increasing risk of toxicity for water ecosystems, but Brown and Yan (2015) suggest that trophic status of lakes should be considered when assessing the acute toxicity threat from chloride. For example, food quantity was found to have a significant effect on egg production and neonate release in lab scale experiments conducted on *Daphnia* (*D. pulex* × *D. pulicaria*) isolated from McFarlane Lake, near Sudbury, Ontario, Canada. Chloride demonstrated lower chronic LC50 values when the particulate carbon (C) level was below 0.8 mg C/L (C indicates the quantity of *Pseudokirchneriella subcapitata* that the *Daphnia* were fed with), indicating oligotrophic conditions. The results suggest that oligotrophic lakes are more vulnerable to chloride inputs, such as road salt, than eutrophic lakes (Brown and Yan, 2015).

3.2.6. Fish

Freshwater fish species demonstrate a wide range of salt tolerance, depending on fish species, life stage, salt concentration, temperature and duration of the experiment. Exposure to high salinity levels affects osmosis and respiration in freshwater fish. Many studies on the effects of road salt on fish are laboratory experiments on usually acute or chronic toxicity. The lowest NaCl concentration at which 50% mortality was observed in rainbow trout (*Salmo gairdneri*) in a 24-hour experiment was 5.496 mg/L (Kostecki and Jones, 1983). Significant mortality was also reported in species such as minnows, bluegill sunfish (*Lepomis macrochirus*), channel catfish (*Ictalurus punctatus*) and goldfish (*Carassius auratus*) at salinities ranging from 11.50 to 20.00 mg/L NaCl during short-term exposure (Kszos et al., 1990; Waller et al., 1996). However, Camargo and Tarazona (1991) noted a lack of mortality in rainbow and brown trout fingerlings exposed to salinities 800–1000 mg/L NaCl for eight days. Selected results from laboratory studies on the effects of road salts on fish from 1934 to 1999 are presented in Evans and Frick (2001).

Studies indicate that freshwater fish reduce their food intake and conversion and show slower growth when exposed to high chloride levels (Boeuf and Payan, 2001). One of the more sensitive species to high salinity are those of the trout genus. In one study, the highest densities of brook trout (*Salvelinus fontinalis*) were observed in streams with low chloride concentrations, i.e. below 100 mg/L (Stranko et al., 2013). Hintz and Relyea (2017) studied the effect of various de-icing salts, viz. sodium chloride (NaCl), magnesium chloride (MgCl₂) and calcium chloride (CaCl₂), on the growth and development of newly-hatched rainbow trout (*Oncorhynchus mykiss*). The results showed that while MgCl₂ did not affect the growth of rainbow trout at any concentration, NaCl exposure influenced the growth of rainbow trout at 3000 mg/L of Cl (9% reduced length; 27% reduced weight), while CaCl₂ affected the growth of rainbow trout at 860 mg/L of Cl (5% reduced length; 16% of reduced weight).

It is worth noting, however, that despite the increasing threat of environmental pollution from road salt, there is relatively little research on its effects on fish species.

3.2.7. Ecosystem structure

Beside the individual responses of freshwater organisms to Cl exposure, it is also important to consider the possible effects on the food web as a whole (St Denis et al., 2010). Van Meter et al. (2011b) investigated ecosystem structure in eight urban stormwater detention ponds in the Red Run Watershed (Baltimore, Maryland, USA). Through the end of May, when Cl concentration was diluted after the winter period, a decline in zooplankton density was observed along an increasing chloride gradient; as such, the density of periphytonic and phytoplanktonic algae was found to increase with decreased grazing pressure. At the elevated Cl concentration (645 mg/L), greater adult copepod mortality was observed, resulting in the tadpoles undergoing metamorphosis earlier and demonstrating greater mass as a result of decreased zooplankton competitors (Van Meter et al., 2011a). High concentrations of NaCl or road salt (0.05–0.9% w/v) have been found to inhibit ciliate bacterivory

in a predator/prey experiment conducted on the relationship between a ciliate (*Tetrahymena thermophila*) and a bacterium (*E. coli* XL-1 pEF-gfp) (St Denis et al., 2010).

In further mesocosm studies, Lind et al. (2018) examined the interactions in freshwater communities at three NaCl concentrations (15, 250 and 1000 mg/L of Cl), two nutrient levels (high eutrophic and ambient oligotrophic) and three light levels (high, medium and low). Elevated salt concentrations and nutrient levels were found to increase the abundance of phytoplankton and periphyton. Increasing salt levels reduced the biomass of macroalgae (*Nitella* spp.). Additionally, high salt treatments were associated with lower chlorophyll *a* concentrations, indicating physiological stress; moreover, as the salt level increased from 250 to 1000 mg/L, two of the three zooplankton groups, viz. the cladocerans and copepods, demonstrated reduced abundance, with these negative effects weakening over time. When analysing the effect of salinity on the organisms studied, the authors found no relationship between the increase in salt and nutrient concentrations, which indicates that they did not interact synergistically. Therefore, since an increase in nutrient concentration did not cause the organisms to demonstrate any disproportionate response to an increase in salinity, the authors conclude that the two factors acted additively.

A mesocosm study by Petranka and Doyle (2010) on freshwater pond communities found copepods and cladocerans to be the most sensitive taxa to commercial road salt, i.e. a NaCl and KCl base with small amounts of CaCl₂ and MgCl₂. Both taxa were rare or absent in mesocosms where mean salt concentrations exceeded 1200 mg/L. In addition, growth of wood frogs (*Lithobates sylvaticus*) was suppressed at mean salt concentrations exceeding 4500 mg/L. Interestingly, the abundance of mosquitoes, *Culex restuans*, increased in pools where the mean salt concentrations exceeded 1200 mg/L. However, this observation was not associated with salt concentration; it is more likely that this resulted from the disturbances in the macrozooplankton and amphibian larvae communities, and reflected the lack of competition between the communities and the mosquito larvae.

Sodium chloride exposure can influence the susceptibility of organisms to parasites. Buss and Hua (2018) report that 24-hour exposure of wood frog (*Lithobates sylvaticus*) tadpoles to 1000, 2000 and 3000 mg/L of NaCl increased trematode infection by 68%, 63% and 96%, respectively, compared to tadpoles that were not exposed to sodium chloride. Among tadpoles exposed to the presence of a predator (damselfly), those subjected to 1000 mg/L NaCl demonstrated 200% more encysted parasites than those that were not earlier exposed. However, NaCl exposure did not appear to affect the susceptibility to infection in groups exposed to dragonfly kairomone + parasites, or to dragonfly kairomone + parasites + predator.

To summarise the effect of Cl impact on biota, a number of studies indicate that road salt application causes changes that may contribute to faster eutrophication of waters and an increased risk of cyanobacterial blooms; this has been attributed to flushing nutrients and other pollutants from catchments (Fig. 2), the negative impact of Cl on species which compete or control the biomass of phytoplankton, and a decrease in the efficiency of some self-purification processes (Fig. 3). This is an area that requires further research to understand the full spectrum of effects of Cl on these processes in inland water resources.

3.3. The impact of climate change

As winters become milder, climate change may well be accompanied by a reduction in the application of de-icing chemicals in many regions; however, this may not necessarily remove the threat to water ecosystems. Research conducted in the East and West Branches of the Tiohnioga River (central New York, USA) suggest that even if the application of de-icing salts to roadways were to decrease, elevated Cl levels would be observed in the stream for decades. Results from these field studies and discriminant analysis suggest 20 to 30-year storage of Cl within the watershed in both analysed branches of the

Toughnioga River (Gutchess et al., 2016). Further simulations have shown that these Cl concentrations will continue to rise over the period 2010–2039, following which, according to all Global Climate Models, it will decline as a result of reduced snowfall (Gutchess et al., 2018). Moreover, road salt application was found to lead to decreased productivity of all trophic levels (periphyton, zooplankton, Northern watermilfoil - *Myriophyllum sibiricum*, American ribbed fluke snail - *Pseudosuccinea columella*, and larval American bullfrogs - *Lithobates catesbeianus*) compared to other aquatic stressors, i.e. acidification (pH) and climate change (water temperature and eutrophication), which demonstrated a lower effect, or none at all, in a mesocosm laboratory experiment (Dalinsky et al., 2014).

To ameliorate the consequences of climate changes associated with water cycle changes, there is also a need to increase stormwater retention (UN WATER, 2020). Although many solutions have been proposed and tested, all have been found to influence the flow path of Cl. For example, permeable pavement (PP) systems decrease the concentration of Cl and Na during winter surface runoff, but increase it in groundwater effluent in spring and early-summer due to slower migration of the ions through the PP (Drake et al., 2014). Although PPs disperse Cl pollution, they still increase Cl concentrations in the water flowing through them to high acute toxicity level (>860 mg/L) after the first rain event following a period of snow with road salt application (Borst and Brown, 2014). In both cases, PP effluent typically demonstrated lower concentrations of Cl, but only in short-term studies. Dry water ponds also change the flow path of Cl. Studies conducted in the Red Run watershed of Baltimore County, Maryland, piezometers located in shallow groundwater below the dry stormwater pond reported 18,540 mg Cl/L in February; however, the mean concentration between June 2009 and May 2010 was 3828 mg/L, and the mean value for November 2009 was 798 mg/L, i.e. near the acute toxicity level of 830 mg/L, in a period when the Cl concentration should be lowest (Snodgrass et al., 2017). This resulted in higher Cl concentrations in non-winter months. At an unmanaged catchment ($n = 7$, no stormwater ponds, first sample point in the river continuum) January mean Cl was found to be around 500 mg/L; this value was similar to that observed in the managed catchment ($n = 4$, first sample point in the river continuum with stormwater ponds in catchment) and the combined catchment ($n = 13$, not first point in river continuum). However, over the following months, viz. April, June and August, the unmanaged catchments were found to have below 50 mg Cl/L, compared to 100 mg Cl/L or more in the combined and managed catchments (Snodgrass et al., 2017). Low impact development (LID), which manages stormwater runoff as part of green infrastructure, decreases surface runoff and increases the retention capacity, thus reducing high Cl concentrations; however, it has also been found to increase Cl levels in base flow and in groundwater throughout the year (Gu et al., 2019).

4. Prevent chloride pollution

4.1. Other de-icing agents

As both the surface and groundwater migration of Cl pose a threat for freshwater ecosystems, magnesium and calcium chloride ($MgCl_2$ and $CaCl_2$) have been proposed as alternatives for road salt, albeit more expensive ones. The primary advantage of the two salts is that their dissolution is exothermic; this induces further snow melt, and thus avoids the need for more salt. In addition, they do not displace the cations in soil so readily and do not have such a harmful impact on soil properties. Even so, they may well exert a harmful impact on the water environment.

Brown and Yan (2015) did not report any significant difference between NaCl and $CaCl_2$ application on egg production and neonate release by *Daphnia*: as mentioned above, food quantity appeared to have the greatest influence on Cl toxicity. Sun et al. (2015) examined the effect of the salts on heavy metal leaching from sediments. An

injection of 0.25 mol NaCl increased the concentrations of As in the leachate by 2.2 to 55.3 ppb, Pb-concentrations from 0.51 to 84.1 ppb, and Hg-concentrations from undetectable to 25 ppb; however, an injection of 0.25 mol of $CaCl_2$ and NaCl (at a 6:4 ratio), a commonly-used mix for road salting, increased As concentrations from 0.96 to 23.14 ppb, Pb levels from 0.79 to 749.2 ppb and Hg levels from 0.17 to 0.31 ppb.

A study of the microbial community in WWTPs found NaCl treatment to demonstrate higher inhibition of respiration activity of activated sludge than $CaCl_2$ and $MgCl_2$; however, it also appeared to have the lower impact on the adaptation time of the microbial community. When 1 M solutions were used, $CaCl_2$ prolonged the microbial adaptation period by 2.2 days, $MgCl_2$ by 1.7 days, and NaCl by only 30 h (Vítezová et al., 2016).

Achkeeva et al. (2015) report that chloride-based de-icing agents (NaCl, $CaCl_2$, $MgCl_2$, KCl) were more efficient for melting snow including those without Cl, and that they are also cheaper than alternative agents such as those based on acetates or nitrates. However, in addition to the extra cost, alternative de-icing agents are also associated with other problems. Schuler and Relyea (2018b) tested two such de-icers; a mix of $MgCl_2$ with GeoMelt (beet juice; proprietary blend) (SNI Solutions, Geneseo, IL) and Magic Salt ($MgCl_2$ premixed with a distillation by-product; proprietary blend) (SEACO, Rome, NY) at concentrations of 100, 400 and 800 mg/L. The results indicated that after seven and 21 days, the concentration of dissolved oxygen (DO) decrease significantly in all tested mesocosms with $MgCl_2$ + GeoMelt and $MgCl_2$ + Magic Salt, compared to controls (25 mg/L) and to pure $MgCl_2$ with the same Cl concentration as the test substances. Although such reductions in DO harm most aquatic organisms, mosquitoes breathe air at the surface of the water through a siphon, and were not negatively affected (Schuler and Relyea, 2018b).

Lee et al. (2017) performed acute toxicity tests on nine different de-icing agents, six with an eco-label certificate, with *Daphnia magna*. The lowest EC50, i.e. the concentration where 50% of the population are immobilized, and LC50 values, i.e. the concentration at which 50% of the test organisms die, were noted for $CaCl_2$ and solid de-icer SS (mixture of NaCl + $CaCl_2$ + $MgCl_2$ + CH_3CO_2Na); both had LC50 and EC50 values below 2000 mg/L, indicating that they had the highest toxicity of the tested substances. In addition, two other eco-label solid de-icers (a mixture of $CaCl_2$ + $MgCl_2$ + organic acid, and a mixture of NaCl + $CaCl_2$ + organic-inorganic composite) also had LC50 and EC50 values lower than for NaCl. In comparison, the LC50 and EC50 values of NaCl were found to be around 4000 mg/L (Lee et al., 2017).

Other research on the toxicity on four "eco-friendly" de-icing agents showed that they were not less toxic for *Chironomus dilutes* than NaCl; measured as a ten-day lethal concentration, NaCl was found to have a similar LC50 value (6.61–6.32 g/L) to two other "eco-friendly" salts: calcium magnesium acetate (LC50 = 6.61 g/L) and beet juice extract enhanced de-icing formulation (LC50 = 6.32 g/L). In addition, the third "eco-friendly" de-icing formulation, based on urea, had an even higher toxicity, with an LC50 value of 0.63 g/L (Nutile and Solan, 2019).

Some of the more environment-friendly de-icing agents, like sodium levulinate, calcium levulinate and magnesium levulinate (produced from sorghum grains), had higher freezing points than NaCl, and so were less effective at lower temperatures. However, they should be considered for use in some areas, where winter is not so cold and where winter temperatures may be rising under the influence of climate change (Ganjyal et al., 2007).

The most frequently-considered alternatives for NaCl still contain chloride ions, which might be the reason for the similar, or even more negative, environmental effect noted for them. Other non-Cl agents were typically much more expensive or had other disadvantages that hampered their widespread use.

4.2. Optimizing the use of de-icing

One way of reducing the environmental impact of winter road maintenance is by optimizing the use of de-icing chemicals. One potential

strategy involves the use of an infrared thermometer to detect the freezing of water on an asphalt surface (Riehm et al., 2012) combined with socio-economic models to calculate socio-economic costs depending on winter maintenance strategy (Arvidsson, 2017). In Finland, a 20-year programme was carried out to reverse the increasing Cl pollution of groundwater. The approach included improvements of de-icing device and practices, improvements in meteorological online data services, as well as an education and reward programme for the private contractors responsible for de-icing; this combined strategy resulted in NaCl use falling by approximately 35% during the 2000s in comparison to the period immediately prior, i.e. 1988–1992 (Salminen et al., 2011). Interestingly, this decrease in NaCl consumption was achieved in a period when the total length of roads, needing winter maintenance, increased by 30–40% and the winters become milder: ambient temperatures around 0 °C were becoming more frequent, which increased the demand for NaCl (Salminen et al., 2011).

Providing an appropriate system to support the use of road salt would yield economic benefits. A 0.1 °C error in estimating the difference between frost point and road surface temperature in the region could result in costs of close to 28,000 € each winter, while an error of 0.5 °C could result in a cost of 93,000 € (Riehm and Nordin, 2012).

4.3. Technical solution

Technical alternatives to de-icing chemicals have been explored, such as ground source heat pipes, electric heating cables, infrared heat lamps, heating fluids and electrically conductive concrete (Zhao et al., 2011). However, such heating systems are rarely used due to problems associated with cost (construction and operating), performance and the general situation. Even solutions based on renewable energy, such as a Hydronic Heating Pavement (Mirzanamadi et al., 2018) are not completely suitable, due to the need for materials to be applied to roads and pavements. Technical solutions based on solar energy could decrease the need for road salt use, but there remains a need for life cycle assessments, and further research into construction technology, maintenance technology and operation parameters (Pan et al., 2015).

Another potential strategy for preventing chloride water pollution could be recycling road salts. Craver et al. (2008) investigated the possibility of recycling NaCl from stormwater ponds located near roads using “brine generation units”. The value return (based on net present value) of the brine generation unit after one to four years depends on the volume of used road salt and stormwater runoff accumulated in the pond.

4.4. Nature-based solution

Urban stream restoration projects that restore floodplain connections may positively impact water quality by storing high salinity road run-off during winter overbank events and discharging that water during the year, thereby attenuating seasonal fluctuations in chloride. These observations indicate that groundwater storage and subsequent discharge from floodplain aquifers in connected urban streams can attenuate seasonal fluctuations in chloride caused by road salt, which may be beneficial to aquatic organisms intolerant of large chloride changes over short periods. On the other hand, the seasonal storage of chloride recharged to floodplain aquifers can artificially elevate baseflow chloride concentrations outside of winter (Ledford and Lautz, 2015). Models of the residence time and storage capacity of an aquifer indicate that restoration projects designed to promote floodplain reconnection and the frequency of overbank flooding in winter have the potential to temporarily store chloride in groundwater, and to buffer surface water concentrations and reduce stream concentrations following periods of road salting (Ledford et al., 2016).

Some halophytes (*Atriplex halimus* and *Atriplex hortensis*) could reduce the negative impacts of salt treatment by accumulating Na⁺ from road salt. Both plants accumulate > 50 mg/g DW of Na⁺ within their aerial parts when exposed to a solution containing 2000 mg/L of NaCl (Suaire

et al., 2016), but those plants were not found to accumulate Cl. More complex purification of both Na and Cl might be provided by *Typha angustifolia* (all tested salinity levels from 700 µS/cm to 13,000 µS/cm) and *Juncus maritimus* (at highest tested salinity, i.e. 8000 µS/cm and 13,000 µS/cm); both demonstrated significantly higher removal rate of Cl than control (200 µS/cm) laboratory constructed wetland (CW) (Guesdon et al., 2016). The plants also were found to accumulate more Na in aboveground and underground parts during elevated salinity than in control CW. Similar levels of Cl accumulation was observed only for *Juncus maritimus*; in *Typha angustifolia*, Cl accumulation increased with salinization only in the underground parts (Guesdon et al., 2016).

The potential for anthracite coal, pozzolan, dolomite and limestone to remove NaCl was investigated by de Santiago-Martín et al. (2016). The four were selected on the basis of their potentially efficiency for removing NaCl and trace metals (Cd, Cu, Ni, Zn), availability for sale, low economic cost, physiochemical stability with a minor impact on ecosystems, and being natural and unmodified. The efficiency of those materials was tested at four Cl concentrations: 150 mg/L (C1), 1500 mg/L (C2), 3000 mg/L (C3) and 5000 mg/L (C4). The anthracite coal had the most stable removal ratio of Cl (ranging from 43 to 56% at 22 °C and 37–54% at 4 °C) at all tested concentrations. The removal efficiency of dolomite at 22 °C was lower, with Cl level increasing from 59% in C1, through 43% for C2 and 40% for C3, to only 15% at C4; however, at 4 °C, the efficiency increased from 18% at C1 and 10% at C2 to 29% at C4. Limestone and pozzolan were tested only at 22 °C: at C1, the two materials demonstrated 4% and 21% Cl removal, respectively. These values increased to 45% for limestone at C4 and 61–68% for pozzolan at C2–C4. These results indicate that anthracite coal appears the best substance for removing only Cl, but dolomite was the best for removing all investigated compounds, i.e. NaCl and trace metals (Cd, Cu, Ni, Zn). The most effective way of removing NaCl and trace metals across the range of temperatures and Cl concentrations which could occur in field conditions would be to use a combination of materials with different properties (e.g. sorption capabilities) best suited to the experimental factors, such as initial NaCl concentration and temperature (de Santiago-Martín et al., 2016).

Pyrolyzed biomass (biochar or charcoal) could also be used to decrease Cl pollution. When added to the soil, it has been found to neutralize salt stress for plants, such as *Abutilon theophrasti* and *Prunella vulgaris*. The addition of 5000 g/m² of biochar to the top of the soil completely alleviated salt-induced mortality by those two species induced by 30,000 mg NaCl/m² (Thomas et al., 2013). Developing denitrification solutions based on carbon-rich materials for groundwater and surface water purification from nitrates (Bednarek et al., 2014; Schipper et al., 2010) could also help with road salt pollution.

5. Conclusion

The use of road salt has a significant environmental impact and cannot be ignored. Cl demonstrated considerable mobility, and although this allows it to be widely used as a geochemical tracer to characterize the water flow path and time of flow (Homer et al., 2017), it also hampers its removal from the environment. There is therefore a strong need for greater awareness of the impact of de-icing materials on people and the environment, and for practical measures to be taken to minimise the risks associated with the use of road salt.

The final step of an Ecohydrology-based approach is to propose system solution to address the water quality problems. While society and stakeholders focus mostly on the impact of road salt on the nearest road environment, they forget about the effects on the recipients of urban runoff. Many studies mentioned in the “Monitoring of threats” and “Cause-effect analysis” sections above found the use of NaCl as a winter de-icer to have a range of effects on water quality and water ecosystem structure. While there has been a growing body of research about the impact of Cl on water bodies in recent years, but remains a need to investigate the long-term impact of such pollution on the

abiotic and biotic processes and components in water. There is also a need to investigate alternatives to NaCl de-icers more carefully before they can be applied more widely, as many studies suggest that they could have similar or more negative effects on surface water ecosystems. In addition, the cost-benefit implications of these alternatives remain unclear, as some de-icing agents could be more expensive (cost), but not cause any increase in the levels of some pollutants or decrease the efficiency of self-purification processes (benefits). Optimizing the use of de-icing agents, and including some technical solutions, could reduce the amount of Cl applied during winter, but in some cases they might not provide sufficient protection for water bodies subjected to long-term NaCl application, and an increasing annual mean Cl level. While some materials and pollutant-absorbing plants might play a complementary role as nature-based solutions in reducing Cl pollution, especially diffused sources associated with developing stormwater retention approaches, there has been no more extensive analysis of the potential of these solutions for Cl removal.

There is a clear need to apply a holistic approach and engage different groups of stakeholders, including policy makers, environmental managers, society, scientists and transportation professionals, to prevent the negative consequences of road salt application and the consequent losses in water ecosystems and ecosystem services (Hintz and Relyea, 2019). One such important local initiative is the Salt Resource Management Plan developed by the State Administration of National Roads (SHA), in conjunction with the Maryland Department of the Environment (MDE). This plan consists of several management practices by the state and local jurisdictions to minimise the environmental impact of road salt runoff; it also includes an assessment of new materials, equipment and winter strategies to improve service levels to drivers during winter storms while reducing the use of road salt (Stranko et al., 2013).

Achieving sustainable development in an environment strongly modified by humans requires innovative and systemic solutions. Understanding the dynamics of ecological processes and the hierarchy of factors regulating these processes is crucial for increasing the potential of the environment and limiting the degradation of natural resources (Kaushal et al., 2017; Rasul et al., 2018). Modern environmental remediation should be based on the harmonisation of nature-based solutions (NBS) with engineering activities, and involvement of the local community in the implementation and maintenance of system solutions (Zalewski, 2000). One example of the implementation of ecological biotechnology for the treatment of storm water from urbanised areas is the sequential sedimentation-biofiltration system (SSSB), which has been used on two rivers in the city of Lodz, Poland. The SSSB is a small and relatively shallow reservoir which connects the water discharge system from the street with the natural river system. It contains underground separators which reduce oil substances and suspended solids, a geochemical part comprising gabions filled with stone dolomite and lime material covered with coconut matting, and a biological part overgrown with aquatic plants for the reduction of biogenic compounds (Jurczak et al., 2018; Szklarek et al., 2018). Further solutions to decrease road salt inflow to urban aquatic ecosystems through a sequential sedimentation-biofiltration system are being tested, e.g. use of halophytes (Guesdon et al., 2016) and the application of microbial processes for the biodegradation of chlorinated compounds.

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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RESEARCH ARTICLE



Does winter application of road salt affect zooplankton communities in urban ponds?

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Abstract

Road salt (NaCl) is commonly used as a deicer during winter to improve road safety, resulting in freshwater salinization. Such elevated chloride concentrations may have a strong effect on zooplankton, which are key elements in freshwater food webs. The aim of this study was to investigate the effect of chloride ion concentration and inter-pond environmental variability on zooplankton density in four urban ponds over 3 years differing in winter conditions. Analysis of variance showed significant differences in chloride ion concentration and zooplankton density regarding study year and ponds. Redundancy analysis of column water data showed that both the environmental variables (including chloride ion concentration) and the pond location significantly contributed to the model explaining the variability in zooplankton densities. However, the environment variable (referring to the environmental variables) had a smaller contribution (11%) than the POND variable (related to the spatial configuration of the ponds), which contributed 62%, indicating that the dynamics of zooplankton density depended primarily on ecosystem characteristics. Even so, although chloride content was not the main determinant of zooplankton density, their presence in aquatic ecosystems and potential interactions with other types of pollutants could have negative biological and ecological effects.

Keywords Chloride concentrations · Cladocerans, copepods, rotifers · Freshwater salinization · Winter conditions

Introduction

To ensure road safety in regions that experience harsh winters, road salt, typically sodium chloride (NaCl), is commonly applied during cold periods. The growth in popularity of sodium chloride-based deicers can be attributed to their relatively low cost compared to other salts (such as

calcium chloride or magnesium chloride) as well as their high effectiveness.

Although road salt is important to maintain road safety, there are drawbacks to its usage. Road salt can enter groundwater with runoff, leading to elevated chloride concentrations in drinking water wells (Bester et al., 2006) and surface waters. Elevated concentrations of chloride ions have been observed in lakes (Scott et al. 2019; Winter et al., 2011; Rogora et al., 2015), stormwater retention ponds (Gallagher et al., 2011; Tixier et al., 2012) and rivers (Hubbart et al., 2017; Corsi et al., 2015). Consequently, road salt application is one of the key causes of freshwater salinization syndrome (Cunillera-Montcusí et al., 2022; Kaushal et al., 2021), resulting in increasing salinization: an emerging threat to freshwater biodiversity (Reid et al., 2019).

In freshwater ecosystems, road salt-induced salinity has negative effects on zooplankton and various trophic interactions. On the species level, Martínez-Jerónimo (2007) observed that increasing concentrations of NaCl (between 2 and 8 g/l NaCl) negatively impacted the clutch size, total progeny, longevity, average lifespan and life expectancy,

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net reproduction rate (R_0), population growth rate, as well as survivorship of *Daphnia magna* exposed to salinity. Similarly, survivorship, total progeny and R_0 of *Scapholeberis mucronata* and *Simocephalus vetulus* were shown to decrease at NaCl concentrations between 500 to 625 mg/l (Gökçe and Özhan, 2014). Huber and co-workers (2023) found that chloride concentrations reaching the chronic (230 mg/l) and acute (860 mg/l) threshold levels reduced *Daphnia mendotae* vertical movement rate by 22–47%. Moreover, increased salinity has been shown to affect zooplankton communities and their structure. Elevated concentrations of chloride ions tested in mesocosm studies decreased the abundance of cladocerans and copepods (Lind et al., 2018), triggered a trophic cascade when present together with fish predators (Hintz et al., 2017) and, at high Cl concentrations > 1000 mg/l, caused a shift towards rotifer-dominated communities (Van Meter and Swan, 2014). However, results from laboratory experiments suggest that *Daphnia* populations may develop adaptations to high concentrations of sodium chloride to increase their survival in high salinity habitats (Parlato and Kopp, 2020). It is crucial to assess whether, and to what extent, road salt affects zooplankton communities in their natural environment; it is also important to understand both the differential adaptive capacity of the organisms and the particularly significant regulatory role of zooplankton in freshwater ecosystems. Unfortunately, the effects of winter road salt application on zooplankton species are difficult to verify in the wild. This is particularly so in temperate regions where zooplankton rarely occurs outside the growing season as overwintering populations.

The present study attempts to fill this knowledge gap, also highlighted by Cunillera-Montcusí et al. (2022). Its main goal was to examine the effect of chloride concentrations on zooplankton density observed in four urban ponds of different environmental conditions located in the city of Lodz, Poland. The main focus is on chlorides because they are a component of the most commonly used de-icing salts, such as sodium chloride (NaCl), magnesium chloride ($MgCl_2$) and calcium chloride ($CaCl_2$), thus being the most representative signal of road salt contamination (Woodley et al., 2023). The research was conducted in 3 weather-differentiated years: 2019 being a typical winter in Poland, 2020 having an exceptionally mild winter without snow days and 2021 with a harsh, cold and snowy winter. We hypothesized that zooplankton density will be related to chloride ion concentrations in the water, as higher concentrations of chloride in harsher winters will reduce the density of zooplankton organisms, and that this effect may be modified by pond-specific environmental conditions. Furthermore, we postulate that, due to the different winter weather conditions in each year of the study, as well as the different characteristics of each pond, there will be a significant effect of pond and year

and pond/year interaction on the concentration of analysed ions and zooplankton density.

The study was conducted over 3 full hydrological years to verify changes in road salt concentrations in aquatic ecosystems, during both the winter (resting) and growing season.

Materials and methods

Study area

The study was performed in four urban ponds located in the city of Lodz, central Poland (51°45' N, 19°28' E). The first pond, Arturówek Górny (AG), on the Bzura River, is located in Arturówek—a recreational complex situated on the edge of Łagiewnicki Forest (Fig. 1a). In 2010–2015 the EH-REK project “Ecohydrological rehabilitation of recreational reservoirs in Arturówek” (Łódź) as a model approach to the rehabilitation of urban reservoirs” was responsible for the implementation of nature-based solutions in this area, improving water quality in the pond (Jurczak et al., 2019). Our sampling site was located at the pond inlet. The area around the pond receives a lower amount of road salt than the rest of the selected sites, as the use of road salt was prohibited on the roads in this area; the only likely source was surface transport by vehicles. Arturówek Górny pond also served as the reference ecosystem in our study.

The second urban pond, Zbiornik Wasiaka (ZW), on the Sokołówka River, is located in a residential area consisting of single-family houses (Fig. 1b). The third urban pond, Julianów Górny (JG), again on the Sokołówka River, is located in Adam Mickiewicz Park, close to two paved alleys (Fig. 1c). The fourth urban pond, Zbiornik Zgierska (ZZ), on the Sokołówka River, is located downstream from the Adam Mickiewicz Park area; it is separated from the park by a two-lane street with moderate traffic that runs near to the pond (Fig. 1d). The surface area, mean depth and capacity of each urban pond are presented in Table S1, Supplementary Material.

In Poland, the city municipalities are not required to collect information about the amounts of road salt used in winter. However, we were able to acquire this information for part of the city where, during the winter season 2020/2021, approximately 6.5 tonnes of NaCl was used per kilometer of traffic lane. Data for other seasons were not available.

Meteorological data source and analyses

Daily meteorological data (precipitation and temperature) were taken from national monitoring networks held by the Institute of Meteorology and Water Management-National Research Institute. The nearest station was Lodz-Lublinek (ID 351190465). To determine the long-term averages and

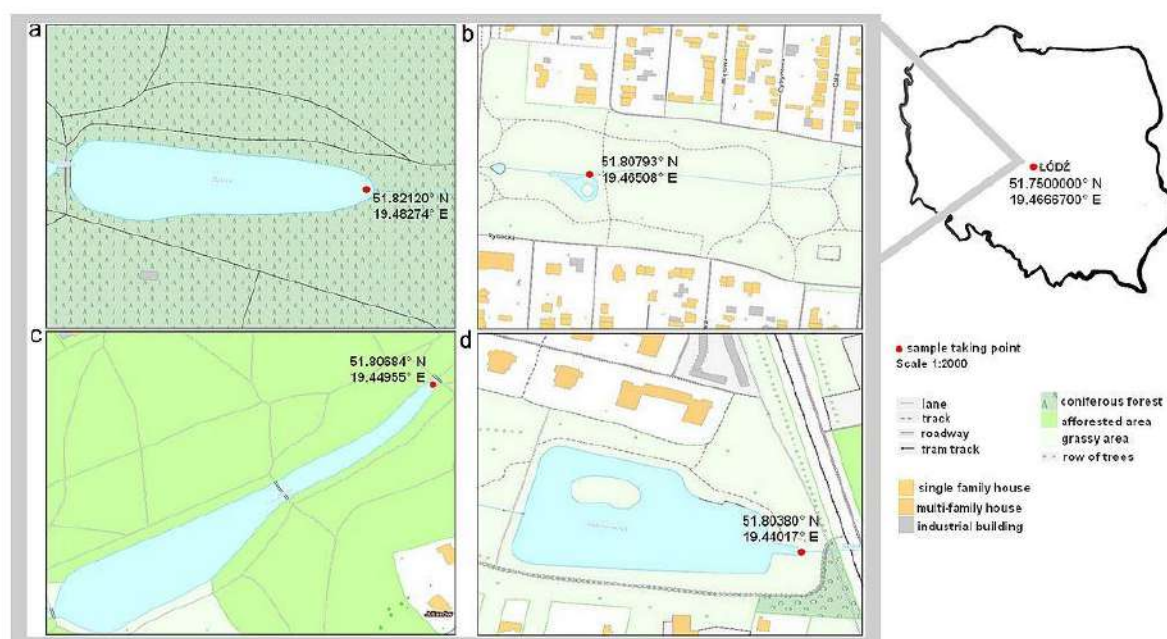


Fig. 1 Maps of the studied ponds and their area. **a.** AG = Arturówek Górny (2017), **b.** ZW = Zbiornik Wasiaka, **c.** JG = Julianów Górny, **d.** ZZ = Zbiornik Zgierska. Coordinates of the Łódź city as well as

all of the sampling points are presented in the decimal degrees format. Source: <https://geoportal.lodzkie.pl/imap>

compare them with the state of the weather during the study period, the data for the period 1951–2020 were taken from the website <https://meteomodel.pl/>, which presents meteorological data from national monitoring programs.

Surface and pore water sampling and analyses

Field monitoring was conducted for three hydrological years: 2018/2019, 2019/2020 and 2020/2021. According to the definition formulated by the Polish Institute of Meteorology and Water Management-National Research Institute, the hydrological year, by convention, begins on 1 November and lasts until 31 October, which serves as the basis for calculating the country's water balance. The end of October/November usually marks the end of rainwater run-off from summer-autumn heavy rainfall and storms, and thus the beginning of the autumn-winter period, when a drop in temperature promotes the development of ice phenomena, and the likelihood of snowfall increases. Column water and pore water samples were taken twice a month for the whole year; samples were taken more often during melting periods to determine maximum chloride concentrations and the period of time for which they remained elevated. Column water samples were taken 20 cm below the surface. Sediment samples were taken from the top 20 cm of sediments by sediment coring device, and then pore water was extracted by centrifugation

(3000 rpm for 10 min) in the laboratory. Column and pore water were analysed in a Dionex[®] ion chromatograph with a cation column (CG18, IonPac CS18, CSRS-ULTRA II) and an anion column (AG22, IonPac AS22, ASRS c ULTRA II) to determine the concentrations of chlorides, sodium, potassium, magnesium, calcium, nitrates, sulphates, ammonium and phosphates.

Zooplankton sampling and analyses

The zooplankton samples were taken every 2 weeks, from the start of the calendar spring until the end of calendar autumn each year. Samples were collected in the limnetic zone at the inflow part of each pond. Water was taken from the 1-m layer below the surface of from each urban pond (sample size 20 l) and then filtered through a plankton net with 50- μ m mesh size. In the laboratory, the samples were concentrated to 10 ml and fixed using Lugol's solution. The zooplankton were subjected to qualitative and quantitative analyses using a glass Sedgewick-Rafter chamber with a 1-ml capacity and a Nikon microscope (magnification 40–100 $\times$). When the abundance of individuals of a given taxonomic group was high (> 150 individuals per subsample), all zooplankton animals in the chamber were counted as standard (the contents of a single chamber were counted). When the abundance of zooplankton in one subsample was

lower, a second subsample was taken and individuals from the next chamber were counted. The density of each found species (for cladocerans and rotifers) or orders (for copepods) was calculated using the equation: $N = XV_z / V_k V_p$, where X = number of counted individuals in the chamber; V_z = volume of the density from which the sample for microscopic determination was taken (ml); V_k = volume of the chamber (ml); V_p = volume of the compacted sample (l).

For copepods, the organisms were only grouped based on their order (Calanoida or Cyclopoida). *Copepod nauplii* were counted separately and were not included in the statistical analyses. Other copepod life stages were not differentiated and counted. Morphological analyses of the collected individuals were performed according to Amorós (1984), Benzie (2005) and Ejsmont-Karabin et al. (2004).

Statistical analyses

Statistical analyses were performed using both Statistica version 13.3 software (StatSoft) (for ANOVA) and CANOCO v. 5.15. (Microcomputer Power, Ithaca, NY, USA) (for RDA). The Shapiro-Wilk normality test was used to determine the data distribution. Parametric tests were used for further analyses (one-way ANOVA and two-way ANOVA, with Tukey's range test as a post hoc test). One-way ANOVA was applied to test the effect of year on the concentration of ions in each individual pond. To test for the effect of years and ponds and their interactions on differences in ion concentration and zooplankton density, we used two-way ANOVA with the years and ponds as categorical variables and ion concentration or zooplankton densities (total and for selected zooplankton groups) as dependent variables. Due to the large differences between years and ponds, the data used for the ANOVA tests were transformed according to the $\ln(x + 1)$ equation. Results were considered significant if the p -value was < 0.05 .

Redundancy analysis (RDA) method was applied to examine the relationships between zooplankton density and environmental variables. RDAs were selected because they enabled the compilation of all selected taxa and environmental parameters (ter Braak & Šmilauer, 2012).

For pore water samples, 12 quantitative variables and one nominal variable (POND) were analysed. Quantitative variables were: average temperature on the day of sampling [temp (d)], average temperature of 7 days before sampling [temp (7)], total precipitation on the day of sampling [precip (1)], total precipitation within 7 days before sampling [precip (7)], chloride [chlorid], phosphates [phospha], sulphates [sulph], sodium [Na], ammonia [amo], potassium [K], magnesium [Mg] and calcium [Ca]. For column water samples, ten quantitative variables and one nominal variable (POND) were analysed. Quantitative variables were: average temperature on the day of sampling [temp (d)],

average temperature of 7 days before sampling [temp (7)], total precipitation on the day of sampling [precip (1)], total precipitation within 7 days before sampling [precip (7)], chloride [chlorid], sulphates [sulph], sodium [Na], potassium [K], magnesium [Mg] and calcium [Ca]. To avoid multicollinearity of variables, the most strongly correlated variables were eliminated based on the Pearson correlation matrix (Pearson's correlation coefficient $r > 0.8$). For both groups of samples, i.e., samples from pore water and samples taken from the column water, the Na variable was strongly correlated with the concentration of Cl ions (*chloride*), and the *temp* (7) variable was correlated with the *temp* (d); therefore, the Na and *temp* (7) variables were omitted from further analysis. In both cases, the Pearson correlation coefficients were > 0.9 .

Further analysis of the collected material was initiated through gradient length calculations via detrended canonical correspondence analysis (DCCA). The length of the longest gradient in the DCCA was equal to 1.0. Therefore, to assess the dependence of zooplankton density on environmental variables, the redundancy analysis (RDA) method was applied. The significance of the RDA axes in explaining the density of zooplankton taxonomic groups was determined, as was the significance of the effects of particular variables on the model of ordering, which was based on the Monte Carlo test with 999 random permutations. The RDA biplot displays the zooplankton and environmental variables as vectors, and dummy environmental variables are displayed as points. The data were $\log(x + 1)$ -transformed, and, in the RDA, centering of the zooplankton data was applied.

To isolate and determine the influence of the location of a given pond on the community of zooplankton in the model, the POND variable was considered a covariate. RDA was first conducted using the entire data set. Subsequently, to isolate the amount of variation explained by pond location effects, we used a partial RDA (ter Braak & Šmilauer, 2012), in which the variable POND served as a covariate, and the ordination was only constrained by environmental variables.

As a complement to the above analysis, variation partitioning for the two groups (fractions) was used (Lepš & Šmilauer, 2012). The first group was the POND (i.e., zooplankton density ~ POND), and the second group was the other variables describing the environmental condition (i.e., zooplankton density ~ the environment variable). The additional fraction was the shared part (i.e., zooplankton density ~ POND + environment).

Biodiversity indexes, including Dominance Index (D), Simpson's Index of Diversity (D') and Shannon-Wiener Diversity Index (H'), were calculated using the Past 4.03. software. Indexes were calculated for total zooplankton as well as two zooplankton groups: cladocerans and rotifers.

Results

Meteorological data

Meteorological data for the city of Lodz, the site of the present research, has been collected since 1951 by the national monitoring service. Some base meteorological statistics were obtained daily (temperature, precipitation and snowcover) for the investigated hydrological years. These are compared to multiyear average values (1951–2020) in Table S2 (Supplementary Material).

All 3 hydrological years had lower year sum of precipitation, shorter non-vegetation period (daily mean temperature $< 5^{\circ}\text{C}$ —range in Poland climate zone), shorter winter (daily mean temperature $< 0^{\circ}\text{C}$) and higher daily mean temperature than the 1951–2020 multiyear average. A cooler period was noted in hydrological year 2019/2020, which was also characterized by the lowest number of days with meteorological winter (daily mean temperature $< 0^{\circ}\text{C}$), days of non-vegetation period, total precipitation in non-vegetation period, total precipitation in meteorological winter and days with snow cover.

Water quality of the analysed urban ponds

Changes in chloride concentrations during the hydrological years

Chloride ion concentrations measured in water column and pore water during 3 hydrological years in the four urban ponds are depicted in Figs. 2 and 3 with the values given after the $\ln(x+1)$ transformation.

Chloride concentrations exceeded the 640 mg/l acute toxicity threshold and persisted for the longest time in the pore water in the ZW pond during the year 2021. These high chloride concentrations were maintained for 124 days, 62 of which were winter days, and the rest were spring days. Chloride concentrations exceeding the 120 mg/l chronic toxicity threshold persisted for the longest time in pore water in both JG and ZZ ponds during the year 2020. In both ponds they were maintained for 348 days, 91 of which were winter days.

Mean chloride and sodium ion concentrations in different years and urban ponds

Over the 3-year study period, the highest mean concentrations of chloride and sodium in column water (as an average for all ponds) were measured in 2021, the year with the coldest winter (mean: 424.26 mg/l and 119.83 mg/l,

respectively). Also, high concentrations were measured in 2019 (mean: 335.39 mg/l Cl^- and 155.78 mg/l Na^+), with the lowest mean concentrations observed in 2020 (61.76 mg/l Cl^- and 25.95 mg/l Na^+). Two-way ANOVA analyses confirmed significant differences in chloride ($p < 0.001$) (Fig. 4a, Fig. S1a, Supplementary Material) and sodium ($p < 0.001$) (Fig. 4c, Fig. S2a, Supplementary Material) concentrations in column water between years.

The mean chloride and sodium concentrations measured in pore water were the highest in 2021 (mean: 1259.97 mg/l and 355.36 mg/l respectively), followed by 2019 (mean: 303.21 mg/l Cl^- and 142.66 mg/l Na^+) and 2020 (mean: 141.86 mg/l Cl^- and 53.53 mg/l Na^+). Two-way ANOVA analyses showed significant differences for chloride ($p < 0.001$) (Fig. 4b, Fig. S1c, Supplementary Material) and sodium (Fig. 4d, Fig. S2c, Supplementary Material) ($p < 0.001$) concentrations measured in pore water between the years.

The highest mean chloride and sodium ion concentrations in column water were observed in the ZW pond (mean: 650.54 mg/l and 238.42 mg/l respectively), followed by the JG pond (mean: 416.98 mg/l Cl^- and 129.91 mg/l Na^+), ZZ pond (average: 207.6 mg/l Cl^- and 72.84 mg/l Na^+) and lastly the reference pond AG (mean: 59.49 mg/l Cl^- and 21.89 mg/l Na^+); no distinction was found between years. Two-way ANOVA analyses confirmed significant differences in chloride ($p < 0.001$) (Fig. 4a, Fig. S1b, Supplementary Material) and sodium ($p < 0.001$) (Fig. 4c, Fig. S2b, Supplementary Material) ion concentrations in column water between the urban ponds.

The highest mean pore water chloride and sodium concentrations were found in the ZW (1757.69 mg/l Cl^- and 544.31 mg/l Na^+), followed by JG (619.63 mg/l Cl^- and 150.41 mg/l Na^+), ZZ (406.22 mg/l Cl^- and 140.69 mg/l Na^+), with the lowest concentrations measured in AG (89.61 mg/l Cl^- and 34.9 mg/l Na^+). Two-way ANOVA analyses confirmed significant differences in chloride ($p < 0.001$) (Fig. 4b, Fig. S1d, Supplementary Material) and sodium ($p < 0.001$) (Fig. 4d, Fig. S2d, Supplementary Material) ion concentrations in pore water between the urban ponds.

Significant year*pond interactions were observed for both chloride (Fig. 4a, b) and sodium (Fig. 4c, d) ion concentrations measured in both column and pore water ($p = 0.01$ for Cl^- and $p = 0.02$ for Na^+ in column water; $p < 0.001$ for Cl^- and $p < 0.001$ for Na^+ in pore water).

The mean, minimum, maximum and standard deviation values of the concentrations of chloride, sodium and other ions (nitrate, phosphate, sulphate, ammonium, potassium, magnesium and calcium) observed during 3 hydrological years, as well as in the four ponds, are shown in Tables S3, S4 and S5 in the Supplementary Material.

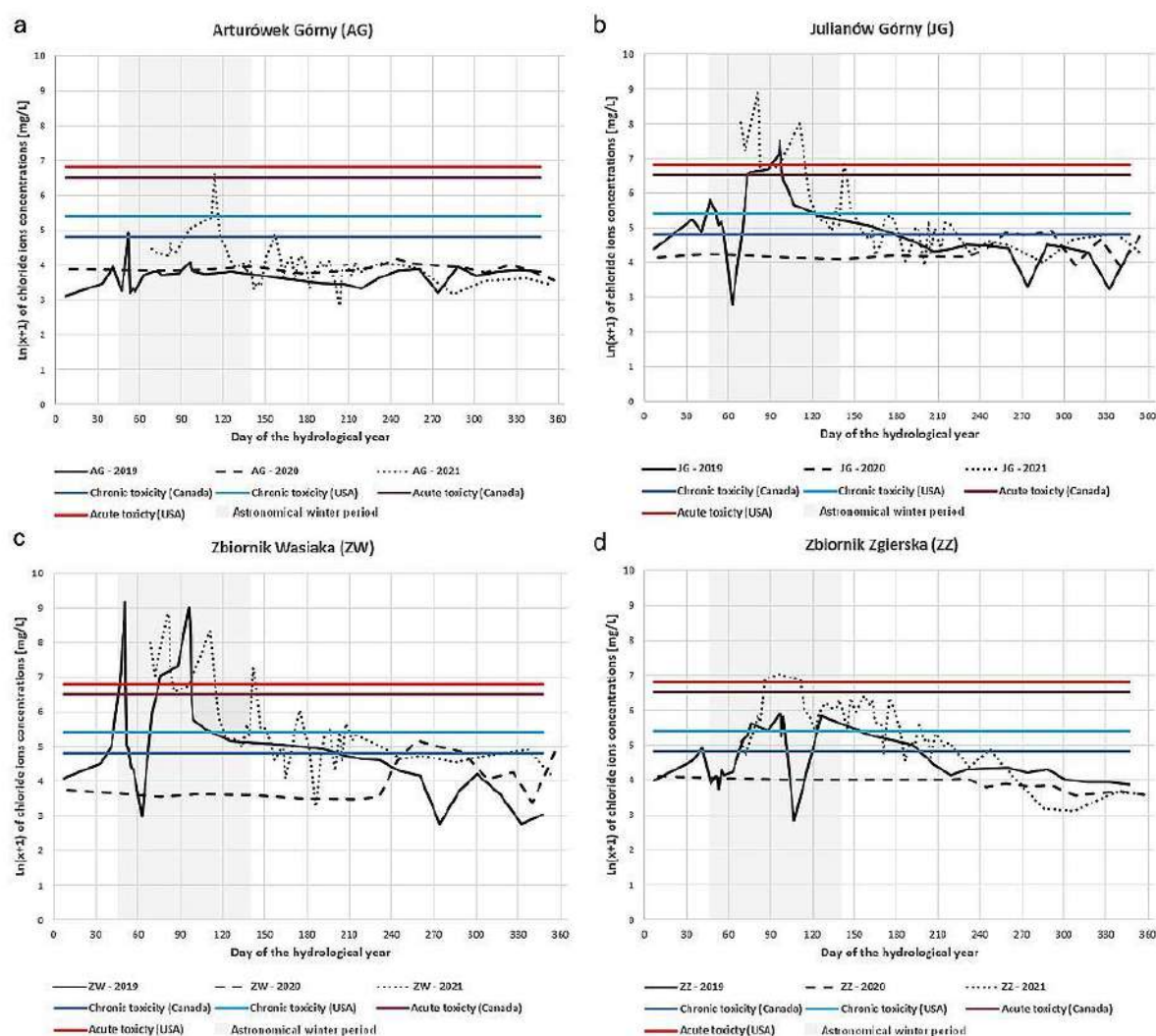


Fig. 2 Changes in chloride ion concentrations measured in the column water in the studied urban ponds (**a, b, c, d**) during the 3 hydrological years: 2019 (solid line), 2020 (broken line), 2021 (dotted line). Horizontal lines indicate the limit values for chronic and acute

toxicities of chlorides in surface water, as established in Canada (CEQG, 2011) and the USA (US EPA, 1988). The grey area indicates the astronomical winter period. All of the data had been transformed according to the $\ln(x+1)$ equation

Zooplankton structure and density

In general, the highest mean total zooplankton densities were observed in ZZ (2742.83 specimens/l in 2019, 1989.92 specimens/l in 2020 and 1527.61 specimens/l in 2021).

The mean values in the reference AG were 254.36 specimens/l in 2019, 353.5 specimens/l in 2020 and 190.29 specimens/l in 2021. The lowest mean densities were observed in JG (42.33 specimens/l in 2019, 17.92 specimens/l in 2020 and 10.36 specimens/l in 2021) as well as in ZW (135.36 specimens/l in 2019, 7.38 specimens/l in 2020 and 2.75 specimens/l in 2021).

Two-way ANOVA for the total zooplankton density showed significant differences between years ($p < 0.01$) and urban ponds ($p < 0.001$). In 2019, total zooplankton density was significantly higher than in 2020 or 2021, with no significant difference between 2020 and 2021 (Fig. 5a). Total zooplankton density was significantly higher in ZZ than in AG, ZW and JG; in addition, total zooplankton density was also significantly higher in AG than in JG and ZW (Fig. 5b).

Two-way ANOVA analysis confirmed the presence of significantly more copepods and cladocerans in 2019 than in 2020 ($p = 0.01$), with no significant differences between 2019 and 2021, or between 2020 and 2021. For the rotifers,

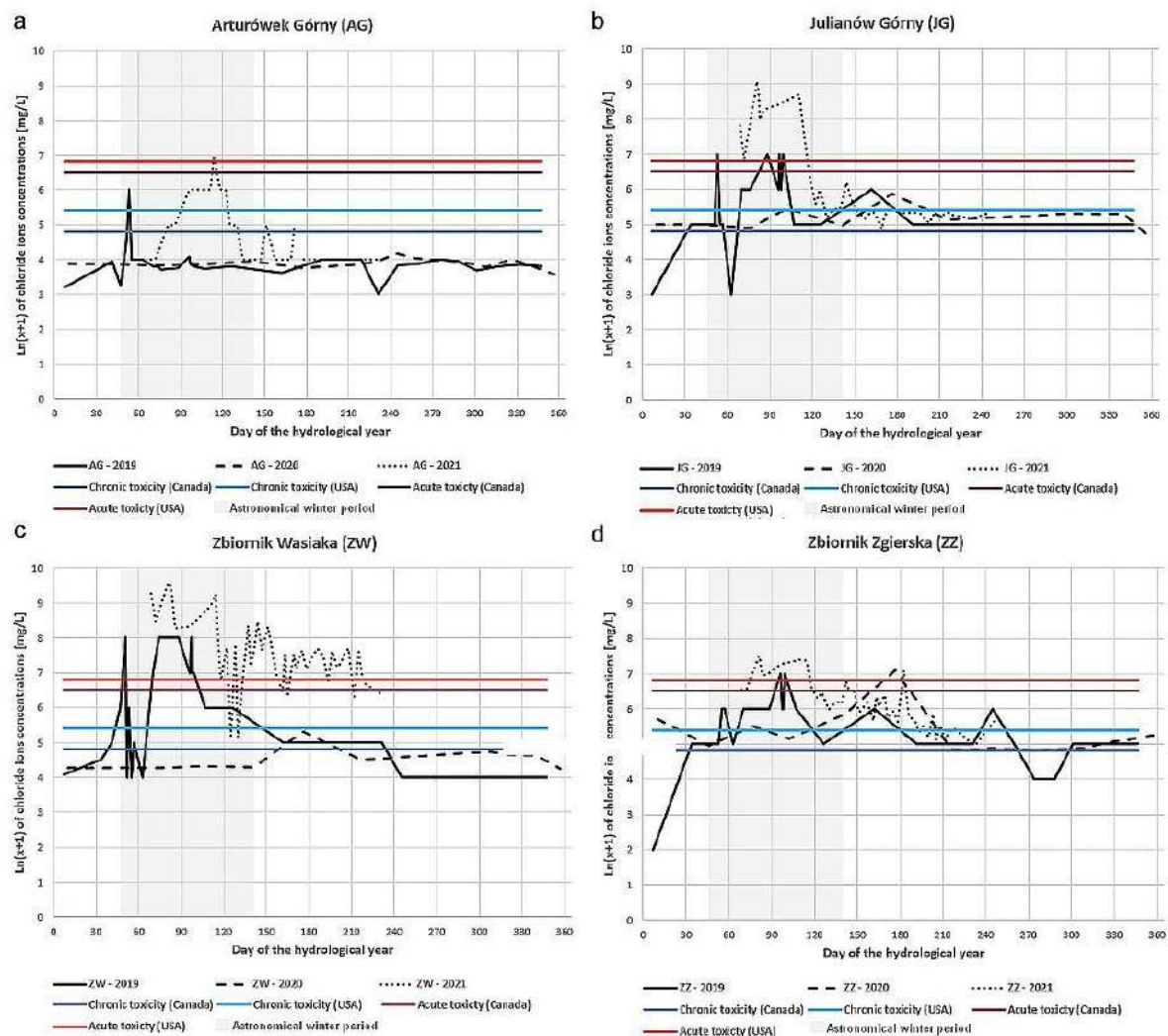


Fig. 3 Changes in chloride ion concentrations measured in the pore water in the studied urban ponds (**a**, **b**, **c**, **d**) during the 3 hydrological years: 2019 (solid line), 2020 (broken line), 2021 (dotted line). Horizontal lines indicate the limit values for chronic and acute toxicities

of chlorides in surface water as established in Canada (CEQG, 2011) and the USA (US EPA, 1988). The grey area indicates astronomical winter period. All of the data had been transformed according to the $\ln(x+1)$ equation

no significant difference in density was observed between years (Fig. 5a). All three groups of zooplankton were the most abundant in ZZ (copepods $p < 0.001$, cladocerans $p < 0.001$, rotifers $p < 0.001$). There were also significantly more rotifers in AG compared with JG ($p = 0.01$) and ZW ($p < 0.001$). AG demonstrated higher density of cladocerans and copepods than ZW ($p = 0.02$ and $p = 0.02$, respectively) (Fig. 5b). For total zooplankton and rotifer densities, there was no interaction between ponds and years ($p = 0.745$ and $p = 0.265$, respectively). For copepod and cladoceran density, interactions were significant ($p < 0.05$).

In terms of zooplankton community structure, the urban ponds studied were mainly dominated by rotifers, with the exception of ZZ during the years 2019 and 2020, when the distribution of rotifers and cladocerans was nearly even. Species that dominated most frequently were *Keratella quadrata* and *K. cochlearis* (as well as *K. cochlearis* in its *tectata* form, albeit to a lesser extent).

Additional information on the characteristics of the zooplankton communities of the studied ecosystems, including the list of all observed species and selected biodiversity indexes, is presented in the supplementary material

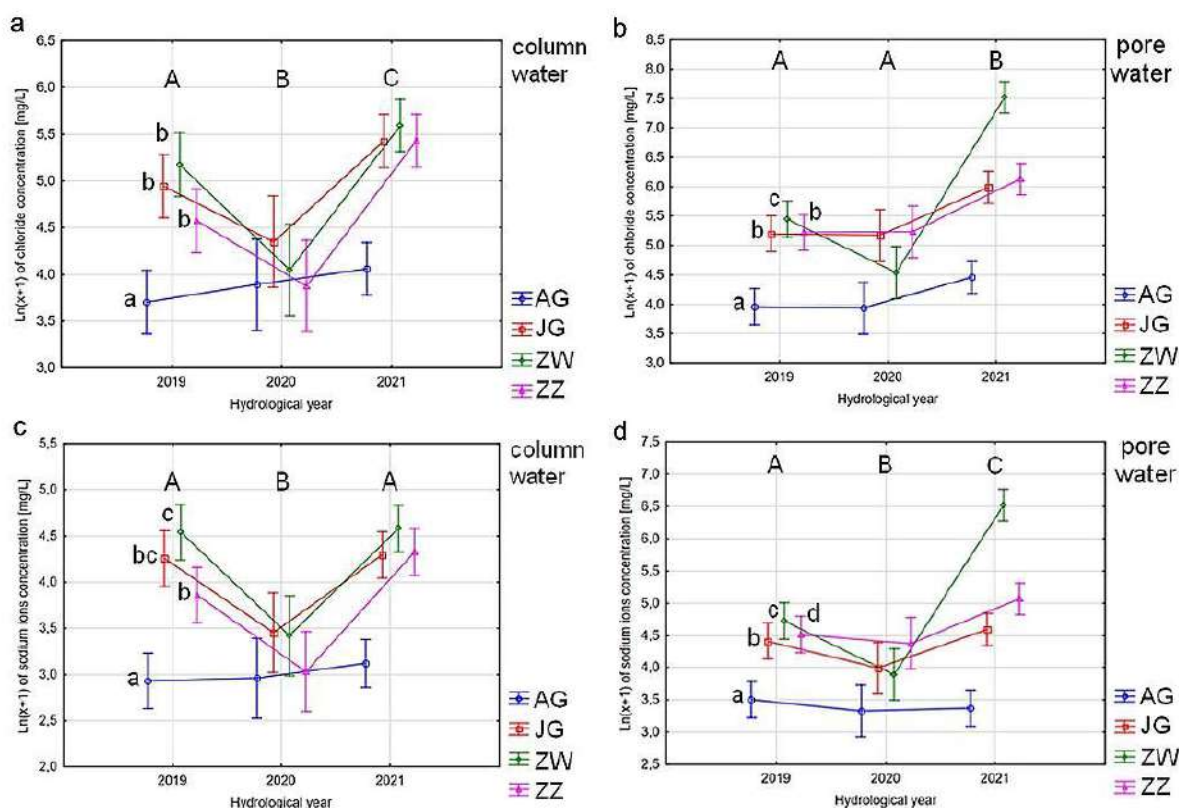


Fig. 4 Chloride (a, b) and sodium (c, d) concentration ($\ln(x+1)$ mg/l) measured in the column and pore water of each pond and each year. Vertical bars represent 0.95 confidence intervals. Uppercase let-

ters indicate significant differences between years; lowercase letters indicate significant differences between ponds

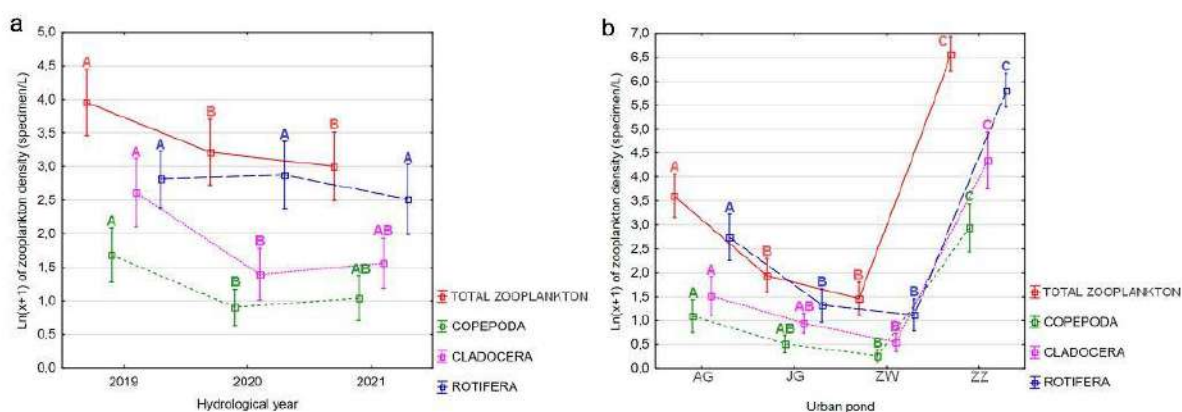


Fig. 5 Mean density of total zooplankton, copepods, cladocerans and rotifers observed during the 3 hydrological years (a) and in four studied urban ponds (b). All of the data had been transformed accord-

ing to the $\ln(x+1)$ equation. Vertical bars represent 0.95 confidence intervals. Different capital letters indicate significant differences between years and urban ponds ($p < 0.05$)

(Table S6, Table S7) together with graphs showing changes in zooplankton densities during the study period (Fig. S3).

Relationship between zooplankton and environmental parameters

For the RDA data collected for the column water, the first ordination axis explained 52.8% of the variability in zooplankton community density. Pond ZZ was most strongly positively correlated with the first axis. The second axis described 2.4% of the observed variability and was related to the Ca variable (Fig. S4a, Supplementary Material). Pond ZZ, Pond AG, K ion concentration and Cl ion concentration had a statistically significant effect (Monte Carlo test) on the model examining the relationships between zooplankton density and environmental variables (Table S8, Supplementary Material). In the partial RDA, the variables K ion concentration, Cl ion concentration and average temperature on the day of sampling [temp (d)] had statistically significant impacts on the model (Fig. 6a). The variables K ion concentration, Cl ion concentration and temp (d) had statistically significant impacts on the model.

In the RDA model for column water samples, covering all variables, cladocerans, rotifers and zooplankton were strongly positively correlated with the Pond ZZ variable. In addition, rotifers and zooplankton were negatively correlated with nitrate (Nitra) and sulphate (Sulph) variables (Fig. S4a, Supplementary Material). When the variable POND was included as a covariate, no significant correlation was found between zooplankton density and environmental variables

(Fig. 6a), although the density of all zooplankton groups demonstrated clear negative correlation with chloride and a positive correlation with potassium. The variation partitioning analysis showed that both the environment variable and the pond factor significantly contributed to explaining the variability in zooplankton density. The environment variable had a smaller contribution to the explained variation (11%) than did the POND variable (62%). The mean contribution (mean square) of the POND variable was > 10 times higher than the environment variable (Table S9, Supplementary Material).

For pore water sample data, the first axis explained 47.3% of the variability in density. Pond ZZ was most strongly positively correlated with the first axis. The second axis described 2.4% of the observed variability and was related to the temp (d) variable (Fig. S4b, Supplementary Material). Pond ZZ, Pond AG and temp (d) had a statistically significant effect (Monte Carlo test) on the model that examined the relationships between zooplankton density and environmental variables (Table S10, Supplementary Material). In the partial RDA, when using the POND variable as a covariate, the first axis explained 4.9% of the variation, and the second axis described 1.6% of the variation (Fig. 6b). In the RDA model for samples from pore water, including all variables, rotifers and zooplankton were strongly positively correlated with the POND ZZ variable. In contrast, cladocerans were moderately correlated with this variable (Fig. S4b, Supplementary Material). When the POND variable was included as a covariate, no significant correlation was found between zooplankton groups and environmental variables

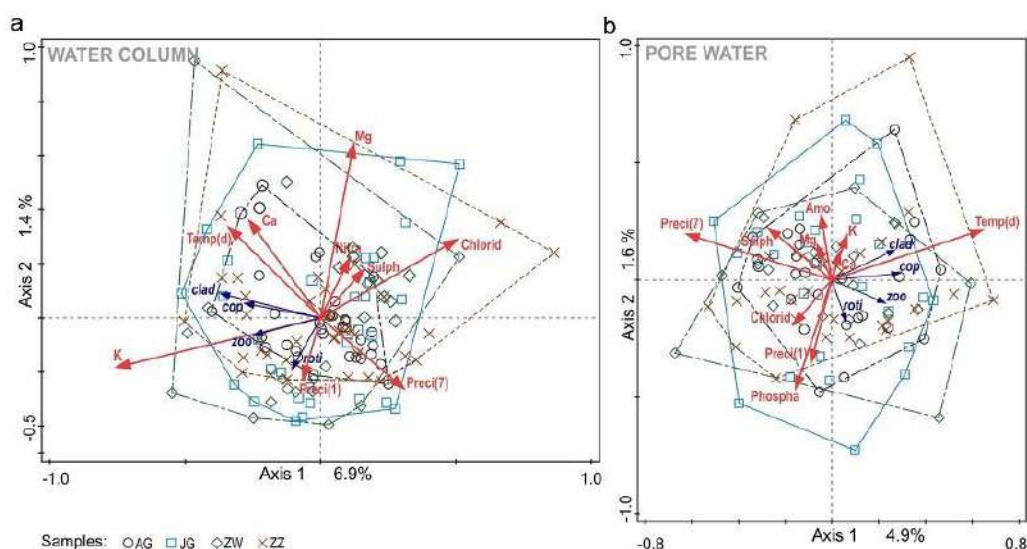


Fig. 6 Association of zooplankton, environmental variables and POND variable for column water samples and pore water samples. Correlation plots obtained by partial RDA using the POND variable

as the covariate. Filled triangle, nominal variables (Pond AG, Pond JG, Pond ZW, Pond ZZ)

(Fig. 6b). The variation partitioning analysis showed that only POND factors significantly contributed to explaining the variability in zooplankton density. The environment variable made a smaller contribution to the explained variation (3%) compared to the POND variable (75%) (Table S11, Supplementary Material).

Discussion

Climate change and freshwater salinization are emerging threats for freshwater biodiversity, as are the cumulative impacts of these and other stressors (Reid et al., 2019). Climate change results in not only an increase in the average temperature but also changes in global and regional water cycles (Douville et al., 2021). Studies have confirmed an ongoing increase in temperature and decrease in snow cover duration during winter in Poland (Tomeczyk et al., 2021) as well as increasing precipitation during the winter season (Szwed, 2018). Our results confirm this trend, with higher daily mean temperature and lower numbers of days with snow cover (Table S2; Supplementary Material).

However, there remains a need to ensure road safety in winter by road salt application. Chloride-based deicer use represents one source of anthropogenic salinization of freshwater ecosystems. Considering its prevalence, as well as possible long-term consequences of road salt usage, the topic of road salt pollution has gained more attention in recent years (Corsi et al., 2015; Hintz and Relyea, 2019; Szklarek et al., 2022). Notably, the maximum Cl^- concentrations in reservoirs after winter and their persistence are influenced by various meteorological (temperature and precipitation) and hydrological (average flow, retention time, deep) conditions. If a wet winter characterized by salt application is followed by a dry spring, the length of the period with elevated Cl^- concentration may persist, thus generating greater ecotoxicity pressure on freshwater communities. The winter-spring characteristics of Cl^- concentration might play a crucial role in the functioning of zooplankton and other organisms, but understanding this relationship needs long-term research. In the present study, Cl^- concentrations above acute toxicity were noted until March in column water (Wasiaka [ZW] pond and Julianów [JG] pond, Fig. 2) and until June in pore water (Wasiaka [ZW] pond, Fig. 3), but longer Cl^- chronic toxicity was observed in all three ponds supplied by road salt runoff.

As salinisation of water due to road salt is not a seasonal problem and can persist throughout the growing season, various studies have examined the effects of road salt pollution on individual species of zooplankton (Hintz et al., 2019; Venancio et al., 2018; Coldsnow et al., 2017) as well as freshwater food webs (Hintz and Relyea, 2017; Lind et al., 2018; Van Meter and Swan, 2011). For example, Van

Meter and Swan (2014) observed that the rotifer family Brachionidae dominates zooplankton communities exposed to elevated chloride concentrations (93%), whereas the same family composed only 27% of the zooplankton community in a low chloride treatment. Van Meter and co-workers (2011) found that elevated chloride concentrations caused a decrease in copepod and cladoceran densities while not affecting the *C. nauplii* or rotifer densities.

The existing studies in the literature play a key role in understanding the complex effect that elevated chloride concentrations may have on zooplankton species and the structure of freshwater foodwebs. However, the majority of the studies concerning the effects of road salt on zooplankton are performed using mesocosm studies or controlled conditions (e.g., toxicity biotests). There are far fewer studies examining the effects of road salt pollution on zooplankton inhabiting natural environments. One valuable study is that of Bielańska-Grajner and Cudak (2014), who examined the effects of salinity on species diversity of rotifers inhabiting 14 anthropogenic water bodies located in Silesian Upland, Poland; however, it focuses on salinization originating from the mining industry and not road salt pollution.

This study is one of the first works to examine the effect of road salt pollution on zooplankton inhabiting the natural environment. We expected that the use of large amounts of road salt, especially during cold winters, would cause a significant increase in chloride concentrations in the studied ecosystems and affect the dynamics of zooplankton density. One of the most important results was obtained by RDA analysis, which confirmed, as we correctly assumed, that chloride concentrations may be relevant to their impact on zooplankton. However, although chlorides were the most important among all factors related to water chemistry, the dynamics of zooplankton density depended primarily on pond-specific ecosystem characteristics. RDA showed that Zgierska (ZZ) pond, Arturówek (AG) pond, potassium (K) and chloride (Cl) had a statistically significant effect on the model examining the relationships between zooplankton density and environmental variables. Potassium and chloride ion content also had a statistically significant impact on the model after using the POND variable as a covariate. Although these ions were not significantly correlated with zooplankton density in this case, a clear negative correlation was observed for all zooplankton groups with chloride. The RDA clearly showed that in the urban ponds studied, the effects of salt and other environmental factors on zooplankton density were masked by significant differences in pond characteristics.

For the Arturówek (AG) pond, characterised by the lowest road salt concentrations, the chloride and sodium ion content in column water or pore water did not appear to have any negative effect on zooplankton. RDA showed that the key factors relevant to this ecosystem were weather conditions.

Thus, our results confirmed that the nature-based solutions used in our reference pond (sedimentation-biofiltration systems; Jurczak et al., 2019) effectively reduce the influx of road pollutants, including road salt, contributing to improved environmental quality.

The situation was different in the other three urban ponds, which were exposed to high road salt inputs. The RDA showed that for these water samples, the density of total zooplankton or the density of individual zooplankton groups was negatively correlated with the concentration of chloride ions, although the strength of this correlation was weakened by the significant differences in the environmental characteristics of the ponds. Notably, the correlations relate to the growing season; wintertime was excluded because of the absence of zooplankton. During winter, the chloride concentrations in the column water samples were above the acute toxicity level in all tested urban ponds in 2021, i.e., the year with high road salt use, and in Julianów (JG) pond and Wasiaka (ZW) pond in 2019, i.e., the year with the typical winter.

Although we predicted a decrease in road salt concentrations during the growing season, chronic chloride toxicity persisted in the urban ponds long after the end of winter and was still observed in May, June and even July in Zgierska (ZZ) pond (Fig. 2). This means that elevated chloride concentrations persisted in the environment during the period of zooplankton hatching and development. Disturbingly high chloride concentrations, exceeding acute and chronic toxicity levels, persisted in the pore water throughout hydrological years 2019, 2020 and 2021 in all urban ponds except the reference Arturówek (AG) pond (Fig. 3). In Zgierska (ZZ) pond, chloride concentrations were elevated, even in the year with a mild winter. The increase in concentrations observed during periods without de-icing suggests that chlorides may have been stored over winter in hydrological reservoirs, such as a shallow groundwater system, and slowly released throughout the year. A similar phenomenon has been observed in streams in the northern US (Corsi et al., 2015).

Although water temperature is an important factor that regulates the abundance and distribution of rotifers (Yin et al. 2018), many studies indicate that variability in rotifer abundance is driven by “bottom-up” forces related to food supply (e.g., Yoshida et al., 2003; Ejsmont-Karabin, 2012). The waters of the studied reservoirs were rich in suspended organic matter (unpublished data), which was especially true for Julianów (JG) pond and Zgierska (ZZ) pond sites. In the former, the source of the organic matter could be, among other, fallen dead leaves which accumulate at the bottom of the pond. During summer, Zgierska (ZZ) pond becomes a habitat of the plants from the *Potamogeton* genus, which heavily cover the water surface and begin to decay during late summer and autumn. It is also possible that organic

matter may have had an anthropogenic source, e.g., from pollutants entering the urban ponds via faulty sewer systems of the nearby residential areas. Rotifers from the *Keratella* genus are known to consume detritus, organic aggregates and bacteria (Gilbert, 2022), which means that two species that dominated in our study, *K. cochlearis* and *K. quadrata*, had favorable feeding conditions. The relationship between the amount of organic matter as a habitat for bacteria and protozoa, a common part of the food of most rotifer species, and rotifer density has been confirmed in field studies, e.g., by Wilk-Woźniak and co-authors (2014) who showed a significant positive correlation between rotifer abundance and the amount of allochthonous organic matter input to the flowing lake Dobczyce (Poland). Moreover, both *K. cochlearis* and *K. quadrata* are eurytopic species; therefore, their dominance is not surprising.

The presence of road salt-derived chlorides in freshwaters should not be underestimated. Depending on the characteristics and degree of pollution/eutrophication of the water bodies, the substances present may interact additively, synergistically or antagonistically with road salt (Woodley et al., 2023). This can pose unforeseen risks to organisms and disrupt the functioning of freshwater ecosystems.

Conclusions

Few studies of the relationship between anthropogenic pollutants, such as road salt, and zooplankton in freshwater bodies have been performed under natural conditions. This may be due to the high variability and dynamics of natural ecosystems, diversity of interacting factors influencing them and complexity of the relationships between organisms and the environment, making it difficult to interpret the results obtained. Furthermore, the problem of water salinity is often downplayed and/or not fully recognized. The results of our 3-year study lead to conclusions that can broaden knowledge of the effects of salinity on aquatic organisms.

Our research shows that the impact of road salt on urban freshwater ecosystems is not a seasonal problem, i.e., it does not only affect the winter period. Elevated concentrations of chloride ions persist in both sediment and water throughout the year and can negatively affect zooplankton during the growing season.

Moreover, our findings are the first from an environmental study to show that zooplankton density is negatively correlated with chloride ion concentrations exceeding chronic toxicity levels in water. This phenomenon was found to be a consequence of cold and snowy winters. However, pond-specific factors (e.g., biotic interactions), which are key to the dynamics of zooplankton density, may have modified the response of zooplankton to the presence of road salt.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00027-023-01009-y>.

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Author contributions AG: Data curation, Formal analysis, Investigation, Visualization, Writing—original draft, Writing—review & editing. SS: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Validation, Writing—original draft, Writing—review & editing, Resources. PF: Formal analysis, Software, Resources, Visualization. KK: Formal analysis, Software, Visualization. AW-F: Conceptualization, Formal analysis, Methodology, Supervision, Validation, Writing—original draft, Writing—review & editing

Data availability The raw dataset generated and analysed during the study are currently available in the Zenodo repository, <https://doi.org/10.5281/zenodo.7924181>

Declarations

Conflict of 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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SUPPLEMENTARY MATERIAL
Does winter application of road salt affect zooplankton communities in urban ponds?

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Table S1. Characteristics of four urban ponds selected in the study. Source: Ecophysiological study, Łódź [Opracowanie ekofizjograficzne, Łódź], 2017.

Name/abbreviation	Arturówek Górny (AG) (Reference)	Julianów Górny (JG)	Zbiornik Wasiaka (ZW)	Zbiornik Zgierska (ZZ)
Surface area [m ²]	10 800	16 400	13 078	17 000
Mean depth [m]	0.93	1.37	n.a.	1.35
Capacity [m ³]	10 000	22 510	2 690	23 000

Table S2. Meteorological data for three analysed hydrological years compared to multiyear mean.

	Hydrological year			Mean (1951-2020)
	2018/2019	2019/2020	2020/2021	
Daily mean temperature [°C]	10.1	10.0	8.8	7.12
Days of meteorological winter*	45	19	55	50.3
Days of non-vegetative period**	114	108	130	137.9

Year sum of precipitation [mm]	411.4	610.2	564.0	655.8
Sum of precipitation in non-vegetative period**	145	88	100	n.a.
Sum of precipitation in meteorological winter*	21	1	39	n.a.
Days with snowcover	28.0	1.0	42.0	54.6***

* daily mean temperature < 0 °C

** daily mean temperature < 5 °C

*** mean snowcover for period 1971-2020

n.a. – not available

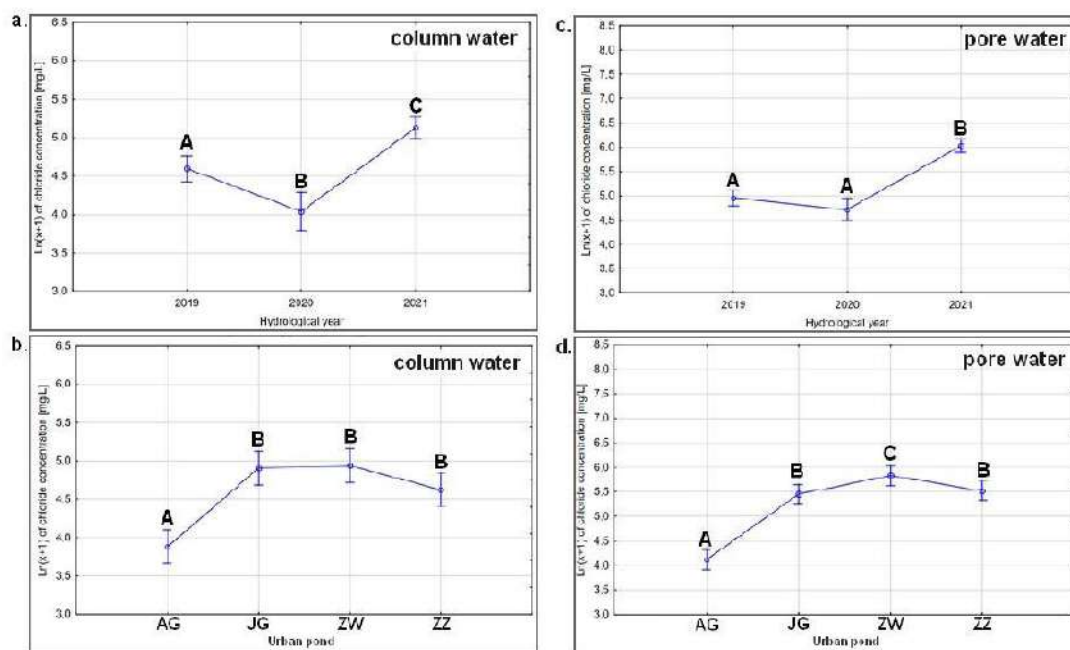


Fig. S1. Chloride concentration ($\ln(x+1)$ mg/L) measured in the column and pore water during each year (a, c) and pond (b, d). Vertical bars represent 0.95 confidence intervals. Uppercase letters indicate significant differences between years and ponds.

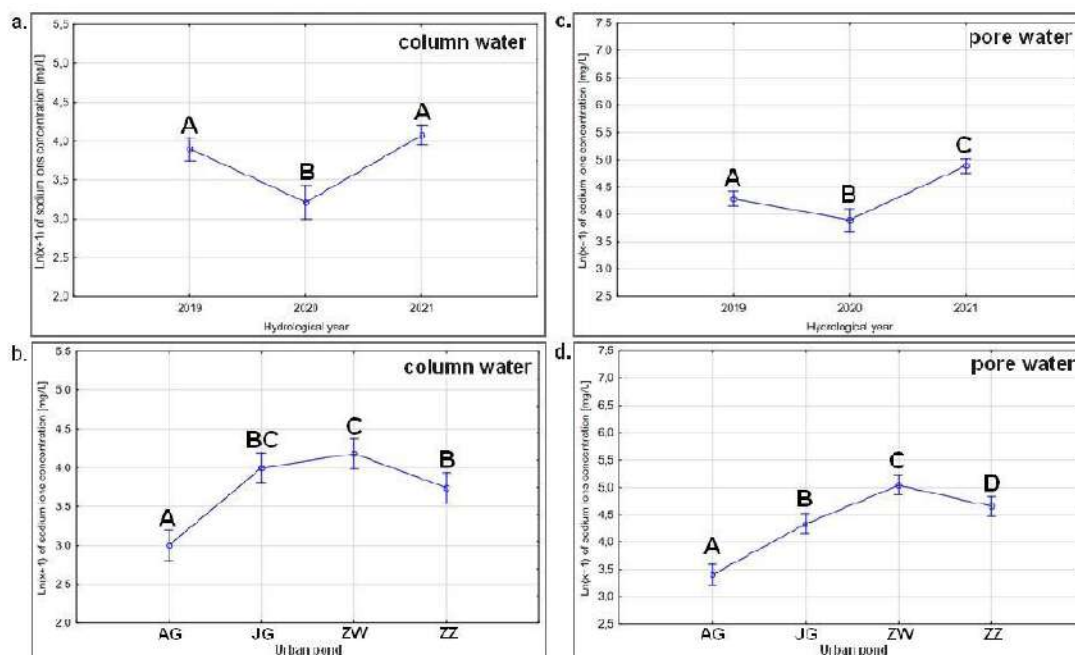


Fig. S2. Sodium concentration ($\ln(x+1)$ mg/L) measured in the column and pore water during each year (a, c) and pond (b, d). Vertical bars represent 0.95 confidence intervals. Uppercase letters indicate significant differences between years and ponds.

Table S3. Mean value (min-max) of chlorides and sodium [mg/L] for pore and column water in each hydrological year. Different letters indicate significant differences between years in one-way ANOVA test ($p < 0.05$).

	CHLORIDES			SODIUM		
	2019	2020	2021	2019	2020	2021
PORE WATER						
AG	64.73 ^A (18.2 - 469.8)	50.74 ^A (43.7 - 81.3)	124.8 ^B (44.2 - 1205.6)	44.44 ^A (12.5 - 284.4)	26.96 ^A (22.9 - 29.2)	29.05 ^A (21.5 - 62.7)
JG	260.92 ^A (16.2 - 1347.3)	181.97 ^A (105.5 - 349.9)	1057.76 ^B (128.7 - 8738)	117.19 ^A (11.2 - 524.7)	53.71 ^A (28.1 - 58.6)	211.88 ^A (0 - 2067.4)
ZW	643.33 ^A (43.10 - 4235.7)	96.34 ^B (68.6 - 203.5)	3231.07 ^C (83.09 - 14374)	292.38 ^A (0.8 - 2160.9)	49.16 ^B (37.4 - 91.5)	921.9 ^C (0 - 3604.3)
ZZ	243.84 ^A (6.06 - 779.9)	238.38 ^A (120.5 - 1206)	589.04 ^B (85.19 - 1812)	116.63 ^A (4.45 - 344.6)	84.31 ^A (58.7 - 246.1)	179.24 ^B (0 - 457.1)
COLUMN WATER						
AG	42.42 ^A (21.3 - 133.5)	48.32 ^{AB} (34 - 63.2)	74.94 ^B (15.7 - 726.8)	18.53 ^A (10.10 - 36.5)	18.74 ^A (12.4 - 23.3)	25.39 ^A (0 - 196.8)
JG	300.16 ^{AB} (15.4 - 1769.2)	81.33 ^B (48.4 - 138.1)	609.18 ^A (49.7 - 7737.7)	124.04 ^A (5.3 - 768.8)	31.56 ^B (17.5 - 54.5)	166.74 ^A (0 - 2063.7)
ZW	871.95 ^A (14.9 - 9344.2)	69.39 ^B (28.2 - 176.7)	691.19 ^A (24.6 - 6827.4)	423.48 ^A (3.8 - 5195.8)	32.22 ^B (13.8 - 59.8)	181.12 ^A (11.4 - 1891.3)
ZZ	127.05 ^A (16.3 - 359.1)	48.01 ^B (33.9 - 59.7)	316.18 ^C (21.7 - 1111)	57.07 ^A (21.97 - 149.6)	21.3 ^B (11.2 - 33.2)	100.86 ^C (0 - 272.8)

Table S4. Mean, minimum, maximum and standard deviation values of chloride and sodium concentrations observed in pore and column water. Total values in each hydrological year, and total values in each urban pond are shown.

CHLORIDES					SODIUM			
PORE WATER								
	MEAN	MIN	MAX	SD	MEAN	MIN	MAX	SD
2019	303.21	6.06	4235.70	562.85	142.66	0.80	2160.90	255.81
2020	141.86	43.70	1206.70	152.91	53.53	22.90	246.10	30.78
2021	1259.97	44.20	14374.40	2424.73	366.68	13.24	3604.30	614.86
URBAN PONDS								
AG	89.61	18.20	1205.60	139.68	34.90	12.50	284.40	35.70
JG	619.63	16.20	8738.20	1384.03	153.75	11.20	2067.40	292.72
ZW	1757.69	43.10	14374.40	2969.37	556.40	0.80	3604.30	757.65
ZZ	406.22	6.06	1812.10	380.37	142.22	4.45	457.10	95.08
COLUMN WATER								
	MEAN	MIN	MAX	SD	MEAN	MIN	MAX	SD
2019	335.39	14.90	9344.20	1125.55	155.78	3.80	5195.80	562.15
2020	61.76	28.20	176.70	32.39	25.95	11.20	59.80	11.26
2021	424.26	15.70	7737.70	992.52	122.39	1.70	2063.70	262.79
URBAN PONDS								
AG	59.49	15.70	726.80	74.18	22.12	7.00	196.80	19.74
JG	416.98	15.40	7737.70	965.41	131.27	5.30	2063.70	287.85
ZW	650.54	14.90	9344.20	1598.31	238.42	3.80	5195.80	675.01
ZZ	207.60	16.30	1111.92	212.49	74.37	1.70	272.80	64.98

Table S5. Mean value (min-max) of potassium, magnesium, calcium, nitrate, sulphate and ammonium ions [mg/L] for pore and column water in each hydrological year. Different letters indicate significant differences between years according to one-way ANOVA ($p < 0.05$).

	K ⁺			Mg ²⁺			Ca ²⁺			NO ³		
PORE WATER												
AG	2019	2020	2021	2019	2020	2021	2019	2020	2021	2019	2020	2021
	16.4 ^A	11.86 ^A	7.61 ^B	4.18 ^A	8.92 ^B	6.33 ^C	166.89 ^A	166.71 ^A	95.66 ^B	0.39 ^{AB}	0.24 ^B	0.63 ^A
	(1.3 - 44.3)	(5.1 - 14.1)	(2.9 - 12.2)	(0.4 - 22.1)	(4.6 - 10.4)	(3.9 - 12.5)	(69.5 - 277.4)	(81.2 - 187.4)	(55.8 - 171.4)	(0.00 - 2.20)	(0.20 - 0.90)	(0.20 - 1.30)
JG	36.18 ^A	34.3 ^A	3.71 ^B	7.28 ^A	13.99 ^B	15.22 ^B	318.54 ^A	318.45 ^A	138.92 ^B	0.47 ^A	1.36 ^A	6.24 ^B
	(2.4 - 98.7)	(0.6 - 44.3)	(0.6 - 11.4)	(0.5 - 32.4)	(9.6 - 24.0)	(4.6 - 43.9)	(39.8 - 783.3)	(97.7 - 389.3)	(48.6 - 275.1)	(0.00 - 1.70)	(0.30 - 5.70)	(0.50 - 38.8)
ZW	45.87 ^A	24.19 ^A	13.67 ^B	12.16 ^A	30.16 ^B	17.62 ^C	315.61 ^A	409.27 ^A	124.92 ^B	0.74 ^{AB}	0.29 ^B	2.33 ^A
	(1.0 - 141.4)	(15.7 - 25.5)	(7.42 - 21.6)	(0 - 37.9)	(12.5 - 83.1)	(4.2 - 46.1)	(2.5 - 812.7)	(89 - 972.9)	(44.6 - 324.6)	(0.00 - 4.00)	(0.10 - 0.90)	(0.10 - 5.61)
ZZ	58.2 ^A	23.18 ^A	33.7 ^B	10.08 ^A	21.85 ^B	15.66 ^B	255.71 ^A	205.19 ^A	111.68 ^B	0.64 ^A	0.63 ^A	2.52 ^B
	(1.58 - 453.0)	(10.2 - 25.7)	(3.45 - 893.3)	(0.33 - 46.2)	(12.1 - 47.7)	(6.2 - 31.5)	(19.43 - 660.1)	(96.8 - 273.5)	(49.5 - 193.2)	(0.00 - 3.10)	(0.40 - 0.70)	(0.00 - 7.24)
COLUMN WATER												
AG	14.4 ^A	10.75 ^B	2.1 ^C	2.33 ^A	2.68 ^A	4.02 ^B	98.17 ^A	58.18 ^B	45.27 ^C	1.84 ^A	0.92 ^A	5.74 ^B
	(3.7 - 22.4)	(0.9 - 19.9)	(0.6 - 7.2)	(0.7 - 15.6)	(1.7 - 4.5)	(1.4 - 15.4)	(48.6 - 179.6)	(29.5 - 84.6)	(24 - 101)	(0.00 - 8.90)	(0.20 - 1.60)	(0.20 - 25.10)
JG	22.14 ^A	18.99 ^A	3.73 ^B	4.16 ^A	7.07 ^A	9.77 ^B	166.14 ^A	125.05 ^A	71.52 ^B	17.88 ^A	25.12 ^B	17.25 ^{AB}
	(2.6 - 49.3)	(3.10 - 35.5)	(0.7 - 32.96)	(0.4 - 24.2)	(1.4 - 20.9)	(0.09 - 23.7)	(17.2 - 330.8)	(48.5 - 163.2)	(23.2 - 170.3)	(0.80 - 40.90)	(14.30 - 52.30)	(5.50 - 28.00)
ZW	22.09 ^A	15.1 ^A	5.25 ^B	6.26 ^A	11.14 ^B	12.11 ^B	148.01 ^A	96.18 ^A	67.05 ^B	7.59 ^A	4.66 ^A	15.39 ^B
	(3.7 - 48.1)	(3.6 - 24.6)	(0.7 - 38.42)	(0.4 - 38.6)	(4.4 - 22.7)	(0.09 - 23.4)	(42 - 332.6)	(37.6 - 130.2)	(19.1 - 153.9)	(0.10 - 25.30)	(0.50 - 17.50)	(1.90 - 34.80)
ZZ	18.79 ^A	14.08 ^B	2.43 ^C	3.79 ^A	4.6 ^{AB}	7.21 ^B	119.6 ^A	57.59 ^B	55.01 ^B	2.62 ^A	0.85 ^B	2.68 ^A
	(2.10 - 29.3)	(2.7 - 26.2)	(0.7 - 18.59)	(0.1 - 24.4)	(3.4 - 7.6)	(0.04 - 19.2)	(38.3 - 248.80)	(34.3 - 71.9)	(25 - 139)	(0.00 - 7.30)	(0.30 - 3.30)	(0.62 - 10.30)

	SO ₄ ²⁻			NH ₄ ⁺			PO ₄ ³⁻		
PORE WATER									
AG	2019	2020	2021	2019	2020	2021	2019	2020	2021
	14.58 ^A	7.54 ^A	18.17 ^A	4.83 ^A	6.79 ^B	4.02 ^A	1.76 ^A	3.15 ^B	1.33 ^A
	(0.00 - 131.30)	(2.00 - 14.30)	(0.10 - 75.10)	(1.20 - 11.40)	(4.20 - 7.80)	(0.50 - 7.50)	(0 - 7.4)	(1 - 11.1)	(0.2 - 4.5)
JG	152.68 ^A	118.76 ^A	111.95 ^A	4.05 ^A	3.21 ^A	2.05 ^A	0.75 ^A	0.45 ^A	1.34 ^B
	(2.60 - 428.50)	(3.70 - 228.10)	(0.20 - 570.00)	(0.10 - 39.20)	(0.40 - 4.10)	(0.20 - 5.60)	(0 - 6.8)	(0.2 - 1.4)	(0.1 - 5.5)
ZW	9.62 ^A	128.62 ^B	18.96 ^A	7.65 ^A	9.78 ^{AB}	13.84 ^B	1.16 ^A	101.73 ^B	5.05 ^B
	(0.00 - 46.50)	(2.90 - 667.70)	(0.00 - 203.00)	(0.00 - 20.10)	(9.10 - 14.30)	(0.90 - 28.00)	(0 - 4.8)	(1.8 - 531.6)	(0.43 - 21.2)
ZZ	70.23 ^A	12.03 ^B	25.2 ^B	5.25 ^A	9.78 ^B	9.27 ^B	2.07 ^A	4.63 ^B	1.73 ^A
	(1.30 - 339.80)	(3.80 - 17.40)	(0.00 - 251.90)	(0.17 - 20.00)	(8.20 - 18.50)	(2.50 - 18.70)	(0 - 13.5)	(1.9 - 14.8)	(0.2 - 5.6)
COLUMN WATER									
AG	54.11 ^A	45.41 ^A	40.74 ^A	0.67 ^A	0.08 ^{AB}	0.11 ^B	0.72 ^A	0.78 ^A	0.3 ^B
	(0.50 - 163.30)	(31.90 - 62.80)	(17.21 - 69.90)	(0.00 - 8.00)	(0.00 - 0.30)	(0.00 - 0.50)	(0.1 - 5.4)	(0.5 - 1.1)	(0.1 - 0.9)
JG	84.08 ^A	94.51 ^A	77.28 ^A	0.68 ^A	0.32 ^B	0.24 ^B	7.34 ^A	0.73 ^A	2.12 ^B
	(7.30 - 143.00)	(55.20 - 146.40)	(19.60 - 180.50)	(0.10 - 2.40)	(0.20 - 0.60)	(0.00 - 0.89)	(0 - 153.2)	(0.1 - 2.2)	(0.14 - 4.1)
ZW	65.27 ^A	64.89 ^A	69.04 ^A	1.9 ^A	0.83 ^B	0.64 ^B	0.95 ^A	1.3 ^A	1.75 ^A
	(6.80 - 127.50)	(20.20 - 149.40)	(14.50 - 134.60)	(0.10 - 6.70)	(0.20 - 1.30)	(0.00 - 1.40)	(0 - 5.3)	(0.2 - 3.0)	(0.15 - 3.70)
ZZ	64.28 ^A	34.21 ^A	60.86 ^A	0.6 ^A	1.00 ^A	0.08 ^B	1.11 ^A	0.66 ^A	0.53 ^A
	(0.00 - 165.80)	(24.30 - 39.60)	(0.28 - 145.70)	(0.00 - 2.40)	(0.00 - 1.40)	(0.00 - 0.51)	(0 - 5.9)	(0.3 - 0.9)	(0.08 - 1.7)

Table S6. List of all species observed in studied ponds for three years. In cases where genera are listed, it was not possible to assign observed organisms to species.

ARTURÓWEK GÓRNY POND			JULIANÓW GÓRNY POND		
2019	2020	2021	2019	2020	2021
Rotifers	Rotifers	Rotifers	Rotifers	Rotifers	Rotifers
<i>Asplanchna priodonta</i>	<i>Asplanchna priodonta</i>	<i>Asplanchna priodonta</i>	<i>Asplanchna priodonta</i>	<i>Asplanchna priodonta</i>	<i>Asplanchna priodonta</i>
<i>Brachionus calyciflorus</i>	<i>Brachionus</i> sp.	<i>Brachionus calyciflorus</i>	<i>Brachionus calyciflorus</i>	<i>Brachionus</i> sp.	<i>Brachionus quadrientatus</i>
<i>Brachionus</i> sp.	<i>Brachionus urceolaris</i>	<i>Brachionus quadrientatus</i>	<i>Brachionus quadrientatus</i>	<i>Brachionus urceolaris</i>	<i>Brachionus</i> sp.
<i>Brachionus urceolaris</i>	<i>Cephalodella</i> sp.	<i>Brachionus</i> sp.	<i>Brachionus</i> sp.	<i>Cephalodella</i> sp.	<i>Colurella</i> sp.
<i>Cephalodella</i> sp.	<i>Keratella cochlearis</i>	<i>Brachionus urceolaris</i>	<i>Cephalodella</i> sp.	<i>Colurella</i> sp.	<i>Keratella cochlearis</i>
<i>Colurella</i> sp.	<i>Keratella quadrata</i>	<i>Filinia longiseta</i>	<i>Colurella</i> sp.	<i>Filinia longiseta</i>	<i>Lecane</i> sp.
<i>Keratella cochlearis</i>	<i>Keratella tetsudo</i>	<i>Keratella cochlearis</i>	<i>Filinia longiseta</i>	<i>Keratella cochlearis</i>	Cladocerans
<i>Lecane</i> sp.	<i>Lecane</i> sp.	<i>Keratella quadrata</i>	<i>Keratella cochlearis</i>	<i>Keratella quadrata</i>	<i>Daphnia</i> sp.
<i>Lepadella</i> sp.	<i>Lepadella</i> sp.	<i>Lecane</i> sp.	<i>Keratella quadrata</i>	<i>Keratella tetsudo</i>	<i>Alona</i> sp.
<i>Platylabus quadricornis</i>	<i>Polyarthra</i> sp.	<i>Lepadella</i> sp.	<i>Lecane</i> sp.	<i>Lepadella</i> sp.	<i>Bosmina longirostris</i>
<i>Polyarthra</i> sp.	Cladocerans	<i>Polyarthra</i> sp.	<i>Lepadella</i> sp.	<i>Platylabus quadricornis</i>	<i>Chydorus sphaericus</i>
<i>Tetradella</i> sp.	<i>Daphnia</i> sp.	<i>Trichotria tetrachis</i>	<i>Monomnata</i> sp.	Cladocerans	<i>Daphnia longispina</i>
<i>Trichocerca</i> sp.	<i>Bosmina longirostris</i>	Cladocerans	<i>Platylabus quadricornis</i>	<i>Daphnia longispina</i>	<i>Diaphanosoma branchyurum</i>
<i>Trichotria pocillum</i>	<i>Chydorus sphaericus</i>	<i>Daphnia</i> sp.	<i>Polyarthra</i> sp.	<i>Chydorus sphaericus</i>	<i>Pleuroxus</i> sp.
<i>Trichotria tetrachis</i>	<i>Diaphanosoma branchyurum</i>	<i>Bosmina longirostris</i>	Cladocerans	<i>Daphnia</i> sp.	<i>Scapholeberis mucronata</i>
Cladocerans	Copepods	<i>Chydorus sphaericus</i>	<i>Bosmina longirostris</i>	<i>Diaphanosoma branchyurum</i>	Copepods
<i>Alona</i> sp.	<i>Cyclopoida</i>	<i>Diaphanosoma branchyurum</i>	<i>Chydorus sphaericus</i>	<i>Simocephalus ventulus</i>	<i>Cyclopoida</i>
<i>Alonella nana</i>	<i>Nauplii</i>	<i>Pleuroxus</i> sp.	<i>Diaphanosoma branchyurum</i>	Copepods	<i>Nauplii</i>
<i>Alonella</i> sp.		<i>Scapholeberis mucronata</i>	<i>Scapholeberis mucronata</i>	<i>Cyclopoida</i>	
<i>Bosmina longirostris</i>		Copepods	Copepods	<i>Nauplii</i>	
<i>Chydorus sphaericus</i>		<i>Nauplii</i>	<i>Cyclopoida</i>		
<i>Daphnia galeata</i>		<i>Cyclopoida</i>	<i>Nauplii</i>		
<i>Daphnia</i> sp.					
<i>Pleuroxus</i> sp.					
<i>Scapholeberis mucronata</i>					
<i>Simocephalus ventulus</i>					
Copepods					
<i>Cyclopoida</i>					
<i>Nauplii</i>					
ZBIORNIK WASIAKA POND			ZBIORNIK ZGIERSKA POND		
2019	2020	2021	2019	2020	2021
Rotifers	Rotifers	Rotifers	Rotifers	Rotifers	Rotifers
<i>Asplanchna priodonta</i>	<i>Asplanchna priodonta</i>	<i>Brachionus quadrientatus</i>	<i>Asplanchna priodonta</i>	<i>Asplanchna priodonta</i>	<i>Asplanchna priodonta</i>
<i>Brachionus quadrientatus</i>	<i>Cephalodella</i> sp.	<i>Keratella quadrata</i>	<i>Brachionus calyciflorus</i>	<i>Brachionus calyciflorus</i>	<i>Brachionus calyciflorus</i>
<i>Brachionus</i> sp.	<i>Colurella</i> sp.	<i>Lecane</i> sp.	<i>Brachionus diversicornis</i>	<i>Brachionus quadrientatus</i>	<i>Brachionus diversicornis</i>
<i>Brachionus urceolaris</i>	<i>Keratella cochlearis</i>	<i>Lepadella</i> sp.	<i>Brachionus</i> sp.	<i>Brachionus</i> sp.	<i>Brachionus quadrientatus</i>
<i>Cephalodella</i> sp.	<i>Keratella quadrata</i>	<i>Platylabus quadricornis</i>	<i>Brachionus urceolaris</i>	<i>Brachionus urceolaris</i>	<i>Brachionus urceolaris</i>
<i>Colurella</i> sp.	<i>Lecane</i> sp.	Cladocerans	<i>Colurella</i> sp.	<i>Cephalodella</i> sp.	<i>Colurella</i> sp.
<i>Keratella cochlearis</i>	<i>Lepadella</i> sp.	<i>Bosmina longirostris</i>	<i>Filinia longiseta</i>	<i>Filinia longiseta</i>	<i>Filinia longiseta</i>
<i>Keratella quadrata</i>	<i>Platylabus quadricornis</i>	<i>Chydorus sphaericus</i>	<i>Keratella cochlearis</i>	<i>Keratella cochlearis</i>	<i>Keratella cochlearis</i>
<i>Lecane</i> sp.	Cladocerans	Copepods	<i>Keratella quadrata</i>	<i>Keratella quadrata</i>	<i>Keratella quadrata</i>
<i>Lepadella</i> sp.	<i>Bosmina longirostris</i>	<i>Cyclopoida</i>	<i>Lecane</i> sp.	<i>Lecane</i> sp.	<i>Lecane</i> sp.
<i>Mytilina ventralis</i>	<i>Diaphanosoma branchyurum</i>	<i>Nauplii</i>	<i>Lepadella</i> sp.	<i>Lepadella</i> sp.	<i>Lepadella</i> sp.
<i>Platylabus quadricornis</i>	Copepods		<i>Polyarthra</i> sp.	<i>Polyarthra</i> sp.	<i>Polyarthra</i> sp.
<i>Polyarthra</i> sp.	<i>Cyclopoida</i>		Cladocerans	<i>Tetradella</i> sp.	Cladocerans
<i>Trichocerca</i> sp.	<i>Nauplii</i>		<i>Bosmina longirostris</i>	Cladocerans	<i>Alona</i> sp.
Cladocerans			<i>Chydorus sphaericus</i>	<i>Alona</i> sp.	<i>Bosmina longirostris</i>
<i>Bosmina longirostris</i>			<i>Daphnia cucullata</i>	<i>Bosmina longirostris</i>	<i>Chydorus sphaericus</i>
<i>Chydorus sphaericus</i>			<i>Daphnia galeata</i>	<i>Chydorus sphaericus</i>	<i>Diaphanosoma branchyurum</i>
<i>Daphnia</i> sp.			<i>Daphnia longispina</i>	<i>Diaphanosoma branchyurum</i>	<i>Pleuroxus</i> sp.
<i>Diaphanosoma branchyurum</i>			<i>Daphnia</i> sp.	<i>Pleuroxus</i> sp.	Copepods
Copepods			<i>Diaphanosoma branchyurum</i>	<i>Scapholeberis mucronata</i>	<i>Cyclopoida</i>
<i>Cyclopoida</i>			<i>Leptodora kindtii</i>	Copepods	<i>Nauplii</i>
<i>Nauplii</i>			<i>Pleuroxus</i> sp.	<i>Cyclopoida</i>	
			<i>Scapholeberis mucronata</i>	<i>Nauplii</i>	
			Copepods		
			<i>Cyclopoida</i>		
			<i>Nauplii</i>		

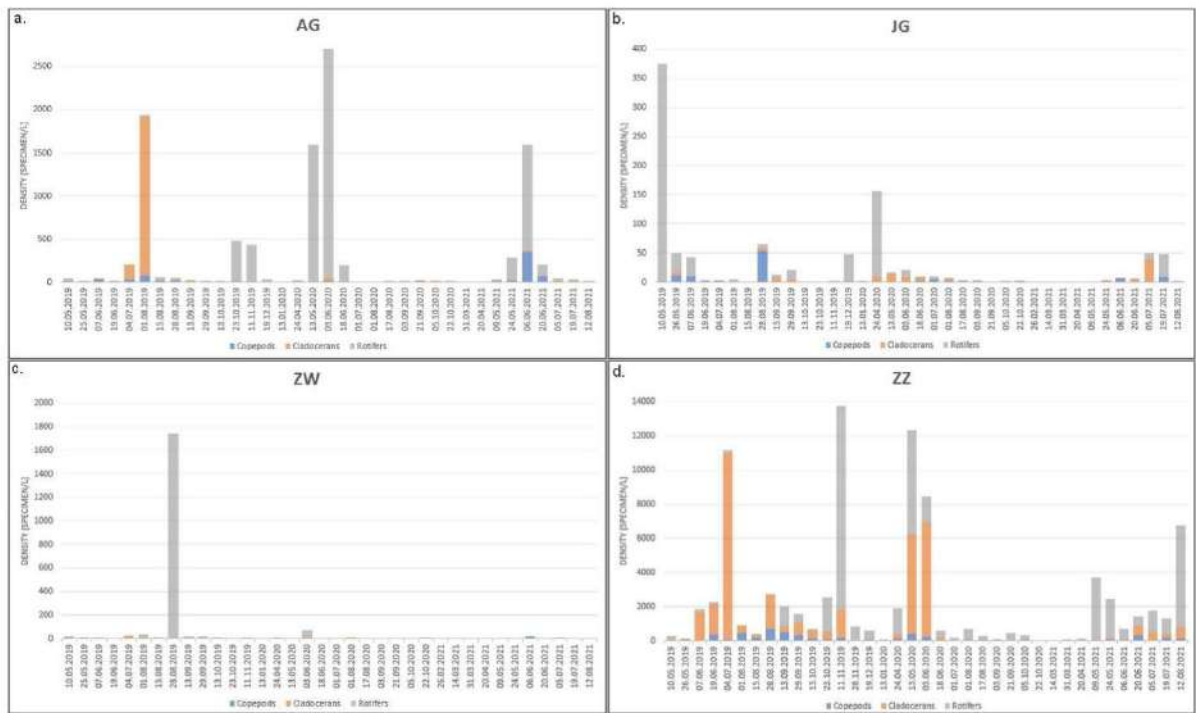


Fig. S3. Dynamics of density change (specimen/L) of adult copepods, cladocerans and rotifers observed in the AG (a), JG (b), ZW (c) and ZZ (d) ponds during the three-year study period.

Table S7. Biodiversity indexes measured for total zooplankton (including cladocerans, copepods and rotifers), in the studied ponds during the three-year period. Yellow highlight indicates the highest value for each index across years and reservoirs.

	2019				2020				2021			
	Total zooplankton											
	AG	JG	ZW	ZZ	AG	JG	ZW	ZZ	AG	JG	ZW	ZZ
Taxa_S	27	19	20	23	14	19	11	20	20	15	11	18
Dominance_D	0.4	0.26	0.68	0.31	0.50	0.39	0.45	0.29	0.45	0.23	0.32	0.42
Simpson_1-D	0.60	0.74	0.32	0.69	0.50	0.61	0.55	0.71	0.55	0.58	0.68	0.58
Shannon_H'	1.313	1.722	0.8396	1.572	0.9015	1.513	1.341	1.597	1.228	1.456	1.634	1.456
	Cladocerans											
Taxa_S	10	4	4	10	4	5	2	6	6	8	2	5
Dominance_D	0.93	0.46	0.42	0.81	0.60	0.39	0.51	0.89	0.32	0.61	0.72	0.48
Simpson_1-D	0.07	0.54	0.58	0.18	0.39	0.61	0.49	0.11	0.68	0.39	0.28	0.52
Shannon_H'	0.2026	1.01	1.101	0.4858	0.7411	1.156	0.687	0.2259	1.249	0.8687	0.4506	0.7893
	Rotifers											
Taxa_S	16	14	15	12	9	13	8	13	13	6	8	12
Dominance_D	0.53	0.35	0.72	0.54	0.52	0.65	0.66	0.28	0.70	0.42	0.21	0.58
Simpson_1-D	0.47	0.65	0.28	0.46	0.48	0.35	0.34	0.72	0.30	0.58	0.22	0.42
Shannon_H'	1.035	1.354	0.6901	1.002	0.798	0.7981	0.8268	1.481	0.7115	1.09	0.7844	1.001

Biodiversity Indexes

The results were obtained using the Past 4.03. software. Total zooplankton includes cladocerans, rotifers and adult copepods. Cladocerans and rotifers section include the values of the indexes only for those two groups.

The highest number of species was observed in the year 2019 in the reference pond AG and reached 27 species, including 10 species of cladocerans and 16 species of rotifers. The number of species in this pond decreased during the year with warm winter 2020 (to 14 species) and then increased in 2021 (to 20). ZZ pond had a similar number of observed species in 2019 – 23 (10 species of cladocerans and 12 of rotifers). The number of species in this pond decreased slightly in 2020 and 2021. Two ponds with the lowest number of species were JG and ZW with 19 and 20 species respectively, and this number also decreased in the year 2020 and 2021. Pond ZW in 2020 and 2021 had also the lowest number of observed cladocerans species, which was only two.

The index of dominance (D) varied between the years and ponds. In 2019, only the ZW pond showed moderate dominance when it comes to total zooplankton. However, this year, high dominance among cladocerans was observed in AG (0.93) and ZZ (0.81) pond. For rotifers, the dominance was moderate in AG, ZW and ZZ ponds, with no species dominating in JG pond. In the year 2020, in all of the ponds there was no species dominance for total zooplankton. Moderate dominance was observed among cladocerans in AG and JG ponds, and high dominance was observed in ZZ pond (0.89). AG, JG and ZW showed moderate dominance among rotifers. In the year 2021, no species dominance was observed for total zooplankton. JG and ZW showed moderate dominance among cladocerans and AG and ZZ showed moderate dominance among rotifers.

The Simpson index showed moderate diversity for total zooplankton for AG, JG and ZZ ponds in the year 2019, then for JG, ZW and ZZ ponds in 2020 and for all of the reservoirs during the year 2021. For cladocerans the Simpson index remained quite low, reaching the highest value in the JG pond during 2019. For the rotifers, the highest value was reached in the ZZ pond in 2020, which indicated moderate diversity.

Similarly, the Shannon Wiener Diversity Index (H') remained low in all of the ponds, during all the years. It reached the highest value in the JG pond in 2019 for total zooplankton, in the AG pond in 2021 for cladocerans and in the ZZ pond in 2020 for the rotifers.

In general, all of the urban ponds examined in the study showed low to moderate diversity, which varied slightly throughout the three years study period. Diversity in the ponds exposed to higher chloride concentrations (JG, ZW and ZZ) were similar to those measured for the reference pond (AG). During 2019 and 2020 high dominance was observed within the cladocerans in the reference pond AG and ZZ pond.

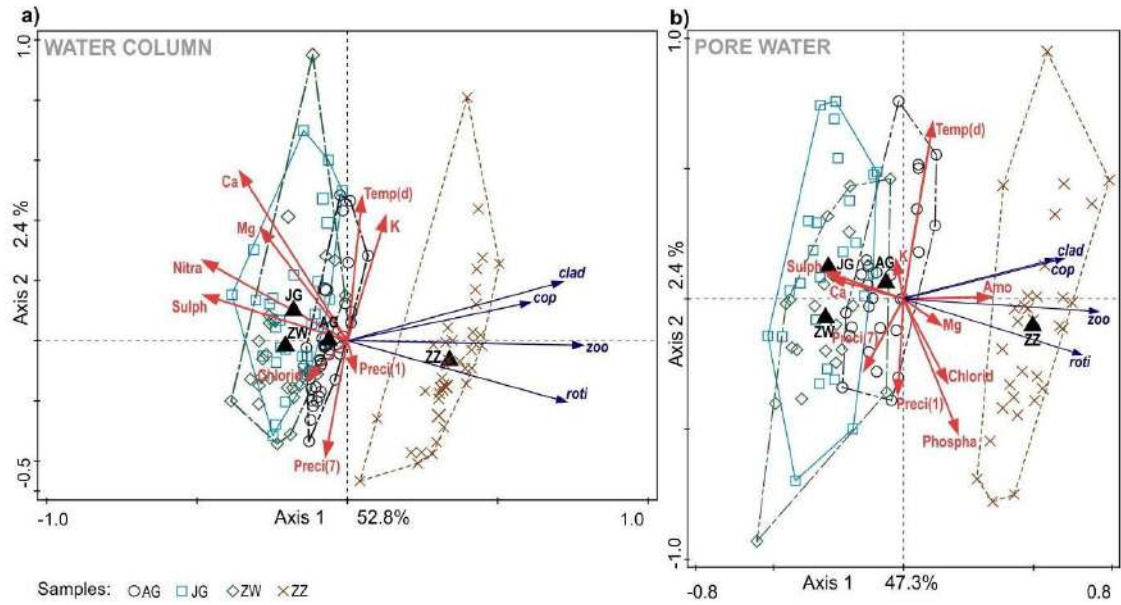


Fig. S4. Association of zooplankton, environmental variables, and POND variable for column water samples and pore water samples. Correlation plots obtained by redundancy analysis (RDA) using the entire data set. ▲, nominal variables (Pond AG, Pond JG, Pond ZW, Pond ZZ).

Table S8. Ranking of the environmental variables appear to influence the density of plankton, based on their marginal and conditional effects on ecological factors; analysis for column water samples.

Variables	λ_1	λ_A	P
Pond ZZ	44.3	44.33	0.001
Nitra	13.0	0.13	0.756
Sulph	12.8	0.29	0.453
Pond ZW	9.9	0.23	-
Pond JG	9.1	0.23	0.572
Ca	7.9	0.42	0.312
Mg	5.9	0.87	0.088
K	1.4	2.01	0.009
Pond AG	1.3	2.80	0.001
Chlorid	1.2	3.51	0.001
Temp (d)	0.9	1.05	0.059
Precip (7)	0.7	0.13	0.765
Precip (1)	0.1	0.06	0.903
λ_1 = fit = eigenvalue with only variable j; λ_A = additional fit = change in eigenvalue; P = significance level of the effect, obtained through Monte Carlo permutations under the full model with 999 permutations.			

Table S9. Summary of variation partitioning using partial RDA of plankton assemblages, with environmental variables and space (pond) as predictors; analysis for column water samples.							
Fraction	Variation	% of Expl	% of All	Mean square	df	F_λ	p
Pond	0.31971	62.3	32.0	0.10296	3	27.7	0.001
Environment	0.05531	10.8	5.5	0.00972	9	2.6	0.002
Pond + Environment	0.13856	27.0	13.9	-	-	-	-
Total Explained	0.51359	100	51.4	0.04651	12	12.5	0.001
All Variation	1	-	100.0	-	131	-	-
Variation – sum of all canonical eigenvalues; % of Expl – contribution of the fraction to the explained variation; % of All– contribution of the fraction to the total variation; Mean square –amount of variation explained in the group of variables per single degree of freedom, i.e. per one constrained axis; df - degrees of freedom; F_λ – the F-ratio statistics for the test on all canonical axis; p – corresponding probability value obtained by the Monte Carlo permutation test, 999 random permutations.							

Table S10. Ranking of the environmental variables appear to influence the density of plankton, based on their marginal and conditional effects on ecological factors; analysis for pore water samples.

Variables	λ_1	λ_A	P
Pond ZZ	36.5	36.47	0.001
Pond JG	9.3	0.19	-
Pond ZW	8.0	0.19	0.759
Amo	5.4	0.54	0.381
Sulph	4.4	0.58	0.327
Ca	3.8	0.39	0.513
Phospha	2.7	0.60	0.337
Temp (d)	1.8	3.19	0.013
Chlorid	1.6	0.23	0.713
Precip (7)	1.3	1.61	0.056
Mg	1.2	0.23	0.714
Pond AG	0.8	2.88	0.004
precip (1)	0.4	0.26	0.675
K	0.2	0.03	0.983

λ_1 = fit = eigenvalue with only variable j; λ_A = additional fit = change in eigenvalue; P = significance level of the effect, obtained through Monte Carlo permutations under the full model with 999 permutations.

Table S11. Summary of variation partitioning using partial RDA of plankton assemblages, with environmental variables and space (pond) as predictors; analysis for pore water samples.

Fraction	Variation	% of Expl	% of All	Mean square	df	F_λ	p
Pond	0.31034	75.0	31.0	0.0991	3	17.4	0.001
Environment	0.01397	3.4	1.4	0.0071	10	1.2	0.213
Pond + Environment	0.08923	21.6	8.9	-	-	-	-
Total Explained	0.41354	100.0	41.4	0.0375	13	6.6	0.001
All Variation	1	-	100.0	-	103	-	-

Variation – sum of all canonical eigenvalues; % of Expl – contribution of the fraction to the explained variation; % of All – contribution of the fraction to the total variation; Mean square – amount of variation explained in the group of variables per single degree of freedom, i.e. per one constrained axis; df – degrees of freedom; F_λ – the F-ratio statistics for the test on all canonical axis; p – corresponding probability value obtained by the Monte Carlo permutation test, 999 random permutations.

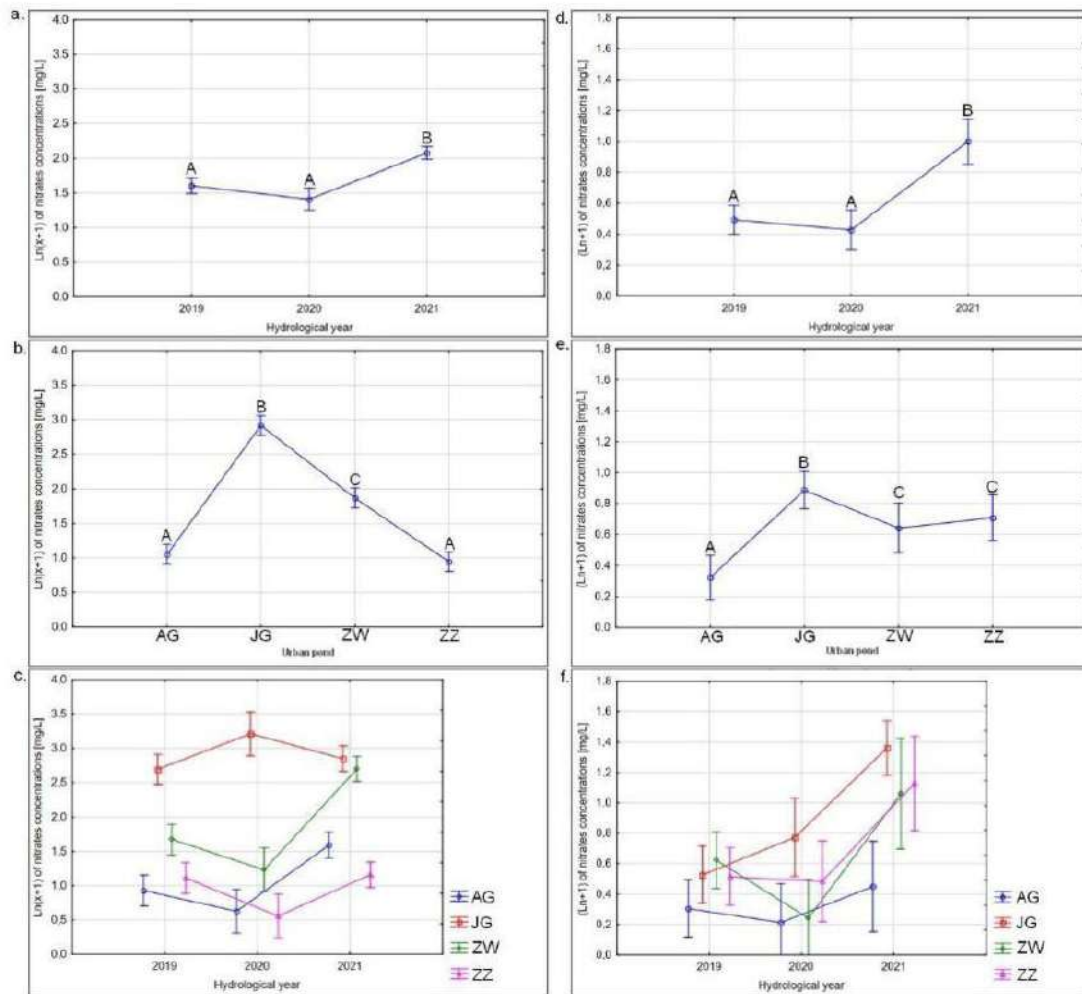


Fig. S5. Nitrate concentration ($\ln(x+1)$ mg/L) measured in the column water (a, b) and pore water (d, e) of each pond and each year (c, f). Vertical bars represent 0.95 confidence intervals. Different capital letters indicate significant differences.

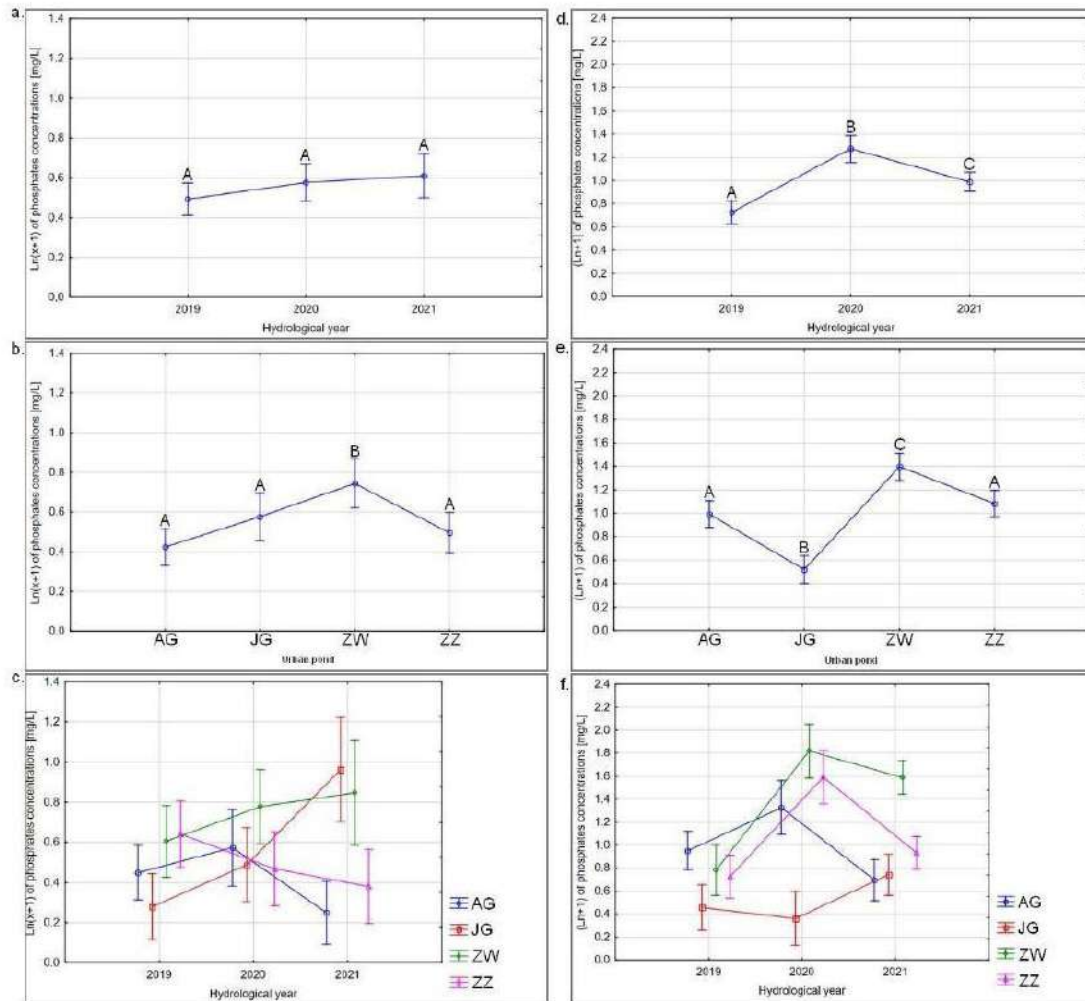


Fig. S6. Phosphate concentration (Ln(x+1) mg/L) measured in the column water (a, b) and pore water (d, e) of each pond and each year (c, f). Vertical bars represent 0.95 confidence intervals. Different capital letters indicate significant differences.

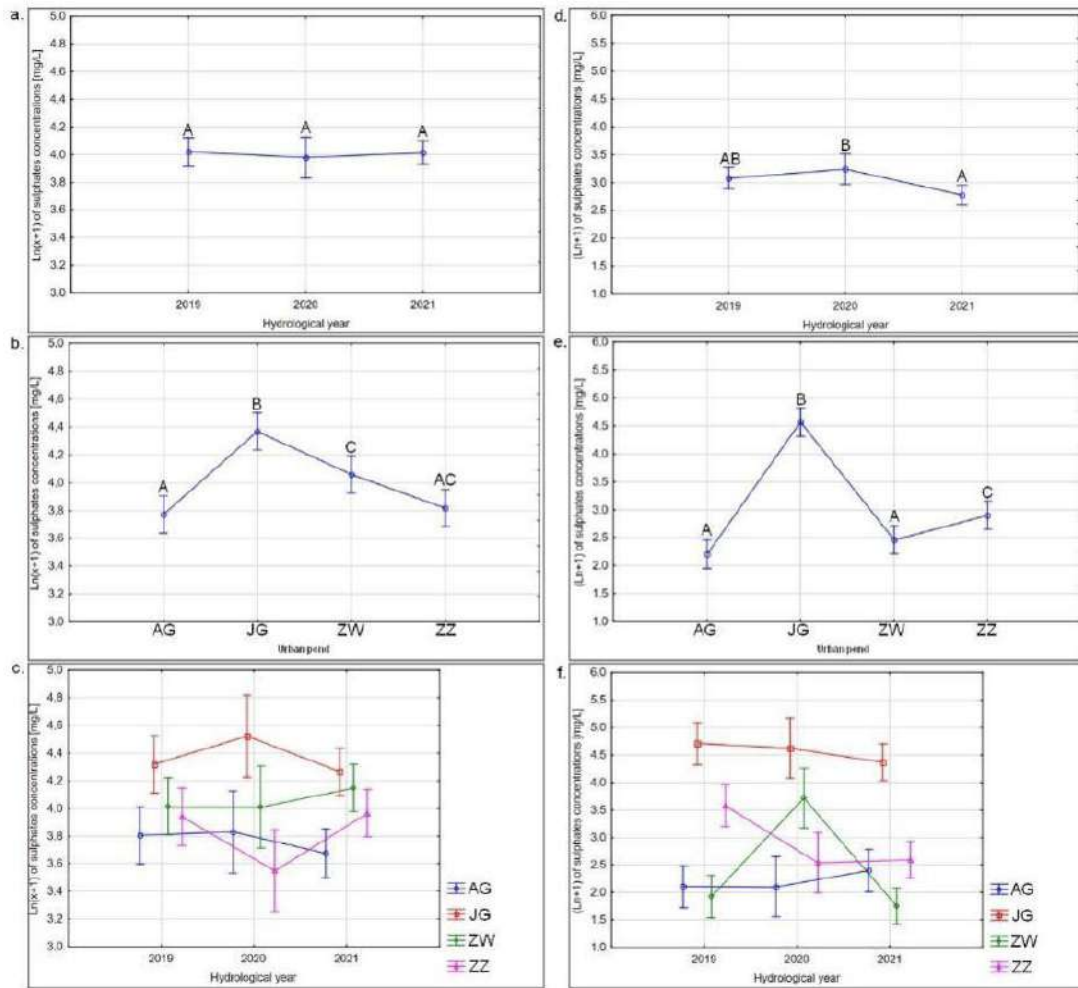


Fig. S7. Sulphate concentration (ln(x+1) mg/L) measured in the column water (a, b) and pore water (d, e) of each pond and each year (c, f). Vertical bars represent 0.95 confidence intervals. Different capital letters indicate significant differences.

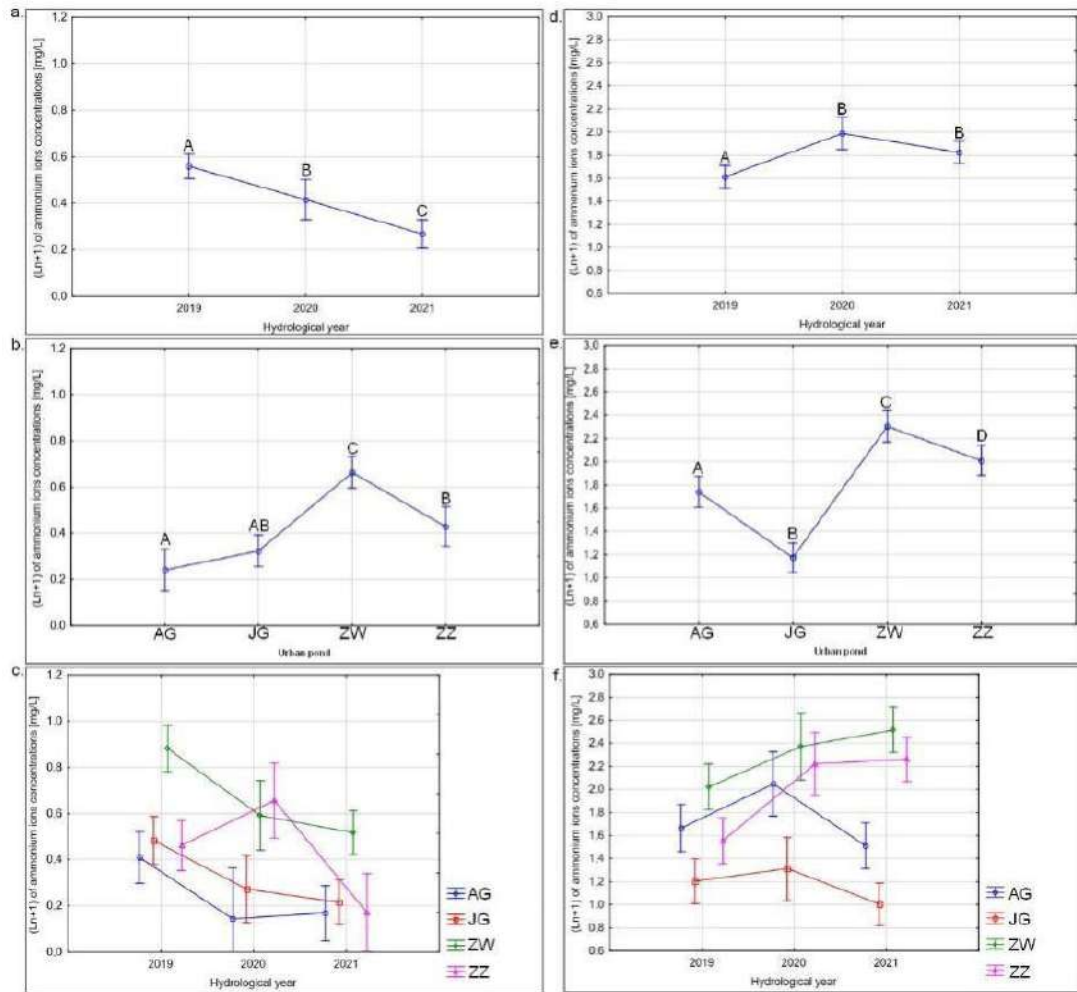


Fig. S8. Ammonium concentration ($\ln(x+1)$ mg/L) measured in the column water (a, b) and pore water (d, e) of each pond and each year (c, f). Vertical bars represent 0.95 confidence intervals. Different capital letters indicate significant differences.

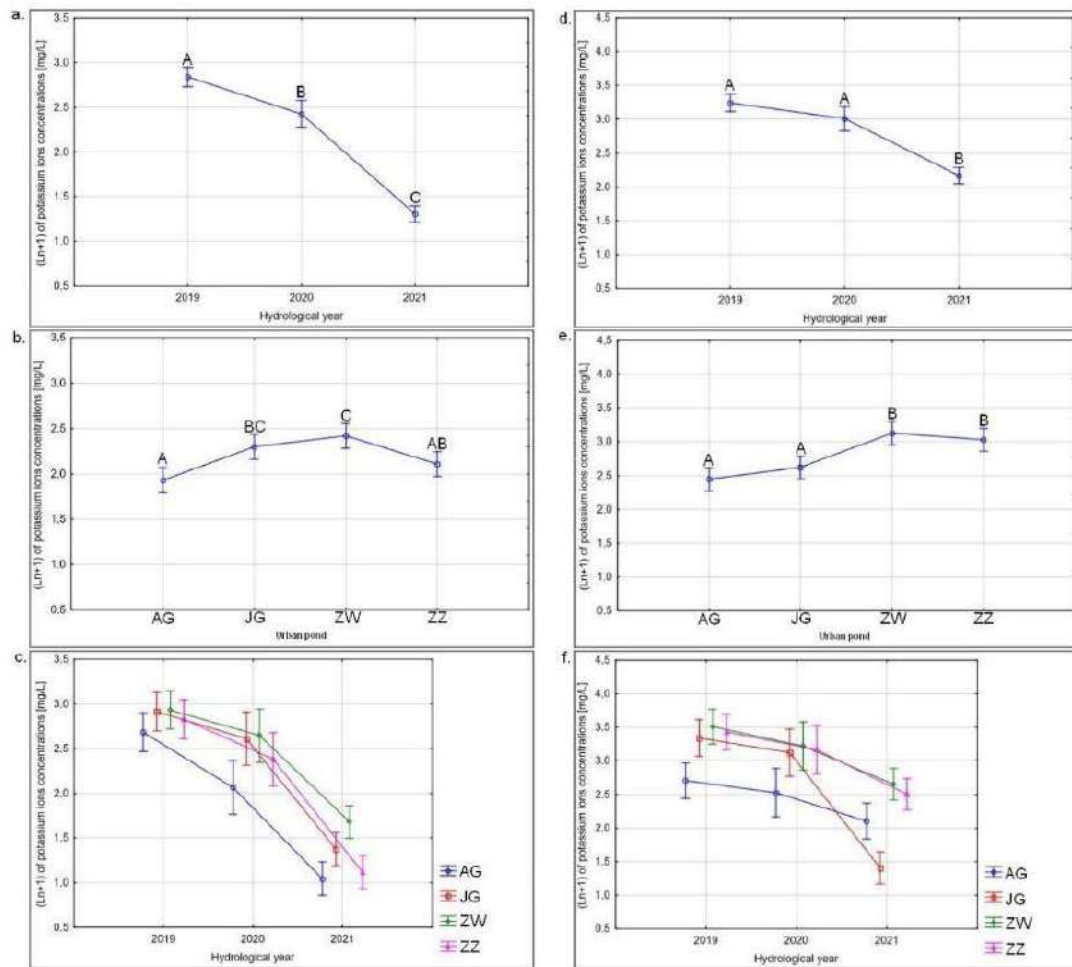


Fig. S9. Potassium concentration (ln(x+1) mg/L) measured in the column water (a, b) and pore water (d, e) of each pond and each year (c, f). Vertical bars represent 0.95 confidence intervals. Different capital letters indicate significant differences.

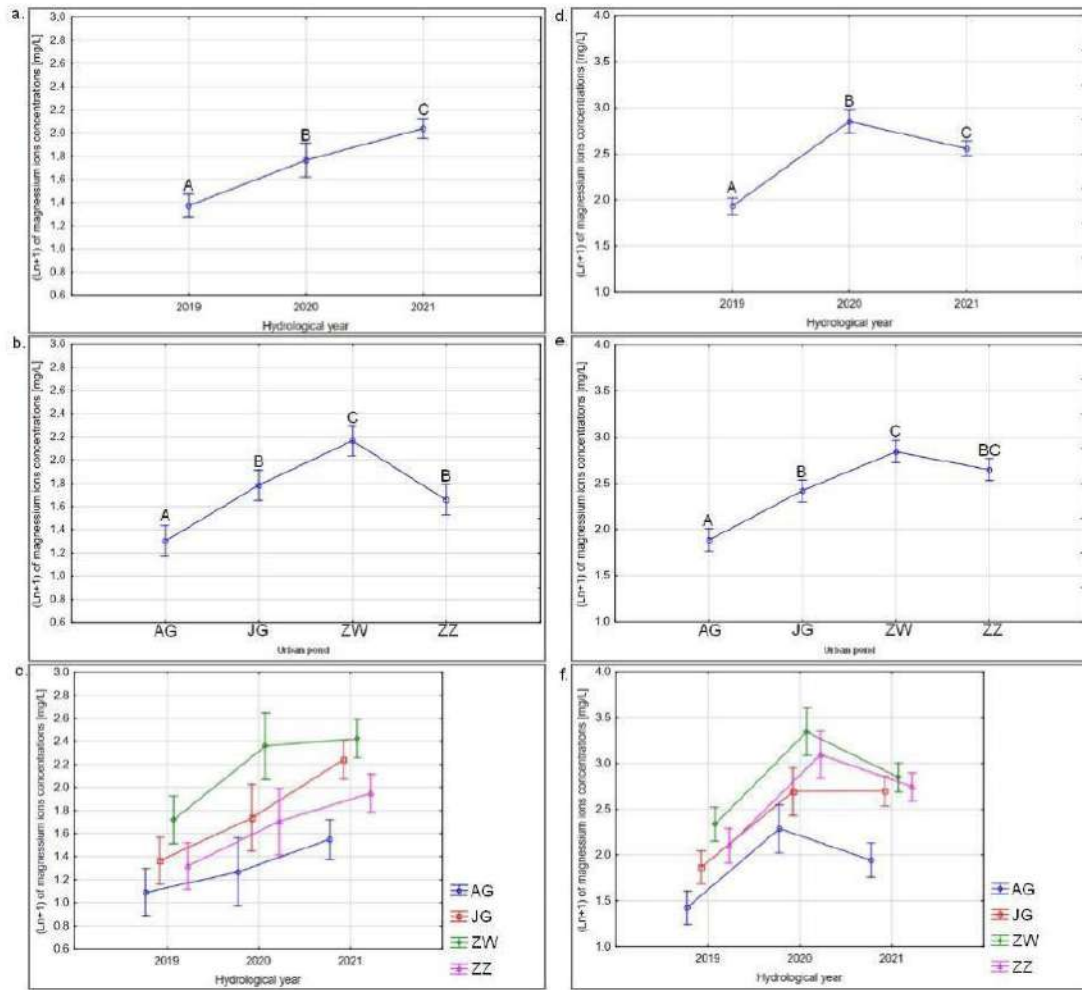


Fig. S10. Magnesium concentration (ln(x+1) mg/L) measured in the column water (a, b) and pore water (d, e) of each pond and each year (c, f). Vertical bars represent 0.95 confidence intervals. Different capital letters indicate significant differences.

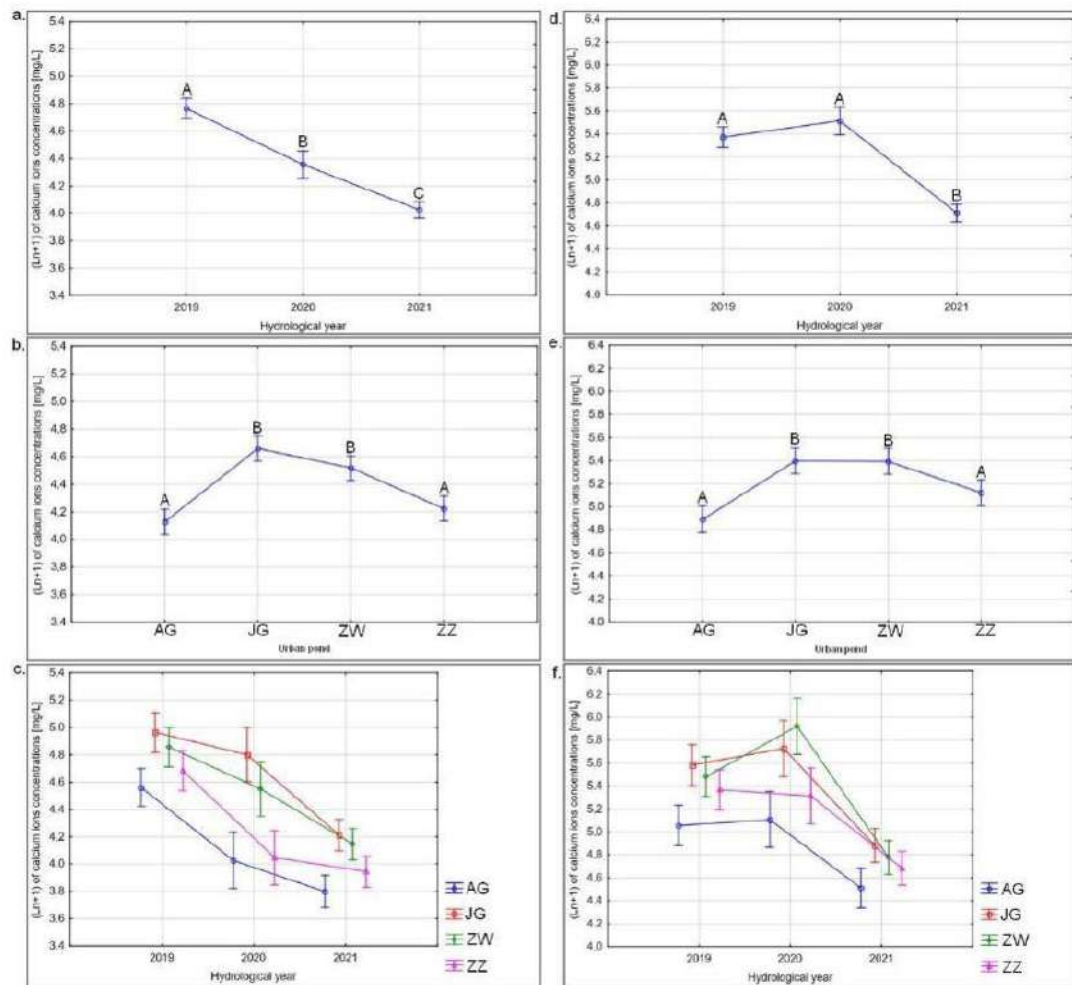
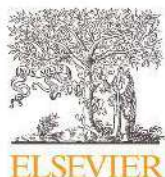


Fig. S11. Calcium concentration ($\ln(x+1)$ mg/L) measured in the column water (a, b) and pore water (d, e) of each pond and each year (c, f). Vertical bars represent 0.95 confidence intervals. Different capital letters indicate significant differences.

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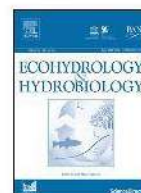
Szklarek S., **Górecka A.**, Salabert B., Wojtal-Frankiewicz A.; *Acute toxicity of seven de-icing salts on four zooplankton species – is there an “eco-friendly” alternative?*; Ecohydrology & Hydrobiology; 2022; 22(4):589-597. DOI: 10.1016/j.ecohyd.2022.08.005.

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Original Research Article

Acute toxicity of seven de-icing salts on four zooplankton species– is there an “eco-friendly” alternative?

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ABSTRACT

While de-icing salts improve safety on roads and sidewalks during winter, they have a negative impact on the environment. This has spurred the search for more environmentally-friendly alternatives. The present study tests the impact of NaCl and six other de-icing salts (pure CaCl₂, CaCl₂+NaCl, “eco-label” CaCl₂, MgCl₂, solid and liquid CH₃COOK), promoted as more eco-friendly alternatives to NaCl, on four zooplankton species from biotests: *Ceriodaphnia dubia* (Ceriodaphtokit), *Daphnia magna* (Daphtoxkit), *Brachionus calyciflorus* (Rottoxkit) and *Thamnocephalus platyurus* (Thamnotoxkit). 24-h EC₅₀ evaluation showed that only solid CH₃COOK was less toxic for all tested species than NaCl. The other tested de-icing salts had similar or even higher toxicity, with CaCl₂, MgCl₂ and liquid CH₃COOK being significantly more toxic for all species than NaCl. Of the tested species, *Thamnocephalus platyurus* demonstrated toxicity response at significantly lower concentration of all used de-icing salts, while *Daphnia magna* had significantly the highest concentrations of toxicity response. Our findings demonstrate that these alternatives are not less toxic in this regard than the commonly-used NaCl and underline the need to verify the accuracy of the term “eco-friendly” on the labels of these products.

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1. Introduction

In many countries, freshwater areas are increasingly subject to salinity input from anthropogenic sources, such as agriculture, mining effluents, industry effluents and urbanization. Indeed, such trends in salt ion concentrations have been observed in aquatic ecosystems in the USA (Corsi et al., 2015; Dugan et al., 2017), Canada (Lembcke et al., 2017), many European countries (e.g. Löfgren, 2001; Salminen et al., 2011; Müller and

Gächter, 2012; Espinha Marques et al., 2021; Niedrist et al., 2021), China (Yu et al., 2014; Zhao et al., 2021) and Australia (Shenton et al., 2022). The salinity level of an area depends on a range of climate, topography, geology, hydrology and catchment characteristics as well as the nature of any anthropogenic sources. In cold regions, one of the main freshwater salinization sources is road salt, commonly used in winter for de-icing roads (Cañedo-Argüelles et al., 2013; Cunillera-Montcusí et al., 2022; Kaushal et al., 2021).

The complex interrelationship between salt ions and chemical, biological and geological parameters, and their environmental and socio-economic consequences, have

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been named Freshwater Salinity Syndrome (FSS). Long-term studies indicate that without urgent remedial action, FSS will continue to increase the salinity of freshwater ecosystems, groundwater and soils at local, regional and global scales, and alter their ionic composition (Kaushal et al., 2021). Despite this, standards for chloride ion concentrations in freshwater ecosystems, i.e. the most standard chemical determinant of salinity, have not generally been established in European countries (Schuler et al., 2018), with a few exceptions, such as Germany and Sweden (Schulz and Cañedo-Argüelles, 2019). In addition, research indicates that chloride concentration limits set in the US and Canada do not sufficiently protect lake food webs. Research to revise existing standards or to establish new legal guidelines is therefore urgently needed to protect freshwater ecosystems from human-induced salinisation (Hintz et al., 2022).

The combined effect of increasing eutrophication and salinization on freshwater ecosystems promotes the formation of large blooms of phytoplankton and periphyton, and the decline of many species of invertebrates and macrophytes (Delaune et al., 2021; Gillis et al., 2022; Honarvar Nazari et al., 2021; Kaushal et al., 2021; Lind et al., 2018; Szklarek et al., 2022). A particularly salinity-sensitive group of organisms is the zooplankton. According to published studies, elevated road salt concentrations cause a decrease in zooplankton reproduction and an increase in mortality, resulting in an overall decrease in zooplankton density (Arnott et al., 2020; Hintz and Relyea, 2017). Furthermore, salinity stress affects planktivore-zooplankton interactions: road salt and predation stress have additive negative effects on the abundance of *Daphnia* (Hintz et al., 2017), and at higher salinities, changes are observed in daphnid life history and behavioural responses to predation (Liu and Steiner, 2017).

As zooplankton play an important role in top-down processes in lakes and other standing freshwaters, their sensitivity to increasing salinity due to various causes such as the use of road salt, may cause disturbances in the trophic cascade. The use of de-icing salts therefore induces a number of direct and indirect effects in the biotic structure, which may alter ecosystem functioning and ecosystem services (Jones et al., 2017; Hintz and Relyea, 2017; Schuler et al., 2017). Consequently, in an era of excessive use of de-icing agents and salinisation of freshwater ecosystems, detailed studies are needed on the complex effects of road salts on the quantitative and qualitative structure of zooplankton communities. Furthermore, as many zooplankton demonstrate species-specific responses to stressors, studies on the responses to elevated concentrations of de-icing salts should include a range of zooplankton genera, rather than focusing on the model genus *Daphnia*.

Despite being the most commonly-used de-icing salt, sodium chloride (NaCl) is responsible for a number of negative effects on the environment around the application site (road, car and infrastructure corrosion, deteriorating vegetation), and alternative road salts are currently sought. However, while it is assumed that these new salts will be less harmful to the human infrastructure and vegetation near the road, their impact on water ecosystems

is rarely considered in studies, if at all. A few have investigated the impact on freshwater organisms of alternative de-icing salts, particularly those based on magnesium and calcium chloride ($MgCl_2$ and $CaCl_2$). Their findings suggest that NaCl alternatives could have a similar or higher toxicity on freshwater species than NaCl (Lee et al., 2017; Nutile and Solan, 2019; Schuler and Relyea, 2018) and are usually more expensive (Achkeeva et al., 2015).

Consequently, little is known about the effects of new de-icing salts promoted as eco-friendly alternatives to NaCl, many of which have been put into use without prior studies of their environmental impact, especially on the freshwater ecosystem. In addition, laboratory studies published to date have tested the effects of pure chemical salts on zooplankton organisms, rather than commercial de-icing salts, which may have a different toxic response; in addition, these studies usually used in-house cultures, which varied genetically from laboratory to laboratory (e.g., Mount et al., 1997; Elphick et al., 2011; Mount et al., 2016). To fill this gap, the aim of this study was to compare the 24-hour toxicity – effective concentration (24-h EC_{50}) of seven different de-icing salts, viz. NaCl and six other commercial de-icing salts promoted as eco-friendly alternatives to NaCl, on four pure laboratory lines of zooplankton species: *Daphnia magna*, *Ceriodaphnia dubia*, *Brachionus calyciflorus* and *Thamnocephalus platyurus* delivered by the Microbiotest company.

2. Materials and Methods

2.1. De-icing salts

Seven different de-icing salts commercially available in Poland were used in the study (Table 1, more info about manufacturer is given in Table S1). The first was sodium chloride (NaCl), the most commonly-used salt worldwide, with an added anti-caking agent. The next three contained calcium chloride ($CaCl_2$): pure $CaCl_2$, $CaCl_2$ mixed with NaCl and corrosion inhibitor, and $CaCl_2$ with an eco-label, also containing a corrosion inhibitor and pH regulator. The fifth salt was based on magnesium chloride ($MgCl_2$), and the last two were based on potassium formate (CH_3COOK); these were sold in two states of aggregation: solid and liquid.

Additionally, the cost (in Euro - €) of removing the ice and snow from 100 m² was calculated. This calculation was based on the average efficiency given by the producer up to -10°C, and the price in Poland per one kilogram of deicer.

The highest concentration of NaCl used in the biotest was set at 15 200 mg/L, which corresponds to the chloride concentrations reported in previous studies in urban areas (Szklarek et al., 2018, 2015) and the highest Cl concentration noted in other field studies (Gallagher et al., 2011; Mayer et al., 2011; Prosser et al., 2017). Therefore, each de-icing salt was diluted in Standard Freshwater (included in each test) to a concentration of 15 200 mg/L and then sent to a certified commercial laboratory for analysis using the methods presented in Table S2. In addition to the ion content of the salts, such as chlorides, sodium, cal-

Table 1

Characteristics of the de-icing reagents used in the study.

The main ingredient of the de-icing salt	Brand name (in Polish)	Other substances	The cost (€) of use on 100 m ² (based on average efficiency from producer of de-icing salt)	De-icing efficiency according to manufacturers' information [m ² /kg]
NaCl	Road salt (<i>sól drogowa</i>)	anti-caking agent	74.0	5.4
CaCl ₂	DONSOL	n.d.	119.7	30.0
NaCl + CaCl ₂	Road DONSOL (Donsol drogowy)	corrosion inhibitor	113.6	20.0
MgCl ₂ (MgCl ₂ ·6H ₂ O)	DONSOL Magnesium (Donsol Magnez)	n.d.	125.0	20.0
CaCl ₂ ("eco label")	Icebreaker (<i>Lodolamacz</i>)	corrosion inhibitor + pH regulator (ammonium phosphate - (NH ₄) ₂ HPO ₄)	161.2	30.0
CH ₃ COOK (solid)	IceGO solid (IceGO granulki)	n.d.	332.4	22.2
CH ₃ COOK (liquid)	IceGO liquid (IceGO granulki)	n.d.	186.5	27.8

n.d. – no data

Table 2Description of the microbiotest applied for assessment the 24-h EC₅₀ of tested de-icing salts reagents.

Test	Test Biota	Species	International Standards and Guidelines	Temperature of incubation
Ceriodaphtokit	Crustacea (Cladocera)	<i>Ceriodaphnia dubia</i>	US EPA 2002.0	25°C
Daphtokit	Crustacea (Cladocera)	<i>Daphnia magna</i>	ISO 6341; OECD 202; OECD 211; US EPA 2021.0	20°C
Rotokit	Rotifera	<i>Brachionus calyciflorus</i>	ISO 19827; ISO 20666	25°C
Thamnotokit	Crustacea (Branchiopoda)	<i>Thamnocephalus platyurus</i>	ISO 14380	25°C

cium, potassium and magnesium, the de-icing salts were also tested for compounds that might be present in the de-icing salts, such as the anti-caking agents sodium ferrocyanide and iron ferrocyanide (Pandolfo et al., 2012). They were also tested for the presence of any anti-corrosion inhibitors. In addition to these analysed chemicals, our study also included basic ions (Cl, Na, K, Mg and Ca) at threefold, fourfold and fivefold dilutions (25.00%, 12.50% and 6.25%) of the initial concentration (15 200 mg of salts per L); the correctness of the prepared dilutions was confirmed using a Dionex® ion chromatograph with a CG18 cation column (IonPac CS18, CSRS-ULTRA II) and an AG22 anion column (IonPac AS22, ASRS e ULTRA II). The test was only started if those results did not differ from the assumed dilution series by more than ± 5%.

2.2. Toxicity tests

Four different zooplankton species were used in the study: *Ceriodaphnia dubia* (Richard, 1894), *Daphnia magna* (Straus, 1820), *Brachionus calyciflorus* (Pallas, 1766) and *Thamnocephalus platyurus* (Packard, 1877). All tests were performed according to the MicroBioTests Inc. protocols (Ceriodaphtokit, Daphtokit, Rotokit and Thamnotokit, respectively) which meet international standards and guidelines (Table 2).

The first step in toxicity assessment was the range finding test. In accordance with the test protocols, individuals were exposed for 24 hours to a dilution series of de-icers, starting from 15 200 mg/L (100.00%) followed by four more concentrations: 7 600 mg/L (50.00%), 3 800 mg/L (25.00%), 1 900 mg/L (12.50%) and 950 mg/L (6.25%). In the

second step, a definitive test was performed to determine the 10% and 50% effect concentration (24-h EC₁₀ and 24-h EC₅₀). Dilution series were prepared, with the values ranging from the lowest dilution producing a 100 % effect to the highest one producing less than 10 % effect, as identified in the range finding test (first step). In most cases, this range was from 7 600 mg/L (50.00%) to 1 900 mg/L (12.50%). However, a more concentrated solution had to be prepared for the CH₃COOK (solid) de-icer, because it did not demonstrate a 100% effect, even at the highest concentration (15 200 mg/L). The maximum concentration was doubled to 30 400 mg/L, with the lower concentrations being 24 320 mg/L, 21 280 mg/L and 18 240 mg/L.

According to the test protocols, standard freshwater was aerated for 15 minutes by bubbling air through a tube connected to an aquarium air pump. Following this, the dilutions of the de-icing salt given above were prepared and these were incubated for two hours to reach the temperature demand by each test protocol (Table 3). For all prepared dilutions, the dissolved oxygen, pH and electrical conductivity were measured in a volumetric flask using a YSI Professional Plus handheld multiparameter meter before test (T0) and after 24-hours incubation (T24).

After a 24-hour incubation, the numbers of dead and immobilized neonates were compared with those of the actively-swimming test organisms in each well. The immobilized neonates were considered to be those not able to swim after gentle agitation of the liquid for 15 seconds, even if they could still move their antennae. According to the test protocol, the results were valid if the number of dead + immobile organisms did not exceed 10 % in the controls; this condition was met in all tests.

Table 3

Chemical results of de-icing salts (for the concentration 15.2 g of salt per one liter). Value +/- is the expanded measurement uncertainty – based on the standard uncertainty multiplied by the factor $k = 2$, providing a confidence level of approximately 95%.

Analyzed parameter	NaCl	CaCl ₂	NaCl+ CaCl ₂	MgCl ₂ (MgCl ₂ ·x6H ₂ O)	CaCl ₂ ("eco label")	CH ₃ COOK (solid)	CH ₃ COOK (liquid)	Unit
Chrome (Cr)	< 4.0	< 4.0	< 4.0	< 4.0	< 4.0	< 4.0	< 4.0	µg/L
Zinc (Zn)	0.67 (±0.07)	0.63 (±0.07)	0.54 (±0.006)	0.96 (±0.10)	0.61 (±0.07)	0.53 (±0.06)	0.61 (±0.07)	mg/L
Sodium (Na)	5 930 (±600)	< 1.0	3 720 (±380)	37.5 (±3.8)	178 (±18)	< 1.0	10.2 (±1.1)	mg/L
Magnesium (Mg)	< 2.0	< 2.0	< 2.0	1 840 (±370)	< 2.0	< 2.0	< 2.0	mg/L
Potassium (K)	< 10.0	< 10.0	< 10.0	45.8 (±9.2)	40.2 (±8.1)	< 10.0	1 890 (±380)	mg/L
Calcium (Ca)	24.5(±3.2)	4 920(±487)	1 350 (±183)	19.5 (±1.4)	4 270 (±453)	28.2 (±3.6)	9.0 (±0.5)	mg/L
Iron (Fe)	< 60.0	< 60.0	< 60.0	< 60.0	< 60.0	< 60.0	< 60.0	µg/L
Arsenic (As)	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	µg/L
Barium (Ba)	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	mg/L
Sulphates (SO ₄ ²⁻)	143 (±22)	< 2.0	74.3 (±11.2)	30.0 (±4.5)	< 2.0	< 2.0	< 2.0	mg/L
Chlorides (Cl ⁻)	9 120 (±1 370)	9 050 (±1 360)	8 240 (±1 240)	5 590 (±840)	8 110 (±1 220)	< 2.0	29.0 (±5.8)	mg/L
Nitrates (NO ₃ ⁻)	< 4.5	< 4.5	< 4.5	< 4.5	< 4.5	< 4.5	< 4.5	mg/L
Bound cyanides	190 (±48)	< 15	< 15	< 15	< 15	< 15	< 15	µg/L
Free cyanides	< 15	< 15	< 15	< 15	< 15	< 15	< 15	µg/L
Bismuth	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	mg/L
pH	7.32	7.62	7.58	7.95	7.72	7.63	7.88	-
Electrical conductivity	29 800	30 900	31 900	22 700	40 500	513	5 200	µS/cm

Following this, the 24-h EC₅₀ and EC₁₀ values for each salt and zooplankton species were determined by the Reg-tox macro in Excel delivered by the company (author Eric Vindimian – available online: http://www.normalesup.org/~vindimian/en_index.html). This method gives the 24-h EC₅₀ and EC₁₀ value with ± 95% confidence intervals. To evaluate the toxicity response of each species to de-icing salts, the "24-h speciesEC₅₀ and EC₁₀" value was determined, i.e., the mean of the 24-h EC₅₀ and EC₁₀ values from the seven tested salts. To assess the possible impact of each deicer on the zooplankton species, the "24-h saltEC₅₀ and EC₁₀" value was determined: this was calculated as the mean 24-h EC₅₀ and EC₁₀ value of all those determined for the four tested organisms. The significance of the difference between 24-h salt EC₅₀ and EC₁₀, and 24-h species EC₅₀ and EC₁₀ were calculated by the Mann-Whitney U-test, with $p < 0.05$ regarded as significant.

3. Results and Discussion

3.1. Chemical analyses of de-icers

Of all of the analyzed de-icers, the traditional road salt (NaCl) had the highest levels of sodium, chlorides, sulphates and bound cyanides (Table 3). Pandolfo et al. (2012) report that sodium ferrocyanide and ferric ferrocyanide are often added to road salts as anti-caking agents; this could account for the higher content of bound cyanides (190 µg/L) detected in the traditional road salt used in this study, which had an anti-caking agent as additional component. The deicer based on CaCl₂ also contained higher levels of chlorides compared to the other substitutes. CaCl₂ and CaCl₂ ("eco label") had the highest calcium ion concentrations: 4 917.8 mg/L and 4 266.5 mg/L, respectively. Finally, the CH₃COOK deicer in solid form showed the lowest levels of all of the analyzed parameters, including potassium (< 10.0 mg/L); the liquid form demonstrated slightly higher values for sodium (10.2 mg/L), potassium (1 890 mg/L) and chlorides (29 mg/L).

3.2. 24-h EC₅₀ - Toxicity of de-icers

Of the tested salts, NaCl was found to be the most toxic for *T. platyurus* and *B. calyciflorus* (24-h EC₅₀ 3 289 mg/L and 3 318 mg/L, respectively). It was less toxic for cladoceran species, with 24-h EC₅₀=4 328 mg/L for *C. dubia* and 5 244 mg/L for *D. magna* (Figure 1).

Of the six purportedly de-icing salts more environmentally-friendly de-icers, only solid CH₃COOK (solid) demonstrated a higher 24-h EC₅₀ value for all four tested organisms. In this case, the lowest 24-h EC₅₀ value was obtained for *T. platyurus* (19 274 mg/L) and the highest for *B. calyciflorus* (22 786 mg/L). Most of the remaining de-icers had a 24-h EC₅₀ lower than NaCl; only CaCl₂ and NaCl+CaCl₂ had little higher 24-h EC₅₀ values (3 727 mg/L and 3 432 mg/L, respectively) than NaCl, observed for *B. calyciflorus* (Figure 1). The lowest 24-h EC₅₀ values for *T. platyurus*, *C. dubia* and *D. magna* were noted for CaCl₂ (871 mg/L, 1 473 mg/L and 2 224 mg/L, respectively). Of the tested organisms, *T. platyurus* had the lowest 24-h EC₅₀ values for six of the tested de-icers (not solid CH₃COOK), while *D. magna* had the highest 24-h EC₅₀.

Similar dependencies were observed for 24-h EC₁₀ (Figure S1). The EC₁₀ values were 5–63% lower than EC₅₀; the smallest difference was noted for CH₃COOK (liquid) for *T. platyurus* and the highest for CH₃COOK (solid) for *B. calyciflorus* (Table S4).

Due to the limited availability of analogous studies, our 24-hour results were compared with those from a Korean laboratory experiment based on *D. magna*, which used a longer exposure time (48 hours) (Lee et al., 2017). The study yielded a 48-h EC₅₀ value of 4 000 mg/L for NaCl, which is similar to our 24-h EC₅₀ result of 4 328 mg/L obtained after only 24 hours (Figure 1). In contrast, Lee and co-workers (2017) obtained a lower value for CaCl₂, with a 48-h EC₅₀ of 1 600 mg/L, compared to 2 224 mg/L after 24 hours in our present experiment. Mount and coworkers (1997) noted lower 24-h lethal concentration (24-h LC₅₀) values for *C. dubia* for NaCl (3 380 mg/L) and for *D. magna* for MgCl₂ (1 560 mg/L), than the 24-h EC₅₀ values

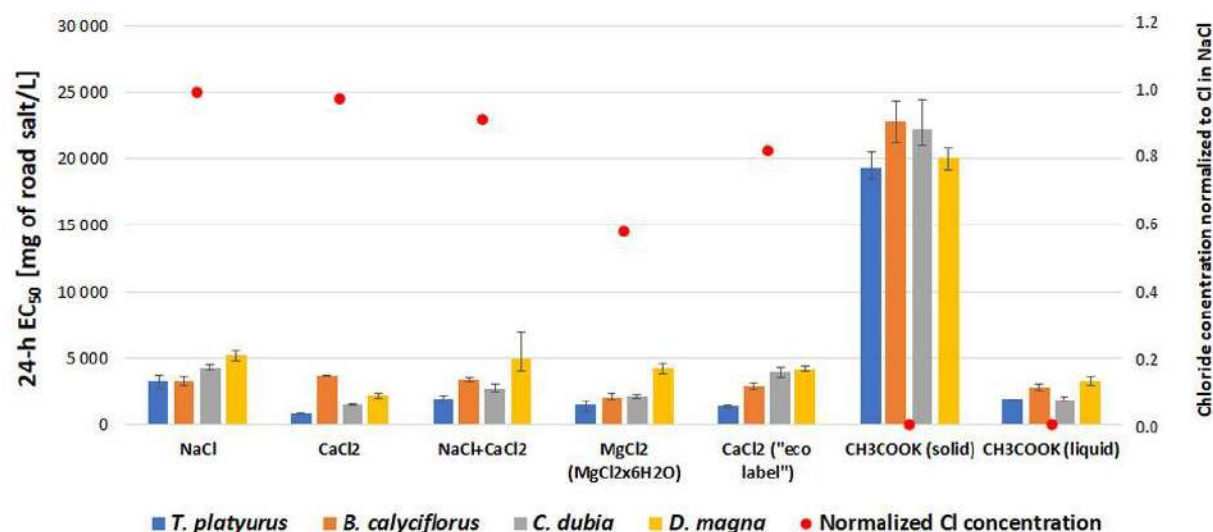


Figure 1. The results of 24h EC₅₀ (mean with confidence intervals $\pm$ 95%) of seven tested de-icing salts for four zooplankton species.

noted in our study (Figure 1). They also reported higher 24-h LC₅₀ values for NaCl, CaCl₂, NaCl+CaCl₂ and MgCl₂ for those species; briefly, CaCl₂ had the highest toxicity impact (24-h LC₅₀) for *C. dubia* and MgCl₂ for *D. magna*, while NaCl had the lowest for both species. In our tests, CaCl₂ had also the highest toxicity for *D. magna* and NaCl the lowest toxicity (24-h EC₅₀) for both species; the remaining three salts had a similar toxicity for *C. dubia*. (Figure 1, Table S5). Those differences may be due to genetic differences between the used culture lines and methodological approaches: Mount et al. (1997) used analytical grade chemicals and observed the LC effect, while our study used commercial chemicals (Table 1 and Table S2) and determined the EC effect.

Moreover, research conducted to determine 144-h LC_x values for mayfly *Colobruscoidea giganteus* (Ephemeroptera: Colobruscoidea) found some difference in toxicity between analytical grade salts and salts commercially used on winter roads (Moulding et al., 2022). These findings, as well as previously-noted differences in the toxic doses of the two types of salt, underline the need to examine the real environmental impact of de-icing salt used for winter maintenance; indeed, commercial salt may have extra ingredients that can pose a toxic hazard either itself, or through antagonistic, synergistic, additive or reversing interactions.

Differences in the toxicity of the seven de-icing salts were evident in the 24-h EC₅₀ calculated for all tested zooplankton species (24-h saltEC₅₀ index) (Figure 2). Solid CH₃COOK had a significantly higher 24-h saltEC₅₀ value (21 089 mg/L) than the other tested salts. Three salts: CaCl₂, MgCl₂ (MgCl₂x6H₂O) and CH₃COOK (liquid) (2 073 mg/L, 2 497 mg/L and 2 515 mg/L, respectively) had significant lower 24-h saltEC₅₀ values than NaCl (4 026 mg/L). The 24-h saltEC₅₀ values for NaCl+CaCl₂ (3 380 mg/L) and CaCl₂ ("eco label") (3 132 mg/L) were not significantly different to those of NaCl (Figure 2). Similar dependencies were observed for 24-h EC₁₀ (Figure S1).

Lee et al. (2017) examined the impact of nine de-icing salts (with six eco-label certificate) on acute toxicity against *D. magna* for 48h exposure. All of tested de-icers contained chloride, but three of the liquid eco-label de-icers had lower Cl content (17.9–21.3%) than the three solid eco-label de-icers (37.6–51.6%), CaCl₂ (44.8%) and NaCl (62.8%). The liquid eco-label de-icers were less toxic to *D. magna* (24-h EC₅₀ from 4 600 to 5 700 mg/L) than the solid eco-label de-icers (1 400 – 3 300 mg/L). In our experiment, no relationship was observed between lower Cl level and lower toxic impact on zooplankton species: MgCl₂ (MgCl₂x6H₂O) had 0.61 times the amount of Cl measured in NaCl (Figure 1, Table 4), but demonstrated significantly higher toxicity (Figure 3). Moreover, CH₃COOK (liquid) had a very low amount of Cl, but similar 24-h EC₅₀ (Figure 1) and 24-h saltEC₅₀ values (Figure 3) to other tested de-icers (except CH₃COOK solid). This might be caused by K cations, which were found to be present at higher concentrations in liquid CH₃COOK compared to the other deicing salts (Table 3); this is particularly significant as K ions employ a different toxicity mechanism, resulting in higher toxicity than other salt cations (Erickson et al., 2018, 2017; Mount et al., 2016). Interestingly, K demonstrated 230 mg/L 24-h EC₅₀ values for *T. platyurus*, 197 mg/L for *C. dubia* and 289 mg/L for *D. magna* (Table S4); these were similar to EC₅₀ values noted in other studies: 126 mg/L for *T. platyurus* (24-h EC₅₀), 129 mg/L for *C. dubia* and 386 mg/L for *D. magna* (both 48-h EC₅₀) (Ivey et al., 2017).

Most ecotoxicity assessments on zooplankton are performed using *Daphnia* spp. (e.g., Martínez-Jerónimo and Martínez-Jerónimo, 2007). In contrast, there is little data about the effect of de-icers on other zooplankton species. The studied zooplankton species demonstrated different sensitivities, as indicated by 24-h speciesEC₅₀ index (Figure 3). The lowest 24-h speciesEC₅₀ value was observed for *T. platyurus*, at 4 317 mg/L, and the highest, i.e., 6 333

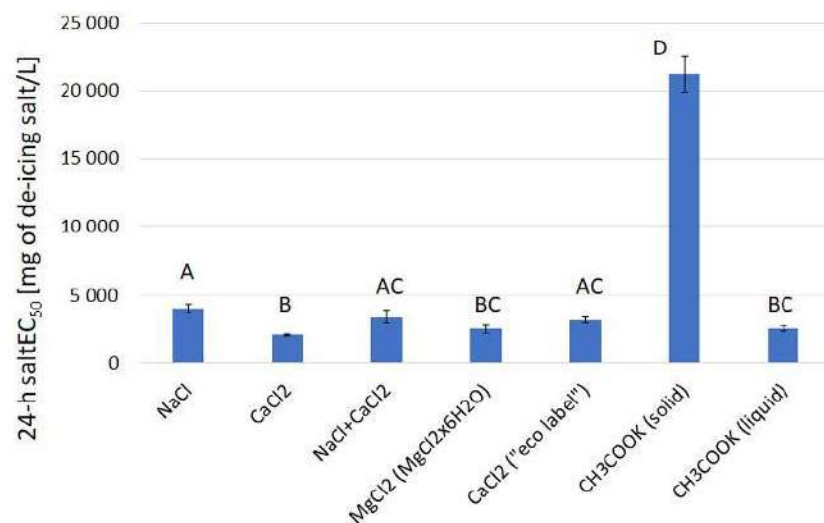


Figure 2. The mean value with confidence intervals $\pm 95\%$ for 24-h saltEC₅₀ (grouped results of four tested zooplankton species) for seven tested de-icing salts. Different letters indicate a significant difference in the Mann-Whitney U-test with $p < 0.05$.

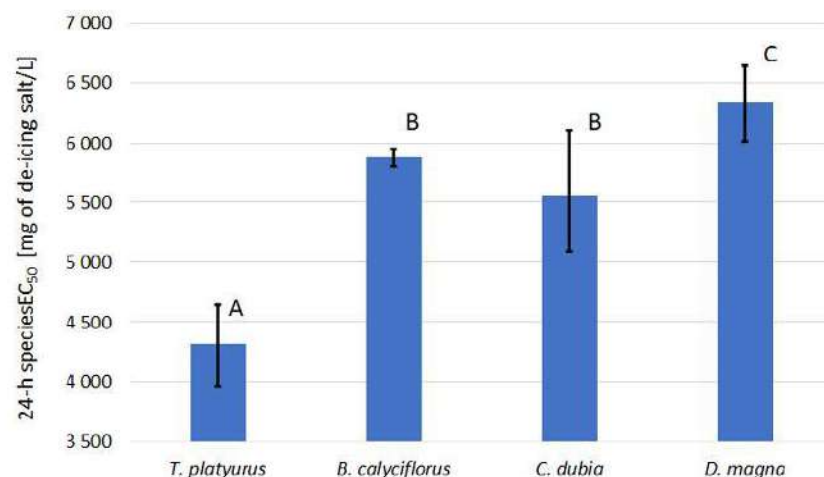


Figure 3. The mean value with confidence intervals $\pm 95\%$ for 24-h speciesEC₅₀ (grouped results of seven de-icing salts) for four tested zooplankton species. Different letters indicate a significant difference, based on the Mann-Whitney U-test with $p < 0.05$.

mg/L, was recorded for *D. magna*. Both values were significantly different from the 24-h speciesEC₅₀ calculated for *B. calyciflorus* and *C. dubia*, which in turn did not differ from each other (Figure 3). For *T. platyurus*, *B. calyciflorus* and *C. dubia*, the 24-h speciesEC₁₀ index value had no significant difference (4 317 mg/L, 3 379 mg/L and 3 589 mg/L, respectively; Figure S3). However, a significantly higher value was observed for *D. magna* than the other analysed species (5 189 mg/L).

Thamnocephalus is considered a very sensitive organism, and in many cases, it appears to be more sensitive than other crustacean species in acute tests. *T. platyurus* demonstrated significantly greater sensitivity to the toxicity of the herbicide atrazine and the insecticides endosulfan sulphate and chlorpyrifos than *Daphnia magna* and *Vibrio fischeri* (Palma et al., 2008). However, we are not aware of any studies, other than those presented here, on

the effects of road salts on *T. platyurus*. Little information exists on the long-term effects of chloride or salt exposure on zooplankton communities Valteau et al. (2020) examined long-term changes in Cladocera assemblages preserved in five lake sediments in Ontario (Canada) impacted by road-salt application. The authors report Cl⁻ concentrations in the range 32.8–90.9 mg/L in those lakes, and note that *Bosmina* spp. were replaced by more generalist scraping feeders (i.e., *Chydorus brevilabris* and *Eurycercus* spp.), unless *Daphnia* spp. were present. This may suggest that *Daphnia* spp. have higher tolerance to road-salt pollution; indeed, a study by Coldsnow and coworkers (2017) found that *Daphnia pulex* populations reared for 2.5 months under elevated salt concentrations (i.e., 100–1 000 mg Cl/L) had higher survival rates than populations previously exposed to natural background levels (15 mg Cl/L). Elphick and coworkers (2011) also found that for *D.*

magna toxicity occurred at higher concentrations of Cl pollution ($LC_{50}=3\ 630$ mg Cl/L) compared to *C. dubia* ($LC_{50}=1\ 068$ mg Cl/L) and *B. calyciflorus* ($LC_{50}=1\ 645$ mg Cl/L). In our present study, *D. magna* also demonstrated higher 24- EC_{50} against Cl (4 667 mg Cl/L) than the other three species (Table S4). The ability of *Daphnia* to adapt evolutionarily to salinity stress may be important in maintaining the stability of the trophic cascade in freshwater ecosystems (Liu and Steiner, 2017).

Other studies indicate that cladoceran abundance significantly decreased with increasing chloride concentration ($r^2=0.69$), while rotifers were more chloride tolerant; these demonstrated a slower decrease in abundance ($r^2=0.31$) with increasing Cl^- level (tested chloride gradient was from 0.41 to 1 500 mg/L) (Greco et al., 2021). In a mesocosm experiment, it was found that at a high Cl concentration (1 067 mg/L), the zooplankton community was dominated by the rotifer family, *Brachionidae* (93%); in contrast, at a low Cl concentration (177.2 mg/L), it consisted of only 27% of *Brachionidae*, about 30% of copepods and about 25% of cladocerans, with the remainder being made up of other rotifers and ostracods (van Meter et al., 2012).

An outdoor experiment comparing the effects of three different de-icing salts (NaCl, $MgCl_2$ and $CaCl_2$) found no significant difference in their effect on population profile. After 12-day exposure to a high chloride concentration (855 mg Cl/L) induced by exposure to the de-icing salts de-icers, all samples demonstrated 87–92% fewer cladocerans and 93–97% rotifers compared to controls (Coldsnow and Relyea, 2021). Schuler et al. (2017) report that exposure to two de-icing salts, GeoMelt (beet juice; proprietary blend) and Magic Salt ($MgCl_2$ mixed with a distillation by-product), resulted in significant higher zooplankton abundance at a concentration of 200 mg Cl/L compare to controls (25 mg Cl/L).

All these findings demonstrate the variable nature of the response of the zooplankton community to de-icing salt pollution. Our own laboratory tests also showed different levels of toxicity response to de-icing salts among organisms not previously exposed to chloride or other pollutants (Figure 1, Figure 3). Further analysis is needed to determine the probable cause of this varying response between the test species.

3.4. Further perspectives

New solutions are needed to prevent the further contamination of water ecosystems and their degradation. One such approach to ensuring sustainable development that balances the need for road safety during winter while reducing anthropogenic pollution involves the use of more environmentally-friendly de-icing salts. However, our results indicate that most of the tested NaCl alternatives had similar or even higher toxicity for the analysed zooplankton species compared to NaCl: only one from six “eco-friendly” alternatives had significant lower toxicity (Figure 1, Figure 3). In addition to the short-term effects of high salt concentrations, many studies report long-term toxicity for zooplankton abundance, egg production and neonate release (Arnott et al., 2020; Brown and Yan, 2015; Coldsnow and Relyea, 2021; Delaune et al.,

2021). It has also been found that the proposed alternatives to NaCl de-icers might have similar toxicity for other freshwater species, including *Chironomus dilutes* (Natile and Solan, 2019), filamentous algae and amphipods (Coldsnow and Relyea, 2021).

While it seems that the Cl-free de-icing salts should be less toxic to the environment, like the solid CH_3COOK studied in our experiment (Figure 2, Table S4), these salts may still might be toxic to the aquatic ecosystem; for example, the organic de-icing salts may increase biological oxygen demand (BOD) de-icing salts, leading to the depletion of dissolved oxygen (Terry et al., 2020); however, the dissolved oxygen concentration did not change significantly during the realised 24-h incubation (Table S3). Our present findings, and those of other studies, indicate that the impact of de-icing salts on the environment remains poorly verified. Their application might result in further degradation of freshwater ecosystems and, together with other factors like global warming and increasing water eutrophication (Donis et al., 2021), encourage a greater incidence of cyanobacterial blooms, as cyanobacteria demonstrate higher tolerance for salinity than other phytoplankton species (Tonk et al., 2007).

The decision to use alternative de-icers also has an economic dimension. While all the tested alternatives had a higher cost, the information given by the manufacturers suggest they are in fact more efficient (Table 1). The most environmentally-friendly de-icer, solid CH_3COOK , was almost five times more expensive than NaCl, but it was also five times less toxic for all tested zooplankton species (Figure 1). This indicates the need for a combined approach comprising both socio-economic research and studies examining the toxic impact of de-icing alternatives on freshwater ecosystems to provide the comprehensive data needed to reduce the negative impact of winter road maintenance on water communities. Such studies would be useful in developing uniform regulations for defining acceptable levels of acute and chronic chloride toxicity in surface waters.

4. Conclusion

This study compares the toxic impacts of NaCl and six alternative de-icing salts on four zooplankton species. Our findings showed that:

- $CaCl_2$, $MgCl_2$ ($MgCl_2 \times 6H_2O$) and liquid CH_3COOK had significantly lower values of 24-h salt EC_{50} (2 073 mg/L, 2 497 mg/L and 2 515 mg/L, respectively), than those noted for NaCl (4 026 mg/L). Only solid CH_3COOK salt had a significantly higher value of 24-h salt EC_{50} (21 089 mg/L).
- Of the four tested species, the lowest toxicity response for all seven de-icing salts was observed for *T. platyurus* – with a 24-h species EC_{50} value of 4 317 mg/L: significantly lower than for the other three species. The highest toxicity response was demonstrated by *D. magna*, with a 24-h species EC_{50} value of 6 333 mg/L: significantly higher than the other three species.

Most of the tested de-icing salts promoted as being more environmentally friendly de-icing salts than the more

commonly-used NaCl might in fact have similar or higher toxicity for zooplankton species. Our results underline the need to perform more comprehensive analysis of the impact of de-icing salts on the entire water ecosystem, which is the final recipient of surface runoff.

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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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:[10.1016/j.ecohyd.2022.08.005](https://doi.org/10.1016/j.ecohyd.2022.08.005).

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Supplementary materials

Table S1. Information about manufacture of each used de-icing salt.

Main ingredient	Brand name (<i>in Polish</i>)	Manufacturer	Address
NaCl	Road salt (sól drogową)	BIO-WAY Sp. z o.o. S.K.A.	Al. Niepodległości 14 p.29 39-300 Mielec
CaCl ₂	DONSOL	Domena Mączka Sp. z o.o.	ul. F. Stefczyka 3, 20-151 Lublin
NaCl + CaCl ₂	Road DONSOL (Donsol drogowy)	Domena Mączka Sp. z o.o.	ul. F. Stefczyka 3, 20-151 Lublin
MgCl ₂ (MgCl ₂ ·xH ₂ O)	DONSOL Magnesium (Donsol Magnez)	Domena Mączka Sp. z o.o.	ul. F. Stefczyka 3, 20-151 Lublin
CaCl ₂ ("eco label")	Icebreaker (Lodolamacz)	Janusz Amanowicz biuro handlowe oraz zarządzanie nieruchomościami	Ul. B. Radziwiłłówny 27, 02-496 Warszawa
CH ₃ COOK (solid)	IceGO solid (IceGO granulki)	Bio-tech Sp. z o.o. Sp. k	ul. łowińskiego 7e 31-752 Kraków
CH ₃ COOK (liquid)	IceGO liquid (IceGO granulki)	Bio-tech Sp. z o.o. Sp. k	ul. łowińskiego 7e 31-752 Kraków

Table S2 Chemical parameters analyzed in the de-icing salts, together with the method used.

Analyzed parameter	Unit	Method
Chrome (Cr)	µg/L	PN-EN ISO 17294-2:2016-11
Zinc (Zn)	mg/L	PN-EN ISO 17294-2:2016-11
Sodium (Na)	mg/L	PN-EN ISO 17294-2:2016-11
Magnesium (Mg)	mg/L	PN-EN ISO 17294-2:2016-11
Potassium (K)	mg/L	PN-EN ISO 17294-2:2016-11
Calcium (Ca)	mg/L	PN-EN ISO 17294-2:2016-11
Iron (Fe)	µg/L	PN-EN ISO 17294-2:2016-11
Arsenic (As)	µg/L	PN-EN ISO 17294-2:2016-11
Barium (Ba)	mg/L	PN-EN ISO 17294-2:2016-11
Sulphates (SO ₄ ²⁻)	mg/L	ISO 15923-1:2013
Chlorides (Cl ⁻)	mg/L	ISO 15923-1:2013
Nitrates (NO ₃ ⁻)	mg/L	PN-EN ISO 13395:2001
Bound cyanides	µg/L	PN-EN ISO 14403-2:2012
Free cyanides	µg/L	PN-EN ISO 14403-2:2012
Bismuth	mg/L	W-METAXFX2

Table S3. Physicochemical data of the prepared de-icer salt at the beginning of the test (T0) and after incubation (T24).

De-icing salt	concentration [mg/L]	SPC [$\mu\text{S}/\text{cm}$]		pH		DO [mg/L]	
		T0	T24	T0	T24	T0	T24
NaCl	15 200	29781	29779	7,32	7,31	8,74	8,72
	7 600	14885	14887	7,30	7,31	8,72	8,70
	3 800	7445	7444	7,31	7,30	8,75	8,72
	1 900	3720	3721	7,29	7,30	8,73	8,71
	950	1859	1857	7,29	7,29	8,76	8,74
CaCl ₂	15 200	30901	30902	7,62	7,60	8,75	8,74
	7 600	15452	15452	7,58	7,57	8,73	8,70
	3 800	7729	7728	7,55	7,55	8,75	8,72
	1 900	3860	3862	7,54	7,55	8,76	8,73
	950	1932	1933	7,50	7,51	8,74	8,71
NaCl+CaCl ₂	15 200	31881	31878	7,58	7,59	8,72	8,70
	7 600	15935	15932	7,51	7,53	8,75	8,72
	3 800	7975	7972	7,49	7,50	8,74	8,73
	1 900	3982	3980	7,50	7,49	8,72	8,72
	950	1990	1993	7,48	7,47	8,74	8,73
MgCl ₂ (MgCl ₂ ·6H ₂ O)	15 200	22711	22704	7,95	7,94	8,75	8,74
	7 600	11350	11353	7,89	7,85	8,73	8,73
	3 800	5678	5676	7,80	7,79	8,72	8,71
	1 900	2840	2837	7,74	7,75	8,71	8,72
	950	2525	2521	7,75	7,77	8,72	8,73
CaCl ₂ ("eco label")	15 200	40450	40448	7,73	7,72	8,73	8,73
	7 600	20225	20226	7,71	7,70	8,74	8,72
	3 800	10110	10107	7,70	7,71	8,73	8,72
	1 900	5058	5054	7,71	7,70	8,74	8,72
	950	2527	2528	7,69	7,70	8,72	8,71
CH ₃ COOK (solid)	30 400	8252	8254	7,68	7,67	8,73	8,72
	24 300	4129	4128	7,66	7,67	8,73	8,71
	21 300	2060	2058	7,63	7,64	8,74	8,72
	18 200	1027	1028	7,64	7,64	8,75	8,72
	15 200	513	511	7,63	7,63	8,74	8,71
CH ₃ COOK (liquid)	15 200	5205	5206	7,89	7,88	8,74	8,72
	7 600	2601	2599	7,87	7,87	8,74	8,72
	3 800	1303	1301	7,79	7,77	8,73	8,71
	1 900	649	648	7,76	7,77	8,74	8,72
	950	323	324	7,74	7,75	8,75	8,73

Table S4. The results of 24-hour EC₅₀ and EC₁₀ (mean with confidence intervals $\pm$ 95%) of seven tested de-icing salts for four zooplankton species.

De-icing salt	<i>Thamnocephalus platyurus</i>			<i>Brachionus calyciflorus</i>			<i>Ceriodaphnia dubia</i>			<i>Daphnia magna</i>		
	Mean	-95%	+95%	Mean	-95%	+95%	Mean	-95%	+95%	Mean	-95%	+95%
24-h EC₅₀												
NaCl	3289	2733	3715	3318	3033	3615	4328	4114	4551	5244	4834	5547
CaCl ₂	871	844	912	3727	3643	3801	1473	1429	1515	2224	2069	2376
NaCl+CaCl ₂	2016	1860	2193	3432	3316	3526	2737	2472	3113	5004	4005	6891
MgCl ₂ (MgCl ₂ ·x6H ₂ O)	1462	927	1873	2094	1894	2380	2160	1990	2328	4313	3889	4658
CaCl ₂ ("eco label")	1370	1284	1476	2913	2717	3167	4024	3602	4375	4224	4043	4395
CH ₃ COOK (solid)	19274	18500	20495	22786	21146	24327	22271	20953	24441	20028	19114	20719
CH ₃ COOK (liquid)	1937	1916	1968	2839	2642	3072	1906	1806	2111	3294	3036	3659
24-h EC₁₀												
NaCl	2 121	1 611	2 863	1 574	1 327	1 816	2 872	2 587	3 130	4 667	3 973	5 292
CaCl ₂	687	579	744	3 386	3 161	3 668	1 103	1 014	1 192	1 409	1 221	1 574
NaCl+CaCl ₂	1 058	852	1 272	2 690	2 337	2 992	1 142	1 037	1 235	4 540	3 436	6 240
MgCl ₂ (MgCl ₂ ·x6H ₂ O)	883	292	1 776	1 036	801	1 361	1 399	1 135	1 626	3 116	2 792	3 352
CaCl ₂ ("eco label")	813	708	921	1 703	1 391	2 038	2 823	2 042	3 391	3 162	2 825	3 454
CH ₃ COOK (solid)	16 247	13 916	18 317	8 487	2 021	22 440	14 205	11 415	18 112	17 111	15 560	18 793
CH ₃ COOK (liquid)	1 848	1 773	1 871	1 430	1 206	1 700	1 581	1 301	1 843	2 325	1 880	2 755

Table S5. 24-h EC₅₀ value for analysed de-icing salts, and their main ions and electrical conductivity, for all used zooplankton species.

Road salt	Species	24-h	24-EC ₅₀ for other parameters					SPC
		EC ₅₀ of	Cl	K	Na	Mg	Ca	
		road salt						
mg/L								
NaCl	<i>T. platyurus</i>	3289	1973		1283		5	6444
	<i>B. calyciflorus</i>	3318	1991		1295		5	6501
	<i>C. dubia</i>	4328	2597		1688		7	8479
	<i>D. magna</i>	5244	3146		2046		8	10274
CaCl ₂	<i>T. platyurus</i>	871	519				282	1771
	<i>B. calyciflorus</i>	3727	2219				1206	7577
	<i>C. dubia</i>	1473	877				477	2995
	<i>D. magna</i>	2224	1324				720	4522
NaCl+CaCl ₂	<i>T. platyurus</i>	2016	1093		493		179	4229
	<i>B. calyciflorus</i>	3432	1860		840		304	7198
	<i>C. dubia</i>	2737	1484		670		243	5740
	<i>D. magna</i>	5004	2712		1225		443	10494
MgCl ₂ (MgCl ₂ x6H ₂ O)	<i>T. platyurus</i>	1462	538	4	4	177	2	2184
	<i>B. calyciflorus</i>	2094	770	6	5	253	3	3127
	<i>C. dubia</i>	2160	794	7	5	261	3	3226
	<i>D. magna</i>	4313	1586	13	11	522	6	6442
CaCl ₂ ("eco label")	<i>T. platyurus</i>	1370	731	4	16		385	3647
	<i>B. calyciflorus</i>	2913	1554	8	34		818	7751
	<i>C. dubia</i>	4024	2147	11	47		1129	10707
	<i>D. magna</i>	4224	2254	11	49		1186	11240
CH ₃ COOK (solid)	<i>T. platyurus</i>	19274		13			36	654
	<i>B. calyciflorus</i>	22786		15			42	774
	<i>C. dubia</i>	22271		15			41	756
	<i>D. magna</i>	20028		13			37	680
CH ₃ COOK (liquid)	<i>T. platyurus</i>	1937	4	241	1		1	663
	<i>B. calyciflorus</i>	2839	5	353	2		2	972
	<i>C. dubia</i>	1906	4	237	1		1	653
	<i>D. magna</i>	3294	6	410	2		2	1128

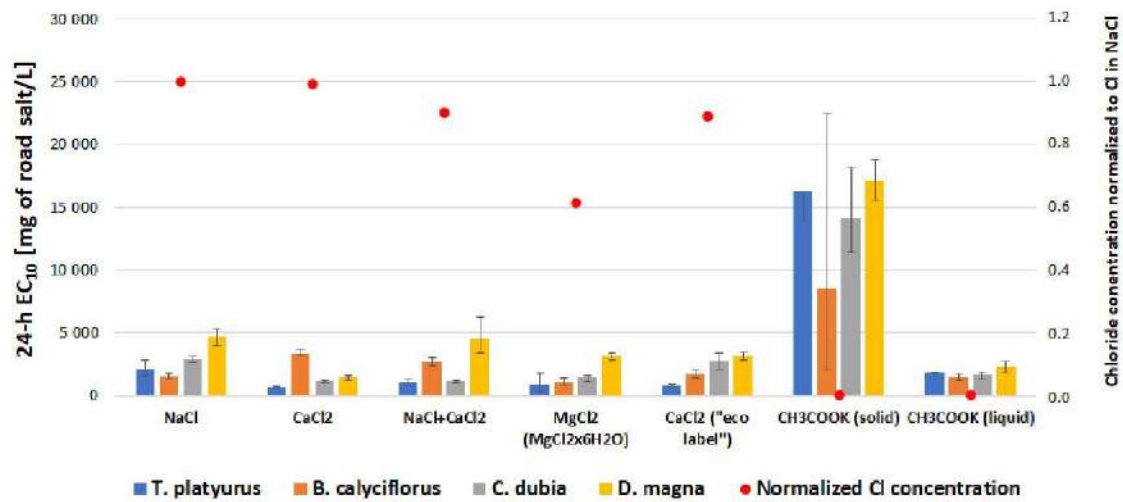


Figure S1 The results of 24h EC_{10} (mean with confidence intervals $\pm$ 95%) of seven tested de-icing salts for four zooplankton species

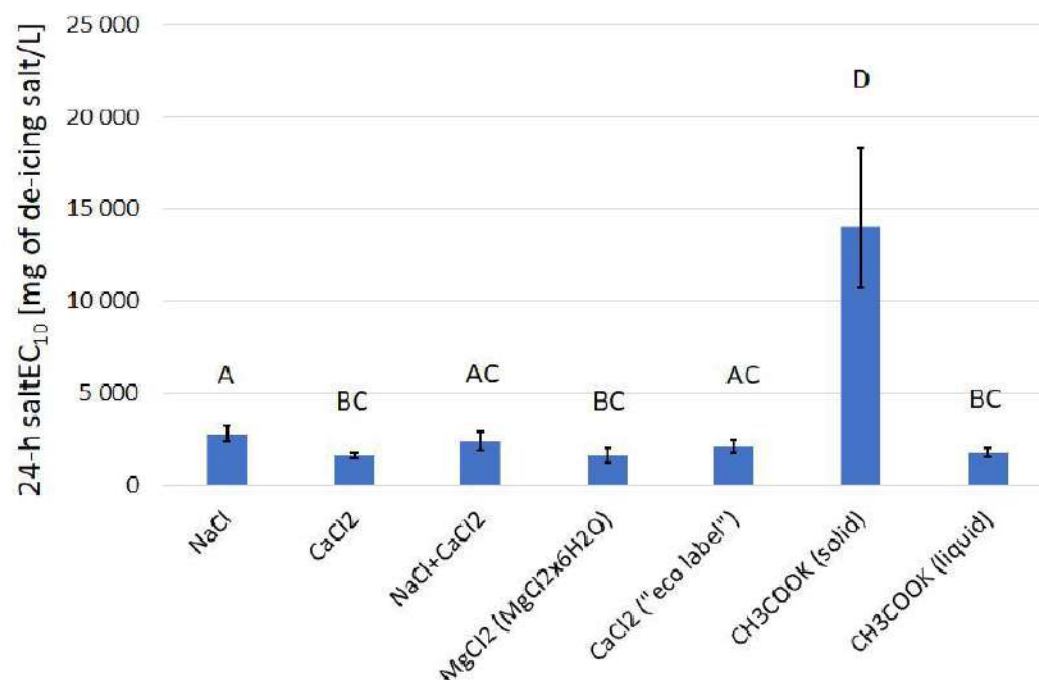


Figure S2. The mean value with confidence intervals $\pm$ 95% for 24-h salt EC₁₀ (grouped results of four tested zooplankton species) for seven tested de-icing salts. Different letters indicate a significant difference ($p < 0.05$; Mann-Whitney U-test).

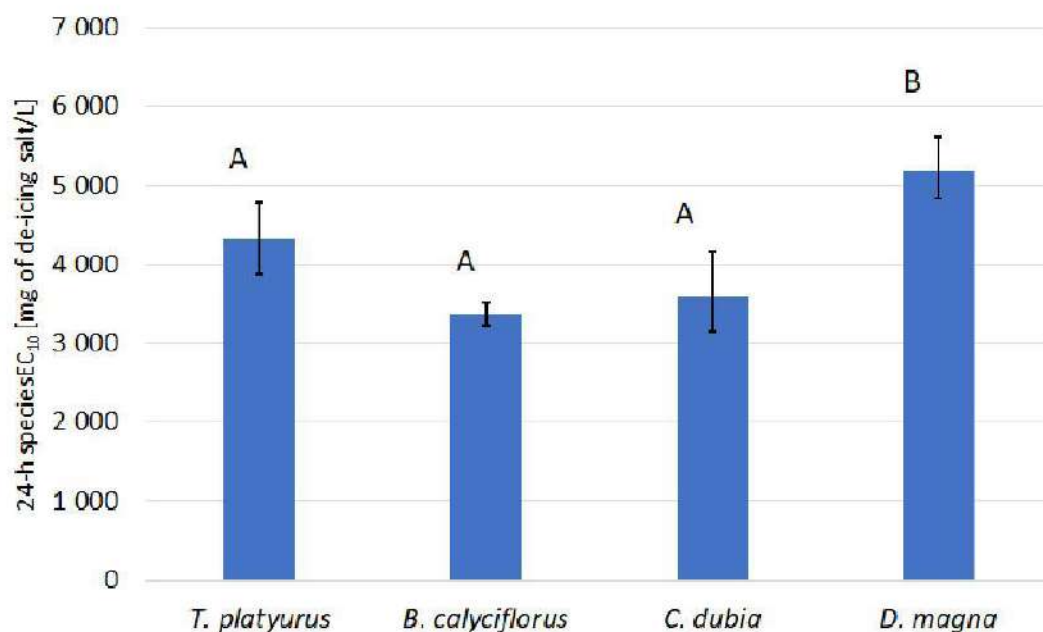
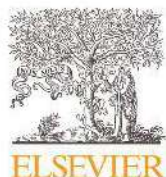


Figure S2 The mean value with confidence intervals $\pm$ 95% for 24-h species EC₁₀ (grouped results of seven de-icing salts) for four tested zooplankton species. Different letters indicate significant difference, ($p < 0.05$; Mann-Whitney U-test).

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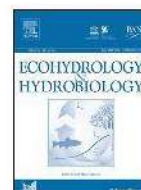
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Original Research Article

The effect of road salt (NaCl) treatment on the hatching success of *Daphnia magna* and *Thamnocephalus platyurus*Sebastian Szklarek^{a,*}, Aleksandra Górecka^b, Piotr Jóźwiak^c,
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ABSTRACT

Salinization is an emerging problem for freshwater biodiversity. Road salt treatment results in salinization of local freshwater, influencing its entire ecosystem throughout the year. The present study examines the impact of road salt on the hatching success of two zooplankton species, *Daphnia magna* and *Thamnocephalus platyurus*, using pure laboratory lines from MicroBioTest. Hatching success was determined without incubation (T0) and after 3, 7 and 28 days of incubation at 5°C (T3, T7 and T28, respectively), at different NaCl concentrations. The low-temperature incubation was intended to simulate winter conditions - 5°C corresponds to the water temperature at the bottom of the ponds during winter in a temperate climate. The number of hatchings decreased with increasing NaCl concentration for *T. platyurus*, and decreased rapidly after a crucial value of 7 600 mg NaCl/L for *D. magna*. At the second hatching (eggs washed after first incubation and put into standard freshwater), *D. magna* demonstrated a low hatching rate regardless of NaCl concentration, while most *T. platyurus* hatchings were observed at the highest NaCl concentration; hence, the highest (15 200 mg/L) and lowest (1 900 mg/L) NaCl concentrations resulted in similar total hatching for the two species. The incubation time for three and seven days in NaCl at 5°C had no significant impact on hatching success; however, T28 resulted in significantly lower hatching success at all NaCl concentrations and controls. No significant differences in hatching success were noted if the eggs were first washed after incubation in NaCl and then put into the standard freshwater for hatching.

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1. Introduction

Of the eight categories of planetary boundaries proposed by Rockström et al. (2009), biodiversity loss, chemical pollution and change in nitrogen and phosphorus cy-

cles are strongly influenced in inland freshwater ecosystems by increasing salinity. Freshwater salinization (FS) also leaches contaminants from soils and sediments and reduces self-purification processes, and hence presents an emerging threat to biodiversity (Reid et al., 2019) (Cañedo-Argüelles et al., 2013; Cunillera-Montcusí et al., 2022; Kaushal et al., 2021; Szklarek et al., 2022a). One source of salinization is the use of salts as de-icing agents.

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Although this issue has received considerable attention in North America, it has been largely neglected in Europe (Cunillera-Montcusí et al., 2022). Long-term research indicates that road salt pollution is not only a winter-spring issue, but one that influences surface water quality throughout the year, with increasing trends in rivers and lakes (Corsi et al., 2015; Dugan et al., 2017). In some cases, elevated chloride concentrations above an acute level of 640 mg/L (Canadian Guidelines - CEQG, 2011) might persist in bottom water until September (Lembcke et al., 2017).

The increased water salinity resulting from the use of de-icing agents can affect freshwater organisms at multiple trophic levels, resulting in changes in trophic structure. This is particularly relevant for the taxonomic groups that are crucial to the food web of lakes (Hintz and Relyea, 2019). One such group is the zooplankton, which act as energy transmitters from primary producers to higher-order consumers in freshwater ecosystems (Lampert and Sommer, 1997). Herbivorous zooplankton can exert pressure via top-down control and determine phytoplankton composition and abundance. The regulating role of zooplankton, particularly evident in mesotrophic ecosystems, influences the entire trophic structure of lakes (Jeppesen, 1998). The top-down effect is weaker in eutrophic lake ecosystems, where phytoplankton can be the dominant primary producer; in such cases, its excessive biomass, known as a water bloom, deteriorates the physico-chemical conditions and has a negative impact on water quality, biodiversity and ecosystem functioning (Amorim and Moura, 2021). Phytoplankton blooms in eutrophic ecosystems can also negatively affect zooplankton species, resulting in changes in dominance patterns or the disruption of zooplankton life history strategies (Moustaka-Gouni and Sommer, 2020).

The anthropogenic eutrophication of waters, cyanobacterial blooms can also arise as a result in increasing salinity of freshwater ecosystems due to road salt runoff (Tonk et al., 2007). Both factors directly and indirectly increase the mortality of zooplankton (Li et al., 2016; Hintz et al., 2022), thus disrupting the zooplankton-phytoplankton energy pathway in the food web of lakes. In addition, as zooplankton provide food for many predatory invertebrates and fish, they can influence production at higher trophic levels. One example can be seen in Lake Michigan in the United States, where reductions in zooplankton abundance and diversity have reduced fish growth and recruitment (Dettmers et al. 2003).

As noted by Hintz and co-authors (2022), current water quality guidelines in the US and Europe do not sufficiently protect aquatic organisms such as zooplankton from the negative effects of road salt. The authors report that water salinity causes significant mortality of zooplankton at the lowest Cl^- thresholds established in Canada (120 mg Cl^-/L) and the United States (230 mg Cl^-/L) and throughout Europe, where Cl^- thresholds are generally higher and only established in some countries (e. g. Sweden and Germany). There is, hence, a need to revise existing guidelines and possibly set new water quality standards, and this requires scientific verification of the resistance of zooplankton species to water salinity. The cladoc-

cerans are particularly sensitive to elevated salt concentrations and respond rapidly to physicochemical and biological changes; they are hence good indicators of environmental conditions and trophic status (Li et al. 2020; Kuczyńska-Kippen et al. 2020; Muñoz-Colmenares et al., 2021).

The evolutionary and ecological perspectives of the impact of salinisation on freshwater ecosystems is poorly understood (Cunillera-Montcusí et al. 2022). Relatively little research has examined the functional, spatial and trophic consequences of freshwater salinization (FS) and the impact of salinisation on the genetic, physiological, community or ecosystem levels of biological organisation. Therefore, the present study examines the effect of road salt (NaCl) on the hatching success of the model organism *Daphnia magna* (Straus, 1820) and *Thamnocephalus platyurus* (Packard, 1877), which demonstrated high sensitivity to seven types of de-icing salts, including NaCl (Szklarek et al., 2022b). A number of experimental studies on the resilience of zooplankton to freshwater salinity are being performed specifically using daphnids (e. g. Liu and Steiner, 2017; Hintz and Relyea, 2019). The aim of this study was to determine the hatching success of the species 1) at different NaCl concentrations without incubation period, and 2) after egg incubation at 5°C ($\pm 2^\circ\text{C}$) at different NaCl concentrations for three, seven and twenty-eight days.

2. Material and Methods

2.1. Hatching procedure

The natural resistance of zooplankton eggs to NaCl pollution was investigated using pure laboratory lines of the zooplankton species *Daphnia magna* and *Thamnocephalus platyurus*. Eggs were exposed for NaCl in the darkness at 5°C – temperature occurred at the bottom of ponds during winter in temperate climate. The lines were supplied as part of the Daphtoxkit and Thamnotoxkit bioassay kits (MicroBioTests Inc., Mariakerke, Belgium). The testing procedure followed the standard operating procedure given by the manufacturer, with the following modifications (steps from 3 to 5 are shown in Figure 1):

1. Prepare standard freshwater (ISO for *D. magna* and EPA for *T. platyurus*).
2. Prepare NaCl dilution series – the highest concentration NaCl-1 (15.20 g of NaCl diluted in one litre of EPA/ISO) was prepared based on the chloride concentration measured in previous field research (Szklarek et al., 2015; 2018) and literature review. The next concentrations were prepared by 50% dilutions with EPA/ISO: NaCl-2 (7.60 g/L - 50.00% of NaCl-1), NaCl-3 (3.80 g/L - 25.00% of NaCl-1), NaCl-4 (1.90 g/L - 12.50% of NaCl-1) and NaCl-5 (0.95 g/L - 6.25% of NaCl-1).
3. Add NaCl concentration to test plate with eggs – one vial with eggs puts to one plate (in three repetitions - $n=3$) per each NaCl concentration per each incubation time. Manufacture don't give information about how many eggs were in each vial, but we made con-

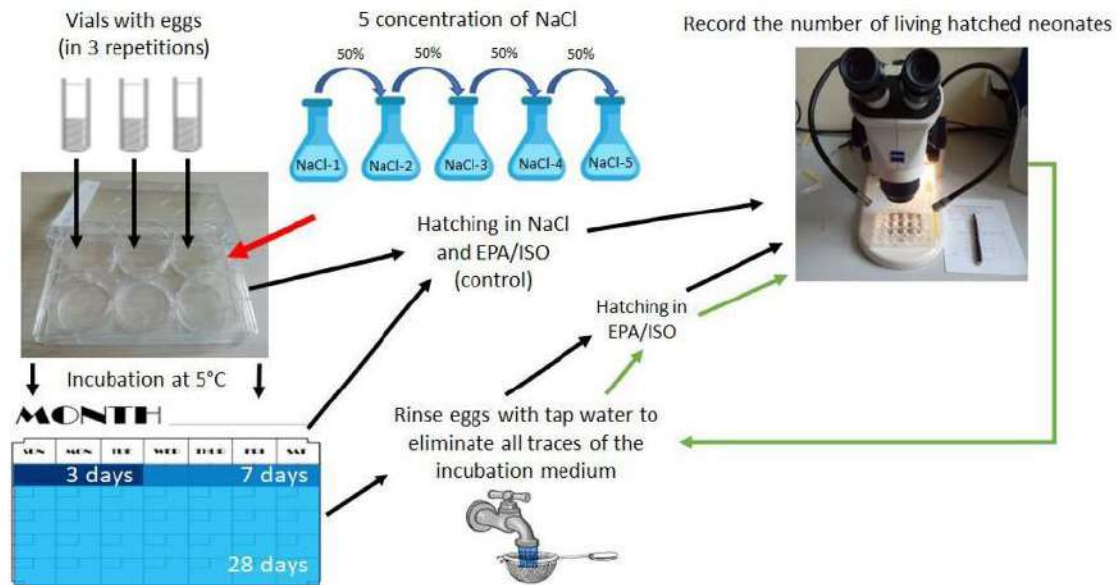


Figure 1. Test scheme for assessing the impact of NaCl on hatching success of *T. platyurus* and *D. magna* (green arrows represent second hatching part of experiment).

trol for each test which give us average of 346 ± 26 SD hatched juveniles for *D. magna* and 427 ± 23 SD for *T. platyurus* (five tests for each species, each with three repetition – see Supplementary hatching data file). *D. magna* was previously rinsed with tap water to eliminate all traces of the storage medium in which they were transported and stored; *T. platyurus* was stored in vials without medium and did not need rinsing. Each rinsing was performed three times.

- 3-1. **First hatching** of the eggs in NaCl (or EPA/ISO for control) without incubation (T0):
 - a. In continuous illumination; 3000-4000 lux for 22 hours at 25°C for *T. platyurus*.
 - b. In continuous illumination; 6000 lux for 72 hours at 25°C for *D. magna*.
- 3-2. Incubate eggs in NaCl (or EPA/ISO for control) at 5°C ($\pm 2^\circ\text{C}$) for 3 (T3), 7 (T7) or 28 days (T28), and then:
 - a. First hatching according to step 3-1.
 - b. Rinsing all eggs in each plate with tap water to eliminate all traces of the storage medium (NaCl or EPA/ISO), put eggs to the test plates with EPA/ISO and allow hatching according to step 3-1. a/b.
4. Record the number of living hatched neonates in each plate – the larvae are considered dead if they do not show any movement during 10 seconds of observation, for *T. platyurus*, or 15 seconds, for *D. magna*. This is in accordance with standard scoring described in test protocols.
5. **Second hatching** – rinsing all eggs from each plate with tap water and put them to the test plates with EPA/ISO and allow hatching according to step 3-1. a/b. Record the hatching success according to step 4.

2.2. Other analyses

The significance of hatching success at both different incubation times and different NaCl concentrations was tested using one-way ANOVA with incubation time and NaCl concentration as the categorical factor and number of hatched individuals as the dependent factor. To test for the combined effect of the incubation time and NaCl concentration we used a two-way ANOVA with the incubation time and NaCl concentration as the categorical factors and the number of hatched individuals as the dependent factor. If significant differences were found in the ANOVA, the Tukey's multiple comparison test was performed. The EC_{50} and EC_{10} values for hatching success were determined by the Regtox macro in Excel; this was delivered by the testing company for the standard procedure (author Eric Vindimian – available online: http://www.normalesup.org/~vindimian/en_index.html). This method gives the EC_{50} and EC_{10} value with $\pm 95\%$ confidence intervals. The results can be compared with the 24-hour EC values obtained in other research for adults of both species using the standard procedure.

Ephippia of *D. magna* previously exposed for three and seven days to different concentrations of NaCl were taken for analysis with an SEM microscope. Thirty ephippia from each concentration were taken, then eggs and embryos of *D. magna* were dissected from ephippia using chemically-sharpened tungsten needles. They were transferred to the SEM stubs covered with a droplet of distilled water. Prior to SEM examination, the stubs were mounted in a temperature-controlled sample holder and frozen at -10°C . The samples were imaged with a Phenom ProX scanning electron microscope. The results observed under the microscope after opening the ephippium were divided into the following categories: 1) empty ephippium, 2) single

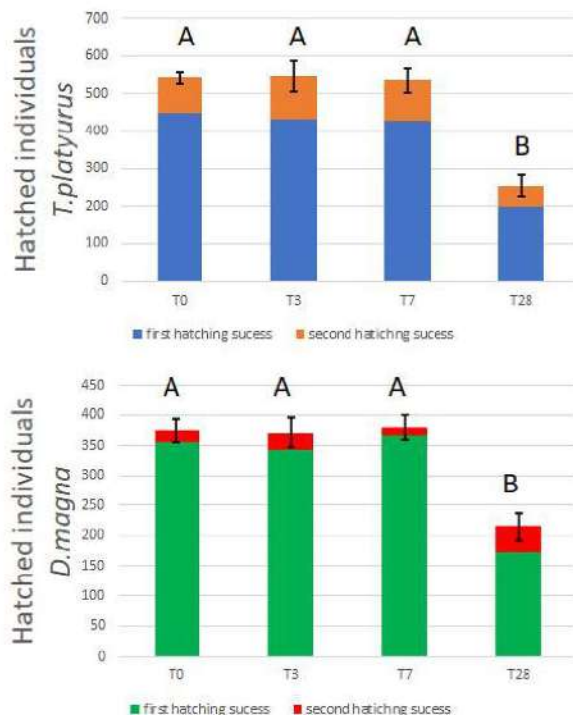


Figure 2. Mean number (with SD) of hatched individuals of *T. platyurus* (up) and *D. magna* (down) in the first hatching and the sum of both hatchings among controls (EPA/ISO medium) subjected to 3, 7 and 28 days of incubation in refrigerator (T3, T7 and T28, respectively) compared to controls without any incubation period (T0). Different letters indicate significant differences (one-way ANOVA test; $p < 0.05$).

egg, 3) two eggs, 4) single embryo, 5) single embryo with visible eye, 6) two embryos, 7) two embryos with visible eye, 8) egg and embryo. In order to determine the effect of NaCl on the ephippia state the distributions of the different ephippia categories observed at different NaCl concentrations were compared with the control using a chi-square test for goodness of fit.

3. Results and Discussion

3.1. Hatching success in control at different incubation time

The main aim of our studies was to investigate the hatching success of two zooplankton species based on eggs from the standardized tests (Daphtoxkit and Thamnotoxkit). The eggs of both species were stored in vials at $5^{\circ}\text{C} \pm 2^{\circ}\text{C}$ before testing: *T. platyurus* in dry conditions and *D. magna* in storage medium. As the addition of EPA/ISO with different NaCl concentrations changed the storage conditions, the first step examined the difference between control samples; the result indicated that eggs could be incubated for up to seven days (T7): no significant differences were observed between controls at T0, T3 or T7 for either species in the first or second hatching (Figure 2). Significant lower hatching success was noted after 28 days of incubation; therefore, the study did not examine hatching success at T28 (whole data in Supplementary 1).

Most previous hatching experiments were performed on environmental samples (e.g. Bailey et al., 2004; Lopatina et al., 2021; Paes et al., 2016; Rogalski, 2015), or examined year-round culturing of live stocks to avoid adaptive tolerance (Jonczyk and Gilron, 2005). However, continuous culture of live test organisms, in good health and in sufficient numbers, can present a technological and financial burden, and the use of spore eggs, from which test organisms can hatch on demand, is an attractive alternative (Persoone et al., 2009). Moreira dos Santos and Persoone (2000) compared the suitability of acute *D. magna* neonates hatched from ephippia for testing, with that of neonates taken from live cultures of laboratory stocks. Tests were carried out for four chemicals. The results, based on test sensitivity and precision, clearly confirmed that the ephippium-based toxicity test is a valuable alternative to the conventional acute *Daphnia* test, and that ephippia is suitable for conducting research on hatching success.

Navis et al. (2013) examined the effect of pesticide on hatching. Their experiment was conducted for 10 days, but the eggs were hatched without incubation at low temperatures, and only 48 eggs were used in each replicate. In contrast, the present study started with 40 eggs per repetition, but with no repeatability of results: hatching success ranged from 0 to 100% in controls, especially for *T. platyurus*. Therefore, to provide repeatability, one vial with eggs was used per repetition. This approach ensured reproducible results of hatching for the controls.

3.2. The impact of road salt on hatching success

In the first experiment, hatching success was investigated without incubation in the refrigerator (T0). The success of the first hatching decreases with increasing NaCl concentration for both species; however, a significantly lower number of hatched individuals (mean 321 ± 79 SD) were noted at 1.900 g NaCl/L (NaCl-4) for *T. platyurus*, and at 7.60 g NaCl/L (NaCl-2) for *D. magna* (mean 103 ± 19 SD). At the highest concentration, i.e. 15.20 g NaCl/L (NaCl-1), no hatching was observed for either species (Figure 3).

More variation was observed for the second hatching. The mean numbers ranged from 10 to 19 for *D. magna* for ISO and road salt concentrations from NaCl-2 to NaCl-5; however, this value increased to 80 ± 10 SD individuals for NaCl-1 (Figure 3). For *T. platyurus*, similar hatching rates were observed for NaCl-5 and NaCl-4, ranging from 79 to 93 hatched individuals (Figure 3), while 179 ± 60 SD hatched individuals were observed for NaCl-3. Hence, for NaCl-3, the sum of both hatchings was not significantly different to those observed for EPA, NaCl-5 or NaCl-4. In addition, the mean number for the second hatching was 263 ± 16 SD individuals for NaCl-2 and 425 ± 15 SD for NaCl-1; the sum of both hatchings of NaCl-1 was not significantly different to those of NaCl-3 or NaCl-4 (Figure 3).

D. magna hatching success declined sharply when the critical concentration was exceeded – for NaCl it was from 7.60 g/L (Figure 3). This value is similar to noted toxic response for *M. macrocopa* for which survival ratio decreased between 4 and 6 g/L of road salt based on NaCl (respectively from 87% to 17%) (Lopatina et al., 2021). Moreover, after six months of incubation with this road salt, hatching

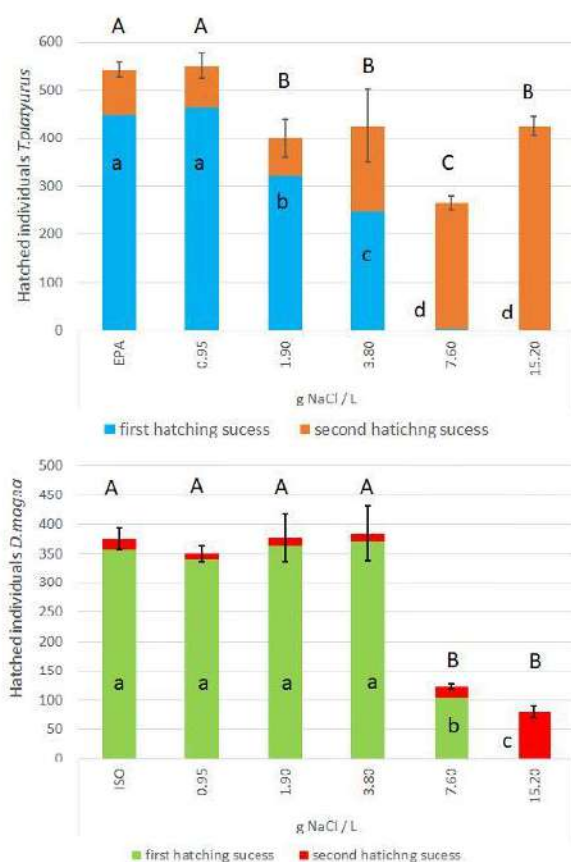


Figure 3. Mean number (with SD) of hatched individuals of *T. platyurus* (up) and *D. magna* (down) at first and sum of both hatching: control samples and five NaCl dilutions. Different letters indicate a significant difference (one-way ANOVA test; $p < 0.05$) – small letters for first hatching, big letters for sum of both hatching.

success also decrease from about 85% at salinity 5 g/L to lower than 5% above 10 g/L salinity (Lopatina et al., 2021). A similar response was noted also for other pollutants. Navis et al. (2013) observed for pesticides; no significant difference in hatching success was found between controls and samples with 50 – 1000 µg/L fenoxycarb; however, hatching was significantly inhibited for 2000 µg/L (6.3%) and 4000 µg/L (0%) (Navis et al., 2013). A similar rapid decrease was also observed in a study on adult *D. pulex*, where survivorship decreased from 5 g NaCl/L (70% survival) to 6 g NaCl /L (Latta et al., 2012). In contrast, for *T. platyurus*, hatching success decreased with increasing NaCl concentration. The difference between species might be due to the fact that *D. magna* demonstrates higher tolerance for NaCl than *T. platyurus* (Szklarek et al., 2022b).

Research conducted on egg bank from Imboassica lagoon, located in the urban area of the city of Macae (Rio de Janeiro State, southeastern Brazil) found hatching success to decrease significantly with increasing salinity (Santangelo et al., 2014). The authors also noted higher hatching rates (20–50 more hatched individuals) after moving the eggs from saline concentrations from 4.0 to

32.0 g/L to freshwater for another 10 days of hatching. These results were similar to the second hatching success noted for *D. magna* in our study (Figure 3). *T. platyurus* could have some mechanism which prevents it from hatching in high salinity; this would account for the higher sum of both hatchings at NaCl-1 than NaCl-2, and the fact this was not observed for *D. magna*. However, *T. platyurus* is not commonly used in ecotoxicological tests, and further research is needed.

Some of the more interesting ecotoxicological studies have been performed using the Thamnotoxkit test as a diagnostic tool in assessing the potentially toxic effects of airport runoff water on aquatic organisms (Novak et al. 2000; Sulej et al. 2014; Sulej-Suchomska et al. 2021). Sulej-Suchomska et al. (2021) found the highest toxic risk to be in winter and autumn, when most maintenance work, such as de-icing of airport grounds and aircraft, is carried out. The highest toxicity was observed for samples taken just in the de-icing area, near the runway and in the vicinity of the airport terminals. In addition, it is worth noting that these findings also indicate that *T. platyurus* is generally more sensitive to surface water and wastewater contamination than *Vibrio fischeri* and other bioindicators used in bioassays.

3.3. The impact of incubation time and hatching environment on hatching success

The next part of the study examined the impact of egg incubation in different road salt concentrations for three (T3) and seven (T7) days. Generally, our results confirm previous findings indicating that multi-day incubation time appears to have no significant effect of NaCl on hatching success, unlike to multi-year storage in exposition on other pollutants, like heavy metals, organic pollutants and ionizing radiation, which causes egg's death (Zadereev et al., 2019). Results of two-way ANOVA showed that NaCl concentration had significant effect on hatching success in conducted study. No significant interactions between NaCl concentration and incubation time were found to affect hatching success. For *T. platyurus*, at the first hatching, significantly different hatching success were noted between all NaCl concentration, beside NaCl-1 with NaCl-2, and EPA control with NaCl-5. (Figure S1). At the sum of both hatching, no significant differences were found between EPA control with NaCl-5, and NaCl-1 with NaCl-3. (Figure S1).

For *D. magna*, in the first hatching, hatching success noted for NaCl-1 and NaCl-2 were significant different from each other and from other NaCl concentration and ISO control (Figure S2). After the second hatching, the only difference was that the hatching success between NaCl-1 and NaCl-2 was not significantly different.

Möst et al. (2015) report *D. longispina* demonstrated significantly greater hatching success in samples exposed to organic contaminants for four days compared to untreated controls: the contaminants consisted of a 1000-fold increased concentration of a complex mix of 10 organic micropollutants with nominal concentrations between 2 mg/L and 800 mg/L previously detected in the sediments. In the present study, both *D. magna* and *T. platyurus* demon-

Table 1

Percentage of SEM observations made after opening the ephippia, falling into each category.

	THREE-DAY EXPOSURE						SEVEN-DAY EXPOSURE			
	CONTROL	NaCl-5	NaCl-2	NaCl-2 (rinsed)	NaCl-1	NaCl-1 (rinsed)	CONTROL	NaCl-3	NaCl-2	NaCl-1
EMPTY	63.33	33.33	16.67	46.67	30	50	66.67	50	26.67	20
SINGLE EGG	10	26.67	20	26.67	16.67	26.67	13.33	23.33	36.67	10
TWO EGGS	13.33	10	10	6.67	10	6.67	3.33	3.33	13.33	16.67
SINGLE EMBRYO	6.67	6.67	13.33	10	33.33	13.33	3.33	20	0	13.33
SINGLE EMBRYO WITH AN EYE/ANTENNAE	6.67	3.33	30	6.67	6.67	0	6.67	0	10	13.33
TWO EMBRYOS	0	6.67	3.33	0	0	0	0	0	3.33	16.67
TWO EMBRYOS WITH AN EYE/ANTENNAE	0	6.67	0	0	0	0	3.33	0	3.33	0
EGG + EMBRYO	0	6.67	6.67	3.33	3.33	3.33	3.33	3.33	6.67	10

CHI-SQUARED TEST RESULTS										
p value	3 DAYS EXPOSURE						7 DAYS EXPOSURE			
	CONTROL	NaCl-5	NaCl-2	NaCl-2 (rinsed)	NaCl-1	NaCl-1 (rinsed)	CONTROL	NaCl-3	NaCl-2	NaCl-1
		0.064	<0.001	0.132	<0.001	0.048		<0.001	<0.001	<0.001

strated greater hatching success after T3 and T7 for NaCl-5 and NaCl-4 than controls, but no significant differences were observed.

The final step was to investigate the hatching success of *T. platyurus* and *D. magna* eggs washed with water after incubation for three and seven days (T3 and T7). No significant differences in hatching success between NaCl concentrations except sum of both hatching for *T. platyurus* were found (Figures S3 and S4). Moreover, there were also no significant interactions between NaCl concentration and incubation time affecting hatching success (Two-way ANOVA test; $p > 0.05$).

The results indicate that the eggs protected the embryos against NaCl pollution for up to seven days of exposure. However, it appears that NaCl concentration has a greater influence at times when temperature and light conditions are suitable for hatching (Figure 2 and Figure 3).

In order to assess whether and how NaCl concentrations directly affected eggs and embryos, observations of *D. magna* ephippia were carried out using SEM microscopy. Two groups of ephippia (thirty for each salt concentration and for the control) differing in incubation time were tested. The first group included individuals exposed to rising NaCl concentrations (control, NaCl-5, NaCl-2, NaCl-1) for three days (T3) and ephippia from NaCl-2 and NaCl-1 concentrations that were rinsed with water, while the second was exposed to rising NaCl concentrations (control, NaCl-3, NaCl-2 and NaCl-2) for seven days (T7). The observations made after opening the ephippium were divided into eight categories, as described in the Methods, and then analysed using a chi-square test to identify differences between frequencies of the observed categories for different NaCl concentrations, with the control used as a reference. For the T3 group, a significant difference was found between controls and NaCl-2 ($p < 0.001$), as well as between controls and NaCl-1 ($p < 0.001$) and controls and NaCl-1 (rinsed) ($p = 0.048$). For the T7 group, all of the NaCl concentrations significantly differed from the control ($p < 0.001$). This could suggest that exposure to the highest NaCl concentrations during the T3 experiment had an

effect on the egg hatching process, resulting in the eggs or embryos being unable to hatch properly. For the T7 group, this effect also extended to the lower NaCl concentration (NaCl-3), at which significant differences were found with controls. The percentage observations falling into each category, as well as the chi-squared test results (p values), are shown in Table 1.

After three- and seven-days exposure, the control samples demonstrated the highest hatching success, indicated by the highest percentage of empty ephippia. Significant differences in ephippia numbers were found between the control samples and those exposed to NaCl-2, NaCl-1 or NaCl-1 rinsed in the T3 group, or NaCl-3, NaCl-2 and NaCl-1 in the T7 group. These samples more frequently presented ephippia containing a single egg or an embryo showing different stages of development. These results suggest that exposure to NaCl influenced the ephippia and caused hatching difficulties for the *D. magna*: either stopping the development at an early stage or preventing the developed organisms from leaving the ephippium. This could indicate that the ephippial case may not offer total protection against the effects of NaCl. Similarly, a study of the hatching success of dormant *D. magna* eggs after exposure to fenoxycarb (Navis et al. 2013), found that the tissue concentrations of the insecticide did not differ significantly between decapsulated and encapsulated eggs, and proposed that the ephippial case offered limited protection. The chi-square test used in our study showed differences in hatching success of *D. magna* depending on NaCl concentration and incubation time, but we did not observe any damage or deformation of eggs or embryos caused by increasing salinity. Analysis of the photographs did not reveal any visible differences between the eggs and embryos from different salt concentrations, nor any visible features that could suggest damage to the organisms (Figure 4).

3.4. EC_x values for *D. magna*

The mean EC_{10} for *D. magna* was 5.867 g NaCl/L for the first hatching and 4.834 g/L for the sum of both hatch-

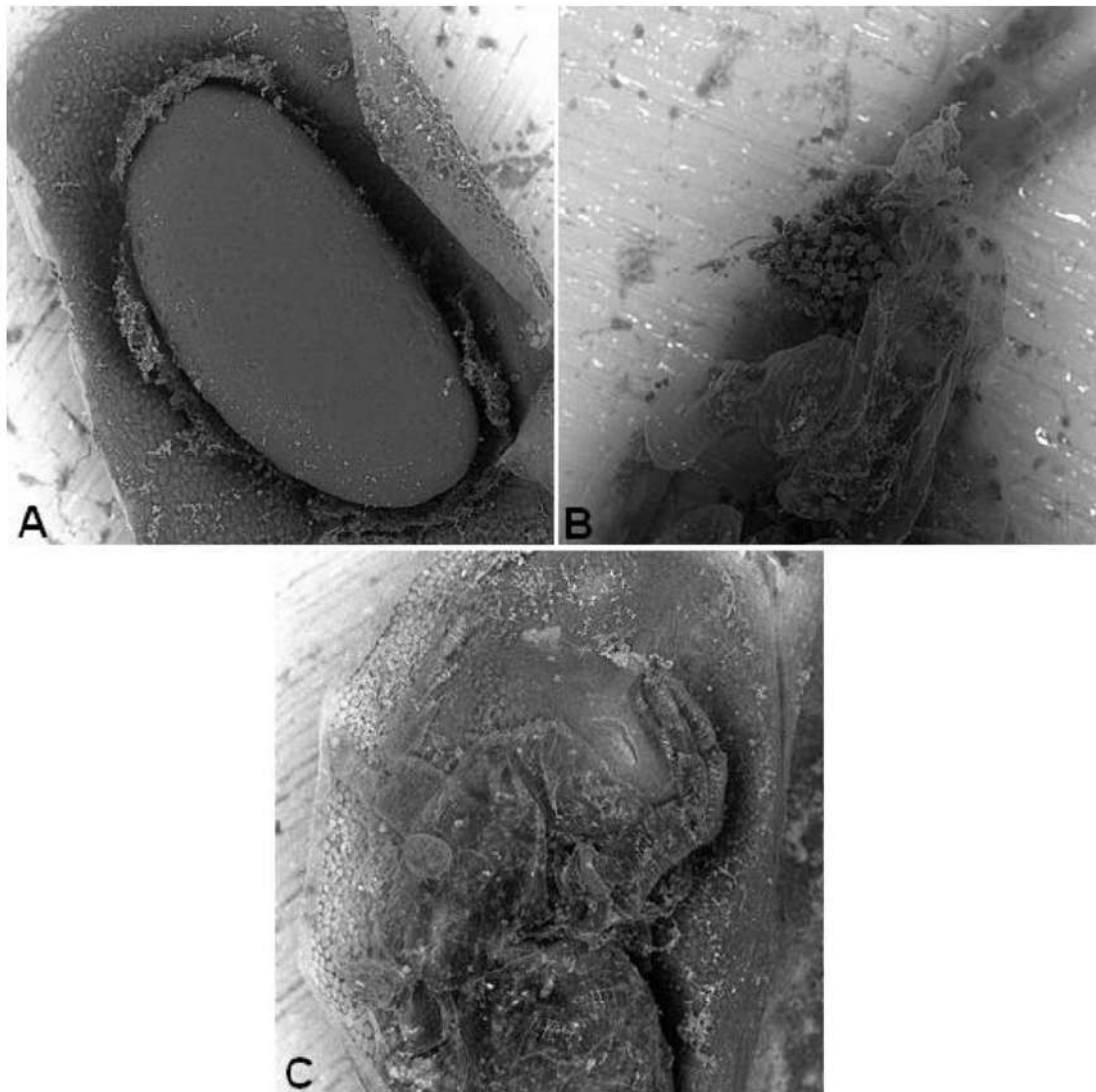


Figure 4. Photographs obtained from the SEM microscope. A: Single egg from NaCl-2, 7-day exposure sample, magnification 530x; B: Embryo with visible eye and antennae from NaCl-2, 7-day exposure sample, magnification 930x; C: Embryo with visible antennae from NaCl-2, 3-day exposure (rinsed) sample, magnification 420x.

ings; these were not significantly different to the 24-h EC_{10} value: 4.667 g/L (Table 2). However, for EC_{50} , a significant difference was found between the 24-h EC_{50} noted for adults (4.667 g/L) and the values for the first hatching (7.041 g/L) and both hatchings (6.921 g/L). In the case of *T. platyurus* no significant difference was found between either EC value (EC_{10} or EC_{50}) for the first hatching and toxicity for adults; in addition, these values were lower than those for *D. magna*. For first hatching of *T. platyurus*, EC_{10} was 1.996 g/L and EC_{50} was 3.799 g/L (Table 2). Due to the increasing hatching success between NaCl-2 and NaCl-1, no EC values for the sum of both hatchings were determined for *T. platyurus* (Figure 3), as these results were not anticipated in the determined EC_x values.

The EC_{10} and EC_{50} values of NaCl for *D. magna* hatching were higher than the EC 24-h values noted for adults

(Table 2). This indicates that a lower number of individuals might survive a short time after hatching, resulting in a lower number of larger grazers in the zooplankton community. The second hatching results for *D. magna* and *T. platyurus* (Figure 3) showed that insufficient dilution of road salt pollution after winter could impact spring zooplankton community structure and the efficiency of top-down control of phytoplankton biomass. Lembcke et al. (2017) noted that in a pond receiving stormwater from a parking lot in Canada, a Cl level above 0.640 g/L, indicating acute toxicity, remained in surface water until June and in over-sediment water until September (above 2.00 g/L from March until June, with a maximum 5.50 g/L in March). Our results suggest that such a value might decrease hatching success of zooplankton species (Figure 3).

Table 2

Mean EC₁₀ and EC₅₀ values (with confidence intervals $\pm$ 95%) for *D. magna* and *T. platyurus* for first hatching and sum of both hatching, 24-h EC values for adults. Different letters mean significant differences (t-test; $p < 0.05$) separately for each species.

		<i>Daphnia magna</i>	<i>Thamnocephalus platyurus</i>
EC ₁₀ [g NaCl/L]	First hatching	5.867 ^A (4.214 – 6.340)	1.996 ^A (1.526 – 2.763)
	Sum of both hatching	4.834 ^A (3.196 – 6.250)	n.a.
	Toxicity for adults*	4667 ^A (3.973 – 5.292)	2.121 ^A (1.611 – 2.863)
EC ₅₀ [g NaCl/L]	First hatching	7.041 ^A (6.391 – 7.234)	3.799 ^A (3.339 – 4.280)
	Sum of both hatching	6.921 ^A (6.613–7.236)	n.a.
	Toxicity for adults*	5.244 ^B (4.824 – 5.547)	3.289 ^A (2.733 – 3.715)

* Results from other research – (Szklarek et al., 2022b)

n.a. – not analysed, because of not standard curve of results – the highest concentration did not have the lowest death ratio – see Figure 3.

Moreover, climate change increases evapotranspiration, changes the precipitation and flow regime characteristics and increases water temperature (Reid et al., 2019; Cartwright et al., 2021); all of these increase the frequency of spring droughts (Swain and Hayhoe, 2015) and increase freshwater salinization (Jones and van Vliet, 2018), and can impact freshwater ecosystem structure (Brock et al., 2005) and increase the biomass of phytoplankton (Lind et al., 2018; Szklarek et al., 2022a). Further studies are hence needed to determine the effect of decreasing NaCl concentration gradients and increasing temperature gradients on hatching success.

4. Summary

Our assessment of the effect of road salt on the hatching success of zooplankton species lies within the research priorities on the ecological and evolutionary importance of freshwater salinity (FS) (Cunillera-Montcusí et al., 2022). It is also the first step to determining the factors influencing zooplankton community functioning and its consequences to ecohydrological processes (Cunillera-Montcusí et al., 2022; Kaushal et al., 2021; Szklarek et al., 2022a).

Zooplankton species, particularly *Daphnia* spp., are very useful in ecotoxicological studies. Tonkopp and Iofina (2007) used *Daphnia magna* as an indicator organism to determine the presence of anthropogenic pollution in freshwater systems in Japan. The high sensitivity of *D. magna* has even led it to be seen as a more ethical and economical substitute for mammals during preliminary toxicity testing of some chemicals (Guilhermino et al., 2000).

Our research uses pure laboratory lines of *D. magna* and *T. platyurus* to investigate the natural mechanism of successful hatching in response to road salt pollution. The determining factor for hatching success in both species was NaCl concentration. This is evidenced by the fact that no neonates were observed at the highest NaCl-1 concentration (15.20 g/L), even though light and temperature conditions were optimal for hatching. In contrast, high hatching success was noted after rinsing the eggs with water, especially in *T. platyurus*. It is possible that in both species, there is a physiological mechanism to prevent hatching under extremely critical conditions. Our re-

sults also confirm previous observations (Szklarek et al., 2022b) that *D. magna* is more resistant to road salt than *T. platyurus*: hatching success was found to decrease gradually with increasing NaCl concentration in *T. platyurus*, but decrease sharply in *D. magna* when the threshold value was exceeded. This may be due to the high adaptive potential of the *Daphnia* genus (Arnér and Koivisto, 1993; Teschner, 1995; Zadereev et al., 2021). A characteristic feature of *Daphnia* biology is its phenotypic plasticity, defined as the ability of a genotype to adjust its metabolism and physiology in response to current environmental conditions (Elmarsafy et al., 2021; Ebert, 2022). This phenotypic plasticity can be seen on both the natural environment and in laboratory studies. For example, Parlato and Kopp (2020) observed that *D. magna* specimens reared under increasing salinity conditions were significantly more resistant to osmotic stress when placed in a 2.30 g NaCl/L solution than specimens previously reared in pure water. Interesting results were presented by Ortells and co-authors (2005) investigating the hatching success of different age-classes of resting *D. magna* eggs from the lake sediment in different salt concentrations. The authors observed that, as in our study, the salinity of the solution appeared to have the strongest effect on neonate survival. Non-adapted genotypes were rapidly eliminated after hatching, while those that survived acquired the ability to function in the range of elevated salinity conditions, due to phenotypic plasticity.

However, over a longer period of time, the presence of an agent in the environment can lead to the development of local adaptations of resistant *Daphnia* clones resulting from genetic differences. Liu and Steiner (2017) found that clonal lines that had been bred for several generations from populations taken from high salinity ponds showed greater survival in acute toxicity tests than lines bred from low salinity ponds.

Some authors (e.g. Ortells et al., 2005) suggest that sexual reproduction and the production of persistent ephippia-type eggs allows dormant forms of *Daphnia* to survive in sediments under high salinity conditions. However, Bailey et al. (2004) observed that higher salinity significantly increased *Daphnia* mortality in ephippia collected from Great Lakes sediments in North America. In our study, although high salinity was not found to dam-

age the ephippia, the salt may have a negative effect on eggs and embryos, as indicated by their lower hatching success in these experimental variants. The variation in resistance to salt concentrations by ephippia taken from the natural environment may be due to genetic adaptations developed over a long evolutionary process. For example, Branstrator et al. (2013) found the hatching success of the cladoceran *Bythotrephes longimanus* resting eggs to not decrease in response to long-term high salinity (up to 4 weeks at 35 g/L salt). This suggests that, despite the species' current preference for fresh water, salinity exposure may have been important in the evolution of *B. longimanus*, originating from euryhaline ponds, and that high salinity conditions are not stressful to its ephippia. In addition, complex interactions between temperature, egg metabolic rate, membrane permeability and dormancy cues, as well as the eggs being protected by overlying sediments, may be influence the rate and quality of cladoceran hatching (Aladin and Potts 1995). Some researchers have examined the close relationship between temperature and salinity: Branstrator et al. (2013) and Gray et al. (2005) found high salinity (up to 32 g/L salt) to have no negative effect on the hatching of cladoceran resting eggs when kept at 4°C; while Bailey et al. (2006) report that zooplankton resting eggs exposed to 8 g/L salinity at 10°C or 30°C had greater hatching success than those at 20°C.

Hence, our results indicate that further research is needed to investigate the effects of road salt and its contaminant mixtures on the hatching success of zooplankton from egg banks. These studies should take into account the various conditions resulting from increasing freshwater salinity and the consequences of climate change for aquatic ecosystems.

Declaration of interests

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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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ecohyd.2023.06.005.

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SUPPLEMENTARY MATERIAL

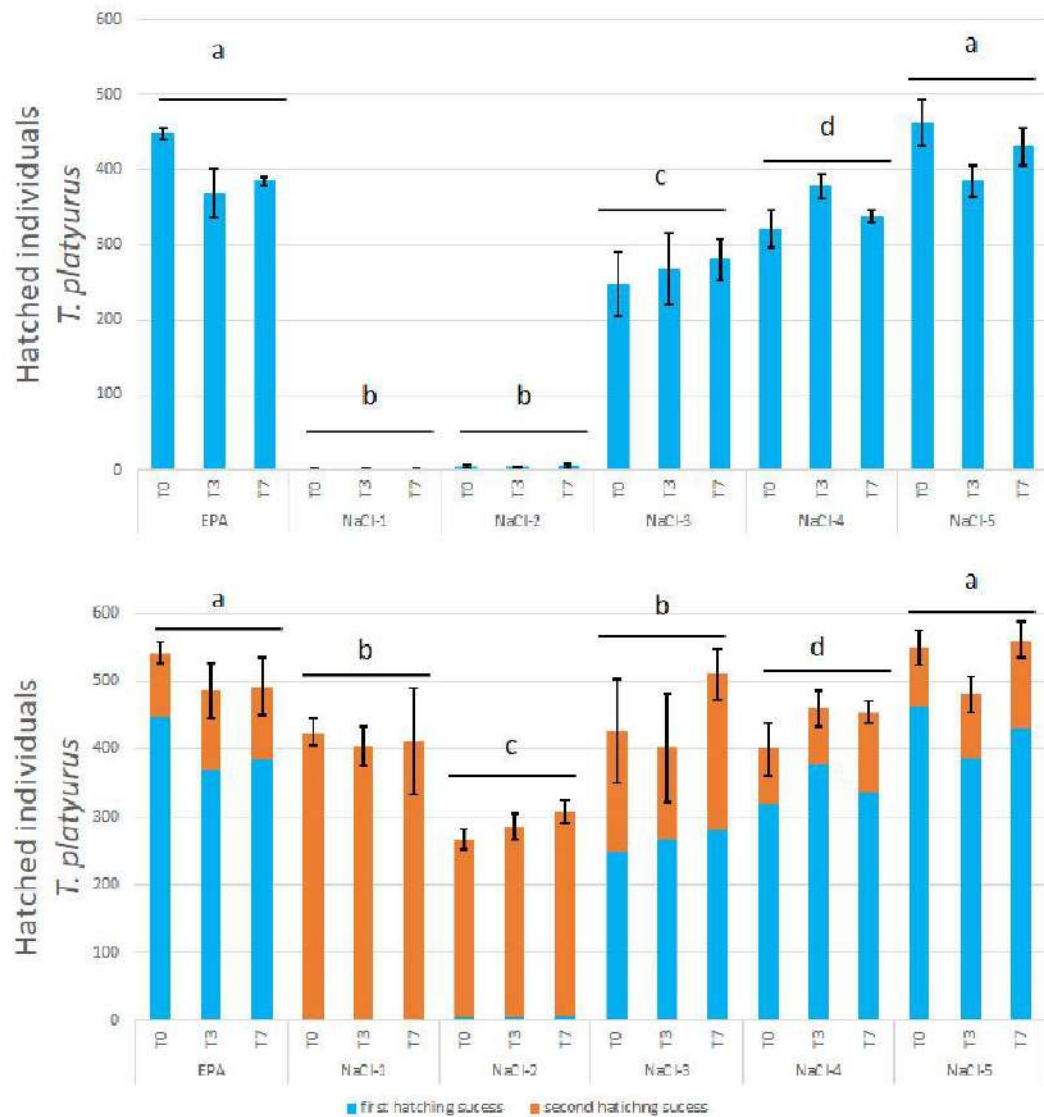


Figure S1 Average (with SD) of hatched individuals of *T. platyurus* at first (upper) and both (down) hatching at control samples and five NaCl dilutions after 3 and 7 days of incubation in refrigerator (T3 and T7, respectively) in comparison to hatching success in samples without incubation period (T0). Different letters mean significant difference between NaCl concentration in two-way ANOVA with $p < 0.05$.

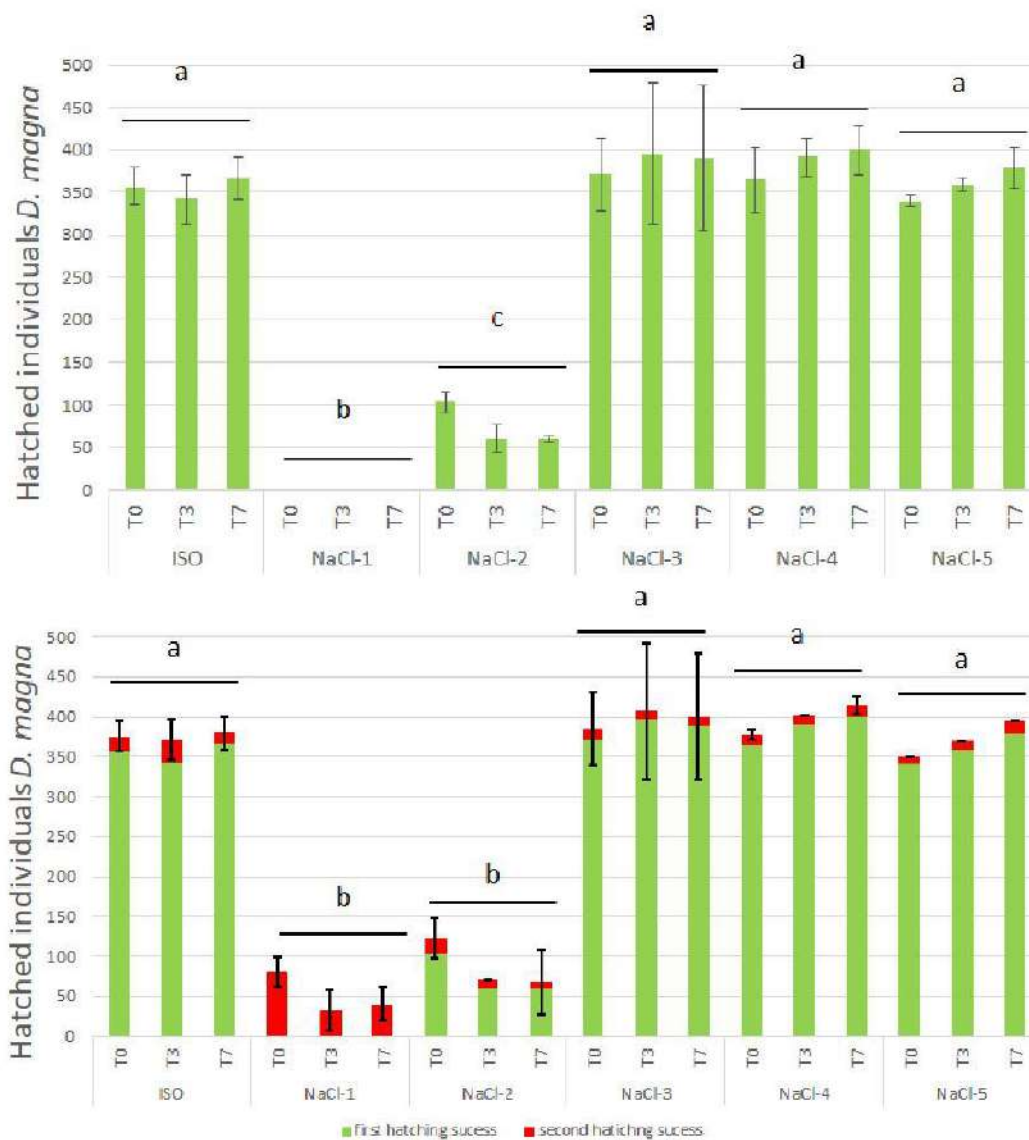


Figure S2 Average (with SD) of hatched individuals of *D. magna* at first (upper) and both (down) hatching at control samples and five NaCl dilutions after 3 and 7 days of incubation in refrigerator (T3 and T7, respectively) in comparison to hatching success in samples without incubation period (T0). Different letters mean significant difference between NaCl concentration in two-way ANOVA with $p < 0.05$.

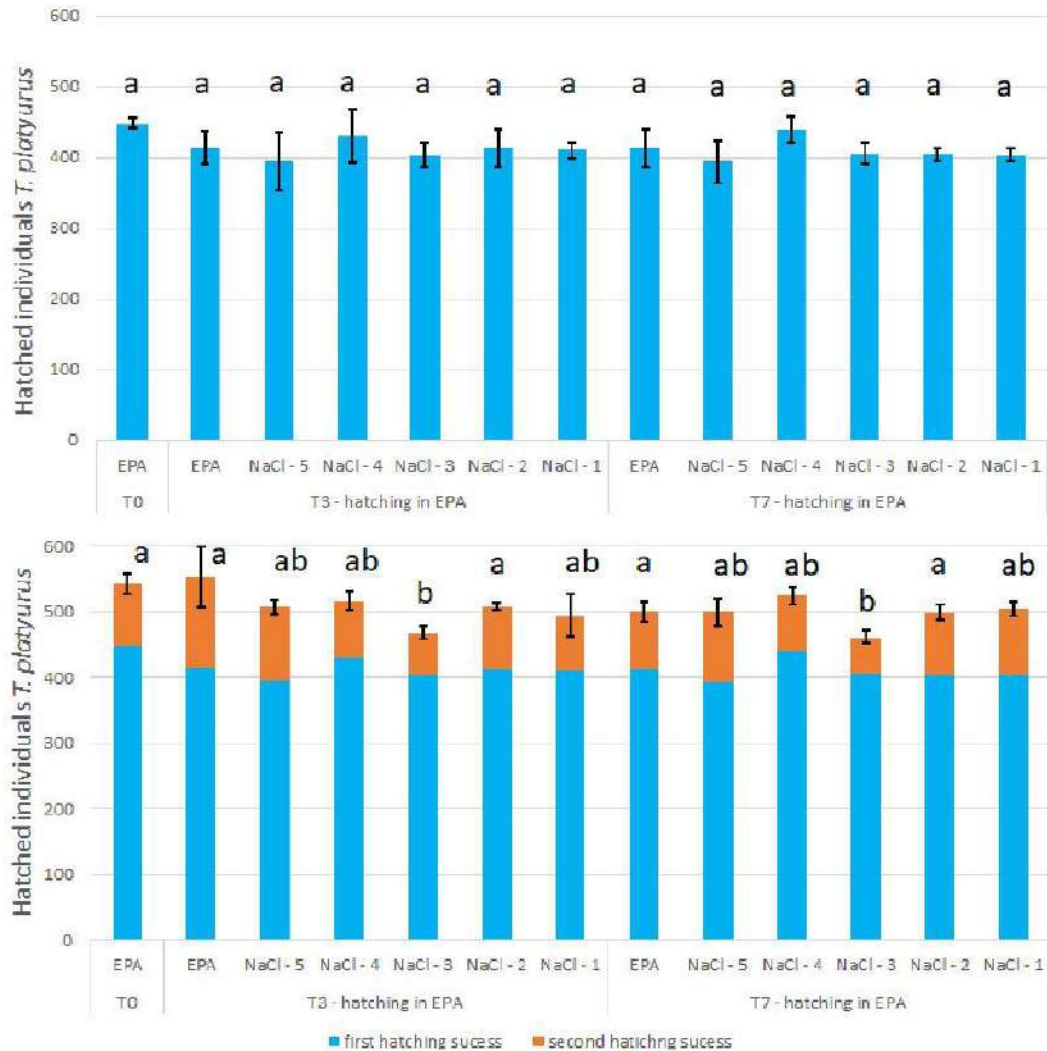


Figure S3 Average (with SD) of individuals of *T. platyurus* hatched in standard freshwater (EPA) after wash eggs after incubation at first (upper) and sum of both hatching (down) at control (EPA) and five NaCl dilutions after 3 and 7 days of incubation in refrigerator (T3 and T7 respectively) compared to results of hatching in EPA without incubation (EPA T0). Different letters mean significant difference between NaCl concentration in two-way ANOVA with $p < 0.05$.

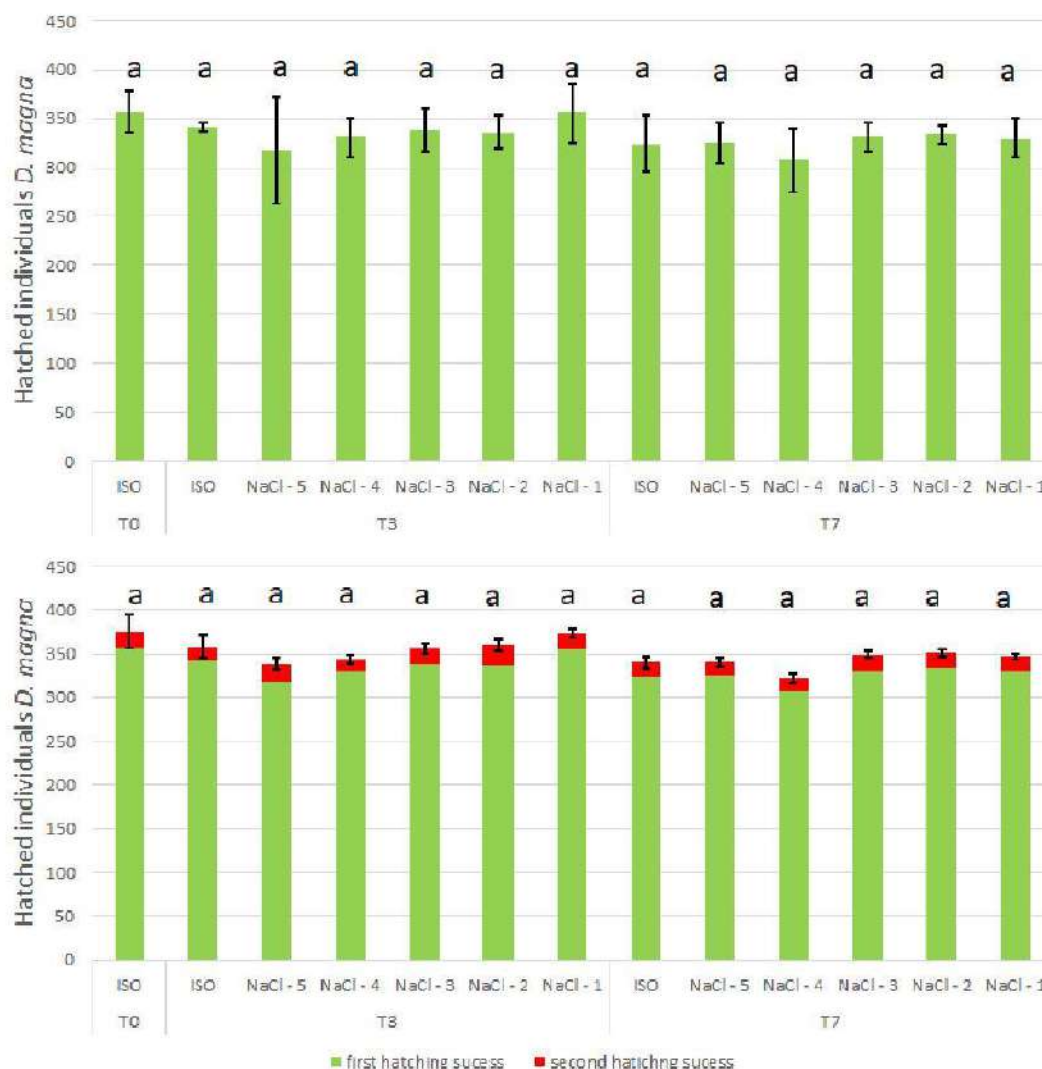


Figure S4 Average (with SD) of individuals of *D. magna* hatched in standard freshwater (ISO) after wash eggs after incubation at first (upper) and sum of both hatching (down) at control (ISO) and five NaCl dilutions after 3 and 7 days of incubation in refrigerator (T3 and T7 respectively) compared to results of hatching in ISO without incubation (ISO T0). Different letters mean significant difference between NaCl concentration in two-way ANOVA with $p < 0.05$.

PUBLIKACJA NR V - MANUSKRYPT

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 **Praca doświadczalna**

Odpowiedź antyoksydacyjna situ rozpięzchłego (*Juncus effesus*) i oczeretu jeziornego (*Schoenoplectus lacustris*) na zanieczyszczenie solą drogową

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1. Wstęp

Postępujące zasolenie naturalnych ekosystemów staje się coraz poważniejszym problemem dotyczącym wszystkie regiony świata. Wtórne zasolenie, tzn. wynikające z działalności człowieka stanowi rosnące zagrożenie dla ekosystemów słodkowodnych i może przyczyniać się do zmian w strukturze i funkcjonowaniu zbiorowisk słodkowodnych organizmów (Cunillera-Montcusí i in., 2022). Również ekosystemy lądowe narażone są na nadmierne zasolenie. Szacuje się, że zasolenie gleb dotyka ponad 10 milionów km² łądów na świecie i przekłada się na spadek jakości usług ekosystemowych, np. zmniejszoną produktywność upraw rolnych (Litalien i Zeeb, 2020). Obok takich czynników jak zwiększająca się liczba ludności, postępujące zmiany klimatu czy niedostatki wody słodkiej, degradacja gleb spowodowana przez zasolenie wymieniana jest wśród przyczyn zagrażających bezpieczeństwu żywnościowemu na Ziemi (Zaman i in., 2016). Do antropogenicznych

przyczyn zasolenia ekosystemów zaliczyć można: irygację terenów rolnych, intensywne nawożenie oraz stosowanie pestycydów, wydobywanie ropy, odpady z przemysłu wydobywczego, papierniczego, metalurgicznego oraz nadmierne stosowanie soli drogowej w celu zimowego utrzymania dróg (Litalien i Zeeb, 2020; Kłosowska, 2010), szczególnie w przypadku regionów klimatu umiarkowanego.

W krajach, w których występują zimy z oblodzeniem i opadami śniegu, stosowanie soli drogowej (NaCl) jest powszechną praktyką, mającą na celu zapewnienie bezpieczeństwa w ruchu pojazdów i pieszych. W dobie szybko postępującej urbanizacji odsetek powierzchni utwardzonych, które wymagają zabezpieczenia w czasie zimy wzrasta. Wraz z topniejącym śniegiem i lodem, jony sodowe oraz chlorkowe przedostają się do wód powierzchniowych, przenikają do gleb i za pośrednictwem wód podziemnych docierają do pobliskich zbiorników wodnych (Rogora i in., 2015, Gallagher i in., 2011, Snodgrass i in., 2017). Warto zauważyć, że zanieczyszczenie solą drogową gleb oraz wód słodkich jest problemem, który nie ogranicza się wyłącznie do miesięcy zimowych. Podwyższone stężenia jonów chlorkowych obserwowane są w zbiornikach miejskich także w miesiącach wiosennych, a nawet letnich (Górecka i in., 2023, w druku).

Zanieczyszczenie solą drogową ma wpływ na wiele organizmów żywych, począwszy od bakterii, aż po organizmy kręgowce (Szklarek i in., 2022; Hintz i Relyea, 2019). Również rośliny są wrażliwe na podwyższone zasolenie. Do skutków takiej ekspozycji zaliczyć można np. zmniejszenie dostępności do wody i tlenu dla korzeni spowodowane zmianami w strukturze gleby; redukcję lub odwrócenie gradientu osmotycznego, co wpływa na zaburzenia w pobieraniu wody przez korzenie roślin i prowadzi do suszy fizjologicznej; zaburzenia w gospodarce jonowej prowadzące do deficytów kationu K^+ ; a także spadek wydajności i zaburzenia procesu fotosyntezy (Litalien i Zeeb, 2020; Kłosowska, 2010). Nieprawidłowości w przebiegu procesu fotosyntezy powodowane przez podwyższone stężenia soli mogą

doprowadzić do nadprodukcji reaktywnych form tlenu (RFT), spadku efektywności procesów naprawczych i w konsekwencji do stresu oksydacyjnego. Reaktywne formy tlenu mogą powodować w komórkach roślinnych m.in. uszkodzenia białek prowadzące do ich inaktywacji, peroksydację lipidów, utlenianie składników kwasów nukleinowych oraz uszkodzenia barwników fotosyntetycznych (Łata, 1998; Kłosowska, 2010).

Rośliny poddane stresowi solnemu wykazują złożone reakcje molekularne, uruchamiające mechanizmy odpornościowe (Zhu i in., 1997). Jednym z takich mechanizmów jest aktywacja systemu antyoksydacyjnego, w którego skład wchodzi enzymy (np. katalazy, dysmutaza nadadtlenkowa, reduktaza glutationowa) oraz niskocząsteczkowe antyoksydanty takie jak np. glutation. Wydajna ochrona antyoksydacyjna może zwiększyć tolerancję roślin na stres solny (Zhu, 2001), co stwarza możliwości wykorzystania odpowiednio dobranych gatunków do tworzenia zielonej infrastruktury w miastach. Pojawia się jednak potrzeba opracowywania nowych rozwiązań, które przyczyniałyby się do zmniejszenia stężeń jonów sodowych i chlorkowych w środowisku, a w szczególności w wodach powierzchniowych. Stąd, istotne jest podejmowanie badań dotyczących potencjału roślin odpornych na zasolenie do oczyszczania ekosystemów słodkowodnych z nadmiaru soli (fitodesalinizacji środowiska wodnego) (Manousaki i Kalogerakis, 2011), gdyż wiedza na temat praktycznego wykorzystania roślin halofilnych jest wciąż ograniczona.

Rozwiązaniami, które mogą być wykorzystane do oczyszczania wody zanieczyszczonej solą drogową są np. sztuczne mokradła (ang. *constructed wetlands*) (Suair i in., 2016; Guesdon i in., 2016). Istotą konstrukcji efektywnie działających sztucznych mokradeł, które pozwoliłyby na niwelowanie zanieczyszczenia solą drogową jest dobór gatunków roślin, które będą tolerować warunki stresowe i jednocześnie skutecznie akumulować jony sodu i chloru w swoich tkankach. Doniesienia literaturowe wskazują na potencjał do fitodesalinizacji takich gatunków jak łoboda solniskowa, łoboda ogrodowa (Suair i in., 2016), łoboda rozłożysta, pałka

szerokolistna (Morteau i in., 2009), palka wąskolistna, sit morski (Guesdon i in., 2016) czy oczeret Tabernemontana (Rozema i in., 2014). Są to jednak gatunki obcego pochodzenia, mogące stwarzać zagrożenie dla rodzimej flory.

Celem niniejszej pracy była ocena potencjału fitodesalinizacji dwóch polskich gatunków roślin: situ rozpięzchłego (*Juncus effesus*) i oczeretu jeziornego (*Schoenoplectus lacustris*). Postawiono następujące pytania: 1) jaki wpływ ma sól drogowa na wybrane parametry stresu antyoksydacyjnego u situ rozpięzchłego i oczeretu jeziornego? 2) w jakim stopniu wybrane dwa gatunki roślin mają zdolność do akumulacji jonów sodu? 3) czy wybrane dwa gatunki roślin mogą być uznane za przydatne w redukcji szkodliwego zanieczyszczenia wód powierzchniowych solą drogową?

2. Materiały i metody

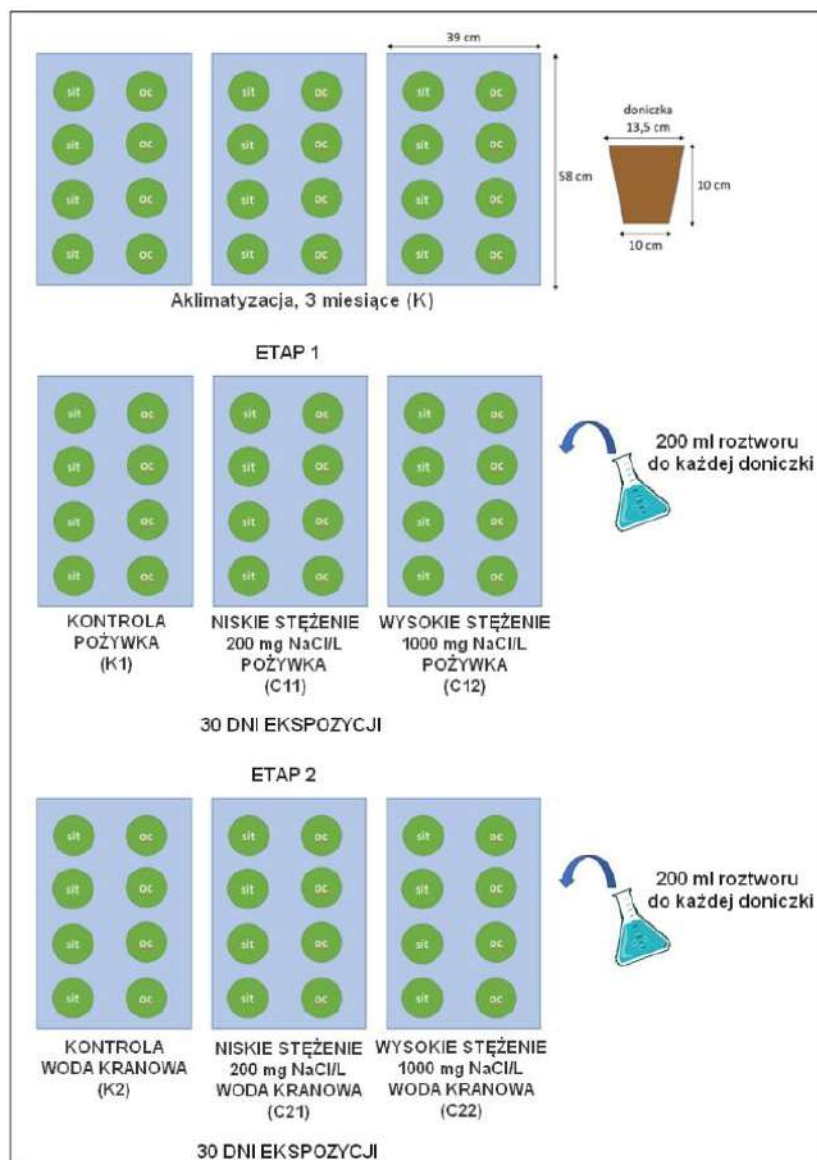
2.1. Rośliny i ich ekspozycja na zasolenie

Do badań wybrano dwa gatunki roślin: sit rozpięzchły (*Juncus effesus*) i oczeret jeziorny (*Schoenoplectus lacustris*). Przy wyborze tych gatunków kierowano się następującymi kryteriami: 1) pochodzenie – rośliny są rodzime dla Polski, 2) wcześniejsze doniesienia o zdolności rodzaju *Juncus* oraz *Schoenoplectus* do znoszenia zasolenia, 3) dostępność zakupu sadzonek. Ekspozycję roślin na zasolenie prowadzono w oparciu o metodykę opisaną przez Morteau i in. (2009). Sit rozpięzchły i oczeret jeziorny zostały zakupione w postaci sadzonek w firmie Perfekt Klik, a następnie zasadzone w doniczkach o średnicy 13,5 cm, wysokości 10 cm. Podłoże stanowiła mieszanka piasku krzemionkowego i podłoża uniwersalnego (skład: torf, perlit, agrosil, dodatek nawozu NPK) w stosunku 1:1. Zasadzono 12 sadzonek z każdego gatunku rośliny. Doniczki umieszczono w trzech plastikowych pojemnikach o wymiarach 39 x 58 cm, tak aby w każdym pojemniku znalazły się cztery sadzonki situ i cztery sadzonki oczeretu. Rośliny podlewano (do pojemników) odstaną wodą kranową przez okres

3 miesięcy, w celu zaaklimatyzowania się roślin. Przez cały okres aklimatyzacji oraz trwania eksperymentu rośliny znajdowały się w namiocie (*grow-box*), gdzie utrzymywana była stała temperatura (26°C , $\pm 3^{\circ}\text{C}$) oraz fotoperiod (16 h światła, 8 h ciemności). Przygotowano trzy warianty eksperymentu: kontrolę, wariant eksponowany na niskie stężenia soli i wariant eksponowany na wysokie stężenia soli. W każdym wariancie były cztery powtórzenia dla każdego gatunku.

Po aklimatyzacji, przez okres 30 dni rośliny kontrolne podlewano mieszanką wody destylowanej z pożywką Hoagland'a (Hoagland i Arnon, 1950) w rozcieńczeniu $\frac{1}{4}$, natomiast rośliny badane podlewano wodą destylowaną z pożywką Hoagland'a w rozcieńczeniu $\frac{1}{4}$ z dodatkiem NaCl, tak aby uzyskać stężenie 200 mg NaCl/L (stężenie niskie) i 1000 mg NaCl/L (stężenie wysokie). Do każdej doniczki wlewano 200 ml przygotowanego wcześniej roztworu.

Po zakończeniu okresu 30 dni, ekspozycję roślin na sól kontynuowano, jednak zrezygnowano z użycia pożywki, aby sprawdzić czy wspomaganie mineralne miało znaczenie w warunkach stresu. Rośliny kontrolne podlewano odstaną wodą kranową, natomiast pozostałe rośliny odstaną wodą kranową z dodatkiem NaCl, tak aby uzyskać podane wyżej stężenia. Do każdej doniczki wlewano 200 ml wcześniej przygotowanego roztworu. Druga ekspozycja na sól drogową również trwała 30 dni. Zastosowano następujące nazwy wariantów badawczych: K – kontrola w czasie aklimatyzacji podlewana wodą kranową, K1 – kontrola podlewana wodą destylowaną z pożywką, C11 – woda destylowana + pożywka + niskie stężenie NaCl (200 mg NaCl/L), C12 – woda destylowana + pożywka + wysokie stężenie NaCl (1000 mg NaCl/L), K2 – kontrola podlewana wodą kranową, C21 – woda kranowa + niskie stężenie NaCl (200 mg NaCl/L), C22 – woda kranowa + wysokie stężenie NaCl (1000 mg NaCl/L). Schematyczny przebieg ekspozycji roślin na zasolenie przedstawiono na Ryc. 1, natomiast rośliny w trakcie doświadczenia przedstawia fotografia Ryc. 2.



Ryc. 1. Schematyczny przebieg ekspozycji roślin na zasolenie. Oznaczenia: sit – sili rozprzeczty; oc – oczeret jeziorny.



Ryc. 2. Fotografia przedstawiająca rośliny w trakcie doświadczenia w namiocie grow-box. Autorstwo zdjęcia: Justyna Nebelska

2.2. Przygotowanie materiału roślinnego do oznaczeń parametrów stresu oksydacyjnego

Nadziemne tkanki roślin pobrano trzykrotnie, tj. 1) przed ekspozycją na zasolenie, 2) po ekspozycji pożywka + sól drogowa oraz 3) po ekspozycji woda kranowa + sól drogowa.

w roztworze buforu fosforanowego (5 mM, pH = 7,4) (1 g mokrej masy liści + 4 ml buforu fosforanowego). Homogenizację materiału prowadzono przy użyciu homogenizatora nożowego (CAT M. Zipperer X 120) w lodzie przez 10 min. Następnie próbki wirowano 15 000 obr/min przez 10 min. Zebrany supernatant przechowywano w temperaturze -20°C (maksymalnie 10 dni) do momentu dalszych analiz.

2.3. Oznaczenie stężenia białka

Stężenie białka w homogenatach oznaczono przy użyciu odczynnika Folina-Ciocalteu metodą opisaną przez Lowry'ego i współautorów (Lowry i in., 1951).

2.4. Ocena stopnia peroksydacji lipidów

Stopień peroksydacji lipidów w homogenacie oznaczono przy użyciu kwasu tiobarbiturowego (TBA) opisanego przez Stocks i Dormandy (Stocks i Dormandy, 1971) z modyfikacją Rice-Evans i wsp. (Rice-Evans i in., 1991). W niskim pH TBA reaguje z produktami peroksydacji lipidów tworząc barwne produkty TBARS (thiobarbituric acid reactive substances). Końcowe produkty reakcji oznaczono przy 535 nm, a stężenie TBARS obliczono stosując milimolowy współczynnik absorpcji ($\epsilon = 156 \text{ mmol}^{-1} \cdot \text{L} \cdot \text{cm}^{-1}$). Stężenie TBARS wyrażono w nmol/mg białka.

2.5. Oznaczenie stężenia zredukowanego glutationu

Stężenie zredukowanego glutationu (GSH) w homogenatach oznaczono przy użyciu odczynnika Ellmana (kwas 5,5'-ditiobis(2-nitrobenzoesowy); DTNB) (Ellman, 1959). Podczas reakcji DTNB z wolnymi grupami tiolowymi zredukowanego glutationu, powstaje optycznie aktywny 2-nitro-5-tiobenzoesan (NTB). Poziom powstającego NTB, oznaczano spektrofotometrycznie przy długości fali $\lambda = 412 \text{ nm}$. Stężenie GSH obliczono w oparciu o równanie krzywej kalibracyjnej przygotowanej dla różnych stężeń zredukowanego glutationu i wyrażono w nmol/mg białka.

2.6. Oznaczenie aktywności katalazy

Aktywność katalazy w homogenatach została oznaczona spektrofotometrycznie w oparciu o metodę Aebi (1984). Stężenie rozkładanego, przez obecną w homogenacie katalazę, nadtlenku wodoru mierzono przez 1 min. przy długości fali $\lambda = 240$ nm. Spadek absorbancji równy 0,036 przyjmowano jako 1 jednostkę aktywności enzymu (1U). Aktywność katalazy w homogenatach wyrażono jako U/mg białka/min.

2.7. Oznaczenie całkowitego nieenzymatycznego potencjału antyoksydacyjnego

Do oznaczenia całkowitego nieenzymatycznego potencjału antyoksydacyjnego zastosowano metodę opisaną przez Benzie i Strain (1996). Metoda polega na redukcji kompleksu żelazotripirydylotriazyny [Fe(III)-TPTZ] do żelaza [Fe-(II)] w niskim pH. Kompleks żelaza [Fe(III)-TPTZ] zredukowany przez antyoksydanty obecne w homogenacie ma intensywnie niebieską barwę z maksimum absorpcji przy długości fali $\lambda = 593$ nm. Nieenzymatyczny potencjał antyoksydacyjny homogenatu został wyrażony w równoważnikach Troloxu wykorzystanego do przygotowania krzywej kalibracyjnej, a uzyskane wyniki podano w nmol/mg białka.

2.8. Analiza zawartości jonów sodowych w tkankach roślin

Badania dotyczące zawartości jonów sodowych w tkankach roślin prowadzono w oparciu o metodykę opisaną przez Iseki i in., 2017. Nadziemne części roślin zostały pocięte i wysuszone w piecu w temperaturze 70 °C przez 12 godzin. Po wysuszeniu rośliny utarto w moździerzu, a następnie pobrano 50 mg materiału roślinnego i dodano do niego 1 ml wody destylowanej. Tak przygotowane próbki wytrząsano wraz z metalowymi kulkami przez 15 minut na homogenizatorze Tissue Lyser II (Qiagen). Następnie próbki odwirowano (10 000 obrotów/min, 10 minut). Pobierano 400 μ l supernatantu i badano stężenie jonów

sodowych (ppm) na mierniku Na LAQUA Twin. Miernik wcześniej skalibrowano, w oparciu o instrukcje producenta.

2.9. Wykonane analizy statystyczne

Analizy statystyczne wyników obejmowały sprawdzenie zgodności rozkładu danych z rozkładem normalnym za pomocą testu Shapiro–Wilk’a. Homogenność wariancji oceniono za pomocą testu Levene’a. Następnie dla danych wykazujących zgodność z rozkładem normalnym i homogenność wariancji przeprowadzono dwuczynnikową analizę wariancji ANOVA, a następnie test post hoc Tukey’a. W przypadku danych niewykazujących zgodności z rozkładem normalnym do analizy statystycznej zastosowano test nieparametryczny Kruskala–Wallisa oraz test U Manna-Whitneya. Istotność statystyczna była akceptowana dla $p < 0,05$. Analizy statystyczne przeprowadzono przy użyciu programu Statistica v. 13.3 (StatSoft Polska, Kraków, Polska). Dane na wykresach przedstawiono w postaci mediany i rozstępu ćwiartkowego (IQR - interquartile range).

3. Wyniki

3.1. Analiza aktywności systemów antyoksydacyjnych u roślin narażonych na zasolenie

Stopień peroksydacji lipidów. Dla situ rozpięzchłego z kontroli podlewanej wodą z pożywką (K1) wykazano statystycznie istotnie mniejszy stopień peroksydacji lipidów w porównaniu z roślinami traktowanymi wodą kranową i NaCl (stężenie 1000 mg/L) (C22). Dla oczeretu jeziornego nie wykazano statystycznie istotnych różnic w stopniu peroksydacji lipidów pomiędzy badanymi próbkami. W przypadku C12, K2 oraz C22 stopień peroksydacji lipidów był wyższy u roślin z gatunku sit rozpięzchły niż u oczeretu jeziornego (Ryc. 3a).

Stężenie zredukowanego glutationu (GSH). W przypadku situ rozpięzchłego nie wykazano statystycznie istotnych różnic w stężeniu zredukowanego glutationu pomiędzy badanymi próbkami. Natomiast dla oczeretu jeziornego wykazano statystycznie mniejsze

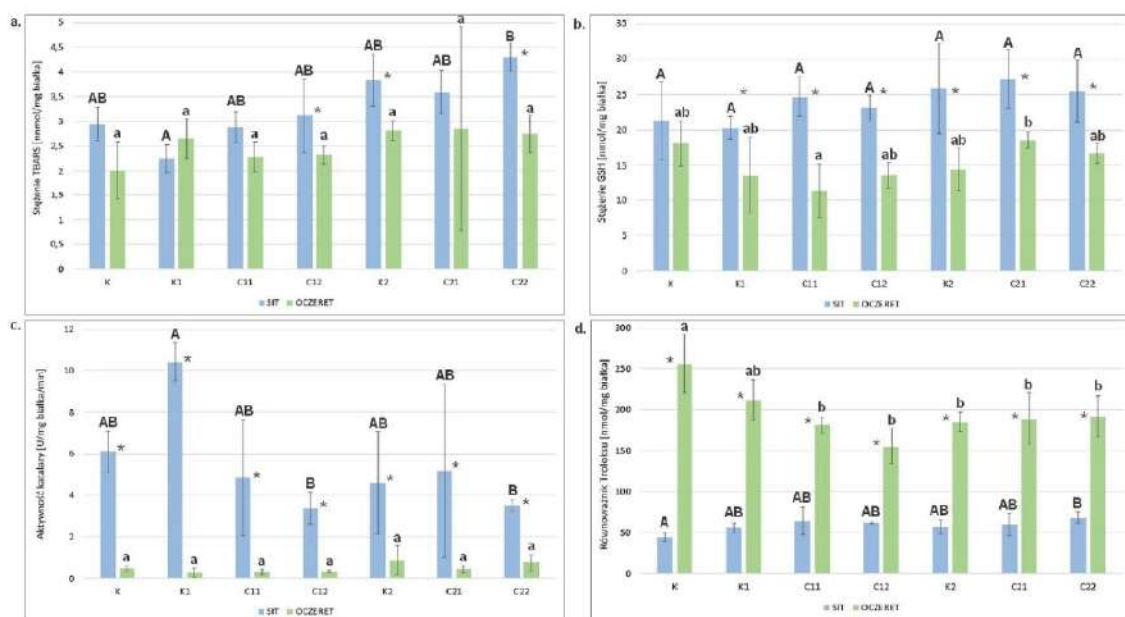
stężenie zredukowanego glutationu w roślinach traktowanych wodą z pożywką i NaCl (stężenie 200 mg/L) (C11) a roślinami traktowanymi wodą kranową i NaCl (stężenie 200 mg/L) (C21). Z wyjątkiem roślin w czasie aklimatyzacji (K), we wszystkich pozostałych kategoriach (K1, C11, C12, K2, C21, C22) stężenie zredukowanego glutationu było statystycznie istotnie wyższe w sicie rozpierzchłym niż w oczerecie jeziornym (Ryc. 3b).

Aktywność katalazy. Sit rozpierzchły z kontroli podlewanej wodą z pożywką wykazał statystycznie istotnie większą aktywność katalazy niż sit traktowany wodą z pożywką i NaCl (stężenie 1000 mg/L) (C12) oraz sit traktowany wodą kranową i NaCl (stężenie 1000 mg/L) (C22). Dla oczeretu jeziornego nie wykazano statystycznie istotnych różnic w aktywności katalazy pomiędzy próbami, jednakże aktywność katalazy w próbach roślin podlewanych wodą kranową (K2, C21, C22) była wyższa niż w roślinach podlewanych wodą z pożywką (K1, C11, C12). W przypadku K, K1, K2, C21 oraz C22 aktywność katalazy była wyższa u roślin z gatunku sit rozpierzchły niż u oczeretu jeziornego (Ryc. 3c).

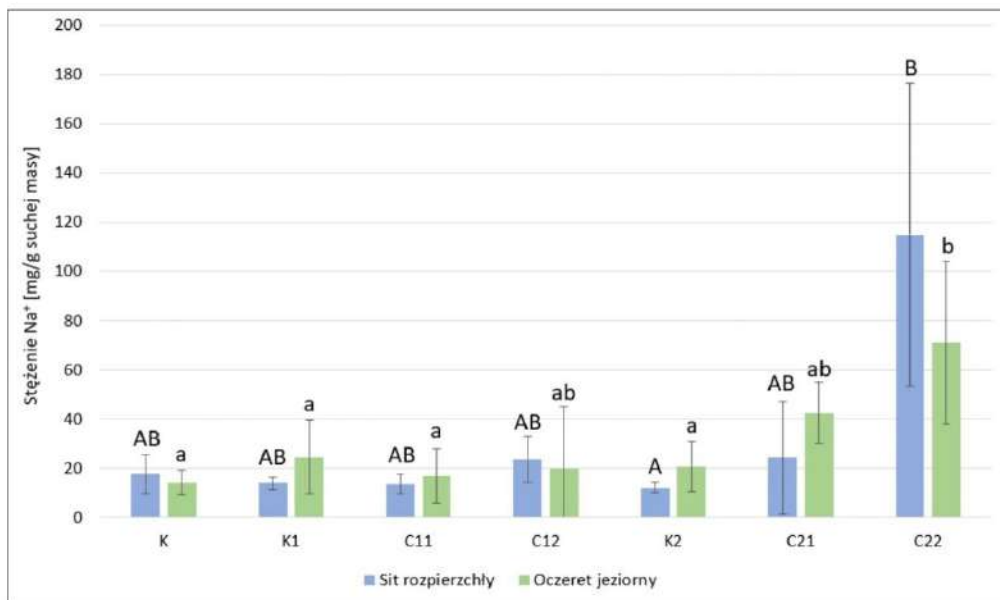
Całkowity nieenzymatyczny potencjał antyoksydacyjny. Dla situ rozpierzchłego w trakcie aklimatyzacji (K) całkowity nieenzymatyczny potencjał antyoksydacyjny był istotnie statystycznie niższy niż w roślinach traktowanych wodą kranową i NaCl (stężenie 1000 mg NaCl/L) (C22). W oczerecie jeziornym w trakcie aklimatyzacji (K) całkowity nieenzymatyczny potencjał antyoksydacyjny był wyższy niż w roślinach traktowanych wodą z pożywką i NaCl (stężenie 200 mg/L) (C11), wodą z pożywką i NaCl (stężenie 1000 mg/L) (C12), roślinach z kontroli podlewanej wodą kranową (K2), roślinach podlewanych wodą kranową i NaCl (stężenie 200 mg/L) (C21) oraz roślinach podlewanych wodą kranową i NaCl (stężenie 1000 mg/L) (C22). Oczeret jeziorny wykazał wyższy całkowity potencjał antyoksydacyjny w porównaniu z sitem rozpierzchłym we wszystkich kategoriach (K, K1, C11, C12, K2, C21, C22) (Ryc. 3d).

3.2. Stężenie jonów sodowych w tkankach roślin

U obu gatunków zaobserwowano wzrost zawartości jonów Na^+ w nadziemnych częściach roślin wraz ze wzrostem zastosowanych w czasie ekspozycji stężeń NaCl . Dla situ rozpięzkiego wykazano istotnie wyższą zawartość jonów Na^+ w roślinach podlewanych wodą kranową z NaCl (1000 mg/L) (C22; średnia 119,3 mg Na^+ /g suchej masy) niż w roślinach z kontroli podlewanej wodą kranową (K2; średnia 13,3 mg Na^+ /g suchej masy). Dla oczeretu jeziornego wykazano istotnie wyższą zawartość jonów Na^+ w roślinach podlewanych wodą kranową z NaCl (1000 mg/L) (C22; średnia 77,7 mg Na^+ /g suchej masy) niż w roślinach w czasie aklimatyzacji (K; średnia 14,1 mg Na^+ /g suchej masy), z kontroli podlewanej wodą z pożywką (K1; średnia 24,3 mg Na^+ /g suchej masy), podlewanych wodą z pożywką i NaCl (stężenie 200 mg/L) (C11; średnia 18,3 mg Na^+ /g suchej masy) oraz z kontroli podlewanej wodą kranową (K2; średnia 22,4 mg Na^+ /g suchej masy). Nie wykazano statystycznie istotnych różnic w zawartości jonów Na^+ pomiędzy dwoma gatunkami roślin (Ryc. 4).



Ryc. 3. Wykres a: stężenie TBARS, **wykres b:** stężenie GSH, **wykres c:** aktywność katalazy, **wykres d:** poziom całkowitego nieenzymatycznego potencjału przeciwutleniającego. Słupki wskazują wartości mediany, wąsy wskazują rozstęp międzykwartylowy (IQR). Różne duże litery nad słupkami wskazują na statystycznie istotne różnice ($p < 0,05$) pomiędzy wariantami dla situ rozprzeczłego, różne małe litery wskazują na statystycznie istotne różnice pomiędzy wariantami dla oczeretu jeziornego. Gwiazdki wskazują na statystycznie istotne różnice pomiędzy dwoma gatunkami w danym wariantcie.



Ryc. 4. Stężenie jonów sodowych w roślinach. Słupki wskazują wartości mediany, wąsy wskazują rozstęp międzykwartylowy (IQR). Różne duże litery nad słupkami wskazują na statystycznie istotne różnice ($p < 0,05$) pomiędzy wariantami dla situ rozpierzchłego, różne małe litery wskazują na statystycznie istotne różnice pomiędzy wariantami dla oczeretu jeziornego.

4. Dyskusja

4.1. Analiza parametrów stresu oksydacyjnego

Ze względu na brak możliwości przemieszczania się, rośliny są organizmami szczególnie narażonymi na zmienne warunki środowiska, w którym żyją. Do czynników abiotycznych mogących wywoływać stres zalicza się m.in. promieniowanie UV, niedostatki wody, obecność metali ciężkich w glebie, temperaturę i zasolenie (Olko, Kujawska, 2011). W toku ewolucji rośliny wytworzyły mechanizmy pozwalające im na regulację stresu oksydacyjnego (Hassan i in., 2017). O tym, w jakim stopniu rośliny będą w stanie zwalczyć toksyczny wpływ reaktywnych form tlenu, decyduje efektywność działania systemu antyoksydacyjnego enzymatycznego i nieenzymatycznego. Do enzymów antyoksydacyjnych należą m.in.: dysmutaza ponadtlenkowa, katalaza oraz peroksydaza i reduktaza glutationowa. Nieenzymatyczny system antyoksydacyjny stanowią z kolei cząsteczki o małej masie cząsteczkowej zdolne do redukcji reaktywnych form tlenu. Do związków o takich właściwościach w komórkach należą przede wszystkim glutation i kwas askorbinowy, ale także tokoferol i karotenoidy (Kłosowska, 2010).

Proces peroksydacji lipidów polega na utlenianiu wielonienasyconych kwasów tłuszczowych. Produkty tych reakcji mogą mieć negatywny wpływ na funkcjonowanie komórek. Peroksydacja lipidów błonowych może wpływać na system transportu błonowego, a także na płynność błony komórkowej i jej depolaryzację (Bartosz, 2003). Poziom produktów reagujących z kwasem tiobarbiturowym (TBARS) w homogenatach situ rozpięchłego poddanego działaniu wysokiego stężenia NaCl z wodą kranową (C22) był znacząco wyższy w porównaniu z kontrolą z pożywką (K1). Znaczących różnic w poziomie TBARS nie zaobserwowano pomiędzy kontrolą z pożywką (K1) a sadzonkami situ poddanym wysokiemu stężeniu NaCl z pożywką (C12). Wskazuje to, że skład pożywki podawanej roślinom pomagał komórkom w przeciwdziałaniu peroksydacji lipidów. Późniejsze odstawienie pożywki

spowodowało znaczący wzrost TBARS świadczący o utlenianiu kwasów tłuszczowych. Stopień peroksydacji lipidów w roślinach wystawionych za zasolenie był wyższy, co wskazuje na to, że zarówno wysokie zasolenie jak i odstawienie pożywki i zastąpienie jej wodą kranową spowodowały większy stres u *situ*. Na ochronne działanie pożywki wskazuje również różna reakcja roślin, które w czasie aklimatyzacji podlewane były wodą kranową, a w czasie eksperymentu stanowiły kontrolę podlewaną pożywką. Zaobserwowano u nich spadek poziomu peroksydacji lipidów po dodaniu pożywki, jednak różnica ta nie była istotą statystycznie. Hassan i in. (2017) zaobserwowali różnice w stopniu peroksydacji lipidów (wyrażonego jako zawartość wolnego dialdehydu malonowego MDA) w zależności od tolerancji gatunku *situ* na zasolenie. Zawartość MDA w sicie członowatym (*Juncus articulatus*), wrażliwym na zasolenie, była wyższa niż u gatunków tolerujących zasolenie (*J. martimus* i *J. acutus*) i wzrastała wraz ze zwiększającymi się stężeniami NaCl. W przypadku oczeretu jeziornego nie wykazano żadnych istotnych różnic w poziomie TBARS pomiędzy analizowanymi wariantami badawczymi. Może to wskazywać na większą odporność tego gatunku na zasolenie. Niemniej jednak, obserwując poziom TBARS u obu gatunków roślin, zarówno pod względem czasu jak i stężenia soli, można przypuszczać, że komórki oczeretu jeziornego są mniej podatne na proces peroksydacji lipidów, a tym samym bardziej odporne na stres.

Glutation (GSH) pełni kluczową rolę w utrzymywaniu homeostazy komórkowej. Do jego funkcji zaliczyć można utrzymywanie grup tiolowych białek w stanie zredukowanym. Poziom grup tiolowych w białkach jest bardzo czułym wskaźnikiem procesów utleniania białek. Modyfikacje struktury białek przez reaktywne formy tlenu mogą powodować utratę ich funkcji i aktywności (Bartosz, 2003). W warunkach fizjologicznych ponad 99% glutationu w komórkach występuje w formie zredukowanej (GSH), jednak w wyniku reakcji GSH

z reaktywnymi formami tlenu, GSH zostaje utleniony do disulfidu glutationu (GSSG) (Bartos, 2003).

W przypadku otrzymanych wyników, nie zaobserwowano spadku stężenia GSH u situ rozpięzchłego – we wszystkich wariantach, tj. zarówno w czasie aklimatyzacji roślin, jak również po ich ekspozycji na zasolenie, stężenie GSH utrzymywało się na takim samym poziomie. Może to wskazywać, że zastosowane stężenia NaCl nie przyczyniały się do redukcji grup tiolowych białek. Wyniki te wydają się niespójne z wcześniejszą obserwacją dotyczącą peroksydacji lipidów u situ rozpięzchłego. Warto jednak zauważyć, że stężenie GSH w komórkach situ rozpięzchłego było wyższe niż to oznaczone u oczeretu jeziornego. Być może zaobserwowany u situ wyższy poziom peroksydacji lipidów doprowadził do wzmożonej produkcji glutationu. W przypadku oczeretu jeziornego, zaobserwowano spadek stężenia GSH w próbach wystawionych na zasolenie (z pożywką) w porównaniu do roślin w trakcie aklimatyzacji, jednak różnica ta nie była istotna statystycznie. Jedyna istotna różnica w poziomie stężenia glutationu u oczeretu jeziornego została zaobserwowana pomiędzy roślinami traktowanymi wodą kranową i NaCl (200 mg/L) (C21) a roślinami traktowanymi wodą z pożywką i NaCl (200 mg/L) (C11).

Dwa zbadane gatunki roślin różniły się znacząco pod względem aktywności katalazy. U oczeretu jeziornego aktywność tego enzymu utrzymywała się na bardzo niskim poziomie i nie różniła się istotnie pomiędzy wariantami. Natomiast u situ rozpięzchłego była ona istotnie wyższa. Dla tego gatunku zaobserwowano również różnice pomiędzy wariantami, tj. aktywność katalazy w kontroli podlewanej wodą z pożywką (K1) była wyższa niż w roślinach traktowanych wodą z pożywką i NaCl (stężenie 1000 mg/L) (C12) oraz wodą kranową i NaCl (stężenie 1000 mg/L) (C22). Spadek aktywności katalazy wraz ze wzrastającymi stężeniami NaCl zaobserwowany został również u *Juncus rigidus* (Abd El-Maboud i in., 2023), a także u trzech gatunków situ (*J. acutus*, *J. martinus*

i *J. articulatus*) przez Hassana i in. (2017). Hassan i współautorzy sugerują, że spadek aktywności katalazy w roślinach może wskazywać na wysoce dynamiczny system antyoksydacyjny badanych gatunków, u których poziom enzymów antyoksydacyjnych zależy od intensywności, a także długości trwania stresu. Autorzy proponują również, że aktywność katalazy mogła być wyższa zaraz po początkowej ekspozycji na NaCl, jednak wraz z upływem czasu (w cytowanych badaniach materiał roślinny do analiz zebrany został po 8 tygodniach), uległa ona obniżeniu.

Nieenzymatyczna aktywność antyoksydacyjna oczeretu jeziornego była wyższa od tej zaobserwowanej u sity rozpierzchłego. Dodatkowo, u oczeretu parametr ten był najwyższy w roślinach w czasie aklimatyzacji. Można to wskazywać, że u oczeretu jeziornego to nieenzymatyczne elementy odpowiedzi antyutleniającej pełniły główną rolę w ochronie rośliny przed stresem spowodowanym zasoleniem, zważywszy również na bardzo niską aktywność katalazy u tego gatunku. Dla oczeretu jeziornego nie zaobserwowano istotnych zmian w poziomie glutationu pomiędzy badanymi wariantami, jednakże nie można wykluczyć, że poziomy innych antyoksydantów takich jak kwas askorbinowy, karotenoidy czy flawonoidy, ulegały zmianie wraz ze wzrostem stężenia NaCl. Na przykład, Zahoor i in. (2012) zaobserwowali spadek zawartości karotenoidów u innego gatunku oczeretu (*Schoenoplectus juncooides*) wraz z rosnącymi stężeniami NaCl. Zawartość antyoksydantów w roślinach może zmieniać się w różny sposób, w zależności od gatunku rośliny oraz jej tolerancji na zasolenie. Hassan i in. (2017) zaobserwowali wzrost całkowitej zawartości flawonoidów u trzech gatunków sity (*Juncus acutus*, *J. maritimus* i *J. articulatus*) wraz z rosnącymi stężeniami NaCl. Natomiast, w badaniach przeprowadzonych na dwóch odmianach marchwi (jedna tolerująca zasolenie, druga wrażliwa na zasolenie) zaobserwowano spadek całkowitej zawartości związków fenolowych (TPC – *total phenolic content*) w odpowiedzi na zasolenie, a także

spadek stężenia kwasu askorbinowego w liściach spowodowany stresem solnym, przy czym spadek ten był wyższy w przypadku odmiany wrażliwej na zasolenie (Kamińska i in., 2022).

Podsumowując, sit rozpierzchły i oczeret jeziorny różniły się w swojej odpowiedzi antyoksydacyjnej na zasolenie. Sit jeziorny charakteryzował się wyższymi stężeniami GSH oraz wyższą aktywnością katalazy, natomiast oczeret jeziorny wykazał wyższy całkowity nieenzymatyczny potencjał przeciwutleniający. Zastosowana w etapie I pożywka mogła przyczynić się do podtrzymania kondycji obu gatunków. Oczeret jeziorny wykazał mniejszą zmienność badanych parametrów stresu oksydacyjnego, co może wskazywać na jego większą odporność na zasolenie w porównaniu do situ rozpierchłego. Pomimo różnic, systemy antyoksydacyjne obu gatunków roślin były wystarczająco efektywne wobec ekspozycji na zasolenie, co sugeruje, że mogłyby one być wykorzystane na terenach narażonych na zanieczyszczenie solą drogową.

4.2. Analiza akumulacji jonów sodu w roślinach

Biorąc pod uwagę wcześniejsze doniesienia o zdolności roślin z rodzaju *Juncus* i *Schoenoplectus* do akumulacji jonów sodu w swoich tkankach (Rozema i in. 2014; Guesdon i in., 2016), w przedstawionych badaniach wybrano dwóch przedstawicieli tych rodzajów, rodzimych dla flory Polski. Zgodnie z przyjętym wcześniej założeniem, zarówno sit rozpierzchły, jak i oczeret jeziorny wykazały zdolność do akumulacji jonów sodu w swoich tkankach. W szczególności dla situ rozpierchłego, akumulacja jonów sodu była znacząca – w roślinach po dwóch miesiącach ekspozycji na wysokie stężenie NaCl (1000 mg/L) wyniosła 119,3 mg Na⁺/g suchej masy. Warto jednak zauważyć, że sit rozpierzchły, który zakumulował więcej jonów sodowych, wykazał większą zmienność w parametrach stresu oksydacyjnego. W przypadku oczeretu jeziornego średnia zawartość jonów sodu w roślinach po dwóch miesiącach ekspozycji na wysokie stężenie NaCl wyniosła 77,7 mg Na⁺/g suchej masy. Wartości te są podobne do tych zaobserwowanych u halofitów, łobody ogrodowej i łobody

solniskowej, u których akumulacja w nadziemnych częściach roślin wyniosła odpowiednio 53 mg Na⁺/g suchej masy i 60 mg Na⁺/g suchej masy po 60 dniowej ekspozycji na stężenie 2000 mg NaCl/L (Suairé i in., 2016).

Akumulacja jonów Na⁺ oraz jonów Cl⁻ może skutkować uszkodzeniem i obumieraniem liści, a objawy te będą zależne od tempa akumulacji jonów oraz efektywności ich sekwestracji w tkankach (Kłosowska, 2010). Zwiększona zawartość jonów sodu może również prowadzić do obniżenia stosunku K⁺/Na⁺, co pociąga za sobą szereg zaburzeń m.in. dotyczących dróg metabolicznych (Kłosowska, 2010). Pomimo że w przeprowadzonych badaniach nie zaobserwowano obumierania roślin wystawionych za zasolenie, akumulacja jonów Na⁺ w sicie rozpiechłym oraz oczerecie jeziornym wymaga dalszych badań, np. wykonania analiz zawartości jonów K⁺ czy Ca²⁺. Dodatkowe analizy pozwolą na lepszą ocenę przydatności tych gatunków do stosowania np. w przydrożnych sztucznych mokradłach lub innego typu Rozwiązaniach Bliskich Naturze (NBS) stosowanych coraz powszechniej w miastach (np. w sekwencyjnych systemach sedymentacyjno-biofiltracyjnych; Jurczak i in., 2019).

5. Wnioski

Wstępne badania zaprezentowane w pracy wskazują, że sit rozpiechły oraz oczeret jeziorny wykazują potencjał do fitodesalinizacji terenów zanieczyszczonych solą drogową. Zbadane parametry stresu oksydacyjnego pokazują, że systemy antyoksydacyjne roślin były w stanie ochronić je w warunkach podwyższonego zasolenia. Odpowiedź antyoksydacyjna tych gatunków różniła się między sobą – oczeret jeziorny wykazał mniejszą zmienność pod względem parametrów stresu oksydacyjnego, co wskazuje na jego wyższą tolerancję na zasolenie. Oba gatunki wykazały również zdolność do akumulowania jonów sodowych, przy czym sit rozpiechły okazał się pod tym względem bardziej skuteczny.

6. Podziękowania

Analizy z użyciem homogenizatora Tissue Lyser II przeprowadzono w Pracowni Obrazowania Mikroskopowego i Specjalistycznych Technik Biologicznych Wydziału Biologii i Ochrony Środowiska Uniwersytetu Łódzkiego.

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Oświadczenia współautorów publikacji

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OŚWIADCZENIE O WKŁADZIE W POWSTAWANIE PUBLIKACJI

1. Szklarek S., Górecka A., Wojtal-Frankiewicz A.; **The effects of road salt on freshwater ecosystems and solutions for mitigating chloride pollution – A review**; *Science of The Total Environment*; 2022; 805:150289.

Oświadczam, że jestem współautorem powyższej publikacji, a swój wkład w jej powstawanie oceniam na 25%. Polegał on na: przeglądzie literatury, przygotowaniu wersji wstępnej manuskryptu, edycji tekstu manuskryptu, przygotowaniu grafik (nr 1, 2, 3).

2. Szklarek S., Górecka A., Salabert B., Wojtal-Frankiewicz A.; **Acute toxicity of seven de-icing salts on four zooplankton species – is there an “eco-friendly” alternative?**; *Ecohydrology & Hydrobiology*; 2022; 22(4):589-597.

Oświadczam, że jestem współautorem powyższej publikacji, a swój wkład w jej powstawanie oceniam na 25%. Polegał on na: wykonaniu doświadczeń z użyciem biotestów, analizie i interpretacji danych doświadczalnych, przygotowaniu tabel (nr 3), przygotowaniu wersji wstępnej manuskryptu, edycji tekstu manuskryptu.

3. Szklarek S., Górecka A., Jóźwiak P., Wojtal-Frankiewicz A.; **The effect of road salt (NaCl) treatment on the hatching success of *Daphnia magna* and *Thamnocephalus platyurus***; *Ecohydrology & Hydrobiology*; 2023.

Oświadczam, że jestem współautorem powyższej publikacji, a swój wkład w jej powstawanie oceniam na 10%. Polegał on na: wykonaniu i interpretacji analiz z użyciem elektronowego mikroskopu skaningowego, przygotowaniu figury (nr 4) oraz tabeli (nr 1), edycji manuskryptu.

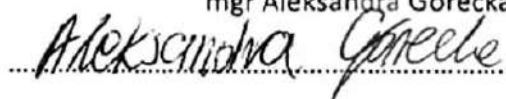
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Oświadczam, że jestem wiodącym autorem powyższej publikacji, a swój wkład w jej powstawanie oceniam na 55%. Polegał on na: sformułowaniu hipotez badawczych, zbieraniu prób w terenie, oznaczeniu ilościowym i jakościowym zooplanktonu, analizie i interpretacji danych dotyczących zagęszczenia zooplanktonu, analizie i interpretacji danych dotyczących chemii wody, przygotowaniu koncepcji manuskryptu, przeglądzie literatury, przygotowaniu wersji wstępnej manuskryptu, edycji tekstu manuskryptu, przygotowaniu tabel (S1, S3, S4, S5, S6, S7), przygotowaniu grafik i wykresów (nr 1, 4, 5, S1, S2, S3, od S5 do S11), przygotowaniu odpowiedzi dla recenzentów.

5. Górecka A., Pieniążek A., Nebelska J., Szklarek S., Wojtal-Frankiewicz A.; Odpowiedź antyoksydacyjna situ rozpięzchłego (*Juncus effesus*) i oczeretu jeziornego (*Schoenoplectus lacustris*) na zanieczyszczenie solą drogową (przygotowany manuskrypt)

Oświadczam, że jestem autorem wiodącym powyższego manuskryptu, a swój wkład w jego powstawanie oceniam na 60%. Polegał on na: sformułowaniu koncepcji badań, sformułowaniu hipotez badawczych, przeprowadzeniu eksperymentu z ekspozycją roślin na sól, przygotowaniem i homogenizacją próbek, wykonaniu analiz dotyczących wskaźników stresu oksydacyjnego w roślinach, wykonaniu analiz dotyczących zawartości sodu w tkankach roślin, analizie i interpretacji uzyskanych danych, wykonaniu części analiz statystycznych, przygotowaniu koncepcji manuskryptu, przeglądzie literatury, przygotowaniu wersji wstępnej manuskryptu, edycji tekstu manuskryptu, przygotowaniu grafik i wykresów.

mgr Aleksandra Górecka

Handwritten signature of Aleksandra Górecka in cursive script, written over a dotted line.

(Podpis)

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4. Górecka A., Szklarek S., Frankiewicz P., Kukuła K., Wojtal-Frankiewicz A.; **Does winter application of road salt affect zooplankton communities in urban ponds? Aquatic Sciences, decyzja o przyjęciu manuskryptu do publikacji otrzymana 14.09.2023 r.**

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.....
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Polskiej Akademii Nauk

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Oświadczam, że jestem wiodącym autorem powyższej publikacji, a swój wkład w jej powstawanie oceniam na 70%. Polegał on na: sformułowaniu koncepcji badań, sformułowaniu hipotez badawczych, wykonaniu doświadczeń dotyczących wylęgów, analizie i interpretacji danych doświadczalnych, przygotowaniu tabel, przygotowaniu wykresów, przygotowaniu koncepcji manuskryptu, przeglądzie literatury, przygotowaniu wersji wstępnej manuskryptu, edycji tekstu manuskryptu, przygotowaniu odpowiedzi dla recenzentów.

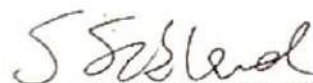
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Oświadczam, że jestem współautorem powyższej publikacji, a swój wkład w jej powstawanie oceniam na 20%. Polegał on na: sformułowaniu koncepcji badań, pobieraniu prób w terenie, przygotowaniu bazy danych dotyczących danych metrologicznych oraz chemii wody, przygotowaniu tabeli (nr S2), przygotowaniu wykresów (nr 2 i 3), przygotowaniu wersji wstępnej manuskryptu, edycji tekstu manuskryptu, przygotowaniu odpowiedzi dla recenzentów.

5. Górecka A., Pieniążek A., Nebelska J., Szklarek S., Wojtal-Frankiewicz A.; Odpowiedź antyoksydacyjna situ rozpięzchłego (*Juncus effesus*) i oczeretu jeziornego (*Schoenoplectus lacustris*) na zanieczyszczenie solą drogową (przygotowany manuskrypt)

Oświadczam, że jestem współautorem powyższego manuskryptu, a swój wkład w jego powstawanie oceniam na 7,5%. Polegał on na: sformułowaniu koncepcji badań, przygotowaniu wersji wstępnej manuskryptu, edycji tekstu manuskryptu

Dr Sebastian Szklarek



(Podpis)

Lyon, 08/09/2023

Bastien SALABERT

Polytech Tours, Génie de l'aménagement et l'environnement

DECLARATION OF CO-AUTHORSHIP

Szklarek S., Górecka A., Salabert B., Wojtal-Frankiewicz A. (2022) Acute toxicity of seven de-icing salts on four zooplankton species—is there an “eco-friendly” alternative? Ecohydrology & Hydrobiology, 22(4) 589-597.

I confirm that I am co-author of the above manuscript and my contribution included carrying out part of the biotests, as well as analyzing the obtained data.

I estimate my quantitative contribution to manuscript preparation at 15%.

Bastien Salabert

A handwritten signature in black ink, appearing to read 'Salabert', with a large, stylized initial 'S'.

Łódź, 07.09.2023 r.

prof. dr hab. Piotr Frankiewicz

Katedra UNESCO Ekohydrologii i Ekologii Stosowanej

Wydział Biologii i Ochrony Środowiska

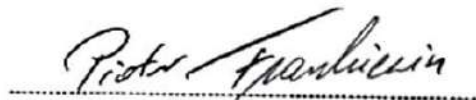
Uniwersytet Łódzki

OŚWIADCZENIE O WKŁADZIE W POWSTAWANIE PUBLIKACJI

1. Górecka A., Szklarek S., Frankiewicz P., Kukuła K., Wojtal-Frankiewicz A.; Does winter application of road salt affect zooplankton communities in urban ponds? Aquatic Sciences, decyzja o przyjęciu manuskryptu do publikacji otrzymana 14.09.2023 r.

Oświadczam, że jestem współautorem powyższej publikacji, a swój wkład w jej powstawanie oceniam na 7,5%. Polegał on na: wykonaniu analiz statystycznych (testy ANOVA), edycji tekstu manuskryptu, przygotowaniu odpowiedzi dla recenzentów.

prof. dr hab. Piotr Frankiewicz



(Podpis)

Łódź, 07.09.2023 r.

prof. dr hab. Krzysztof Kukula

Zakład Ekologii i Ochrony Środowiska

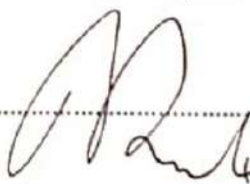
Uniwersytet Rzeszowski

OŚWIADCZENIE O WKŁADZIE W POWSTAWANIE PUBLIKACJI

1. **Górecka A., Szklarek S., Frankiewicz P., Kukula K., Wojtal-Frankiewicz A.; Does winter application of road salt affect zooplankton communities in urban ponds? Aquatic Sciences, decyzja o przyjęciu manuskryptu do publikacji w czasopiśmie otrzymana 14.09.2023 r.**

Oświadczam, że jestem współautorem powyższej publikacji, a swój wkład w jej powstawanie oceniam na 7,5%. Polegał on na: wykonaniu analiz statystycznych (analizy RDA), edycji tekstu manuskryptu, przygotowaniu wykresów (nr 6, S4) oraz tabel (nr S8, S9, S10, S11), przygotowaniu odpowiedzi dla recenzentów.

prof. dr hab. Krzysztof Kukula

.....
 (podpis)

Łódź, 07.09.2023 r.

Dr Piotr Józwiak

Katedra Zoologii Bezkręgowców i Hydrobiologii

Wydział Biologii i Ochrony Środowiska

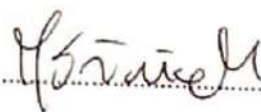
Uniwersytet Łódzki

OŚWIADCZENIE O WKŁADZIE W POWSTAWANIE PUBLIKACJI

Szklarek S., Górecka A., Józwiak P., Wojtal-Frankiewicz A.; The effect of road salt (NaCl) treatment on the hatching success of *Daphnia magna* and *Thamnocephalus platyurus*; Ecohydrology & Hydrobiology; 2023.

Oświadczam, że jestem współautorem powyższej publikacji, a swój wkład w jej powstanie oceniam na 10%. Polegał on na: opracowaniu oraz opisanu w manuskrypcie metodyki wykorzystanej do badań na elektronowym mikroskopie skaningowym oraz wsparcie merytoryczne z zakresu obsługi elektronowego mikroskopu skaningowego.

Dr Piotr Józwiak



(Podpis)

Łódź, 07.09.2023 r.

Dr hab. Anna Pieniążek, prof. UŁ

Katedra Biologii Nowotworów i Epigenetyki

Wydział Biologii i Ochrony Środowiska

Uniwersytet Łódzki

OŚWIADCZENIE O WKŁADZIE W POWSTAWANIE PUBLIKACJI

1. **Górecka A., Pieniążek A., Nebelska J., Szklarek S., Wojtal-Frankiewicz A.; Odpowiedź antyoksydacyjna situ rozpięzłego (*Juncus effesus*) i oczeretu jeziornego (*Schoenoplectus lacustris*) na zanieczyszczenie solą drogową (przygotowany manuskrypt)**

Oświadczam, że jestem współautorem powyższego manuskryptu, a swój wkład w jego powstawanie oceniam na **20%**. Polegał on na: opracowaniu oraz opisie metodyki dotyczącej analiz wskaźników stresu oksydacyjnego u roślin, przygotowaniu i homogenizacji próbek, wykonaniu części analiz statystycznych oraz i interpretacji uzyskanych danych, przygotowaniu wersji wstępnej manuskryptu, edycji tekstu manuskryptu.



(Podpis)

Łódź, 07.09.2023 r.

Justyna Nebelska

Katedra UNESCO Ekohydrologii i Ekologii Stosowanej

Wydział Biologii i Ochrony Środowiska

Uniwersytet Łódzki

OŚWIADCZENIE O WKŁADZIE W POWSTAWANIE PUBLIKACJI

1. Górecka A., Pieniążek A., Nebelska J., Szklarek S., Wojtał-Frankiewicz A.; Odpowiedź antyoksydacyjna siltu rozplzchłego (*Juncus effesus*) i oczeretu jeziornego (*Schoenoplectus lacustris*) na zanieczyszczenie solą drogową (przygotowany manuskrypt)

Oświadczam, że jestem współautorem powyższego manuskryptu, a swój wkład w jego powstawanie oceniam na 5%. Polegał on na: przygotowaniu i homogenizacji próbek.

Justyna Nebelska

(Podpis)