UNIWERSYTET IM. ADAMA MICKIEWICZA W POZNANIU
Wydział Biologii
Zakład Ochrony Wód

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Response of *Chara hispida* L. to restoration treatments using iron and aluminium coagulants

Reakcje *Chara hispida* L. na zabiegi rekultywacyjne z użyciem koagulantów żelazowych i glinowych

Praca doktorska wykonana pod kierunkiem
dr. hab. Tomasza Joniaka

Poznań 2018

PODZIĘKOWANIA

Chciałbym podziękować mojemu promotorowi panu *dr hab. Tomaszowi Joniakowi* za okazane zaufanie, cierpliwość, pomoc i cenne rady we wszystkich aspektach mojego doktoratu. Jestem bardzo wdzięczny za pomoc badaniach terenowych i laboratoryjnych w trakcie wszystkich lat pracy. Dziękuje również, za wszystkie wyjazdy terenowe podczas których nauczyłem się niezmiernie dużo o funkcjonowaniu różnych ekosystemów wodnych oraz zdobyłem doświadczenie w pracy hydrobiologa.

Jestem bardzo wdzięczny wszystkim współautorom za całą pracę i wkład w publikacje wchodzące w skład rozprawy doktorskiej. Szczególnie chciałbym podziękować panu *prof. UAM dr hab. Maciejowi Gąbce* oraz panu *dr hab. Tadeuszowi Sobczyńskiemu*. Dziękuje za całe okazane wsparcie oraz wszystkie dyskusje naukowe, które pomogły w powstaniu niniejszej pracy.

Panu *prof. dr hab. Ryszardowi Gołdynowi,* mojemu pierwszemu opiekunowi podczas studium doktoranckiego, dziękuje za wsparcie, życzliwość oraz możliwość uczestniczenia w moich pierwszych konferencjach naukowych.

Dziękuje kierownikowi Zakładu Ochrony Wód panu *prof. UAM dr hab. Piotrowi Klimaszykowi* oraz wszystkim *Pracownikom Zakładu Ochrony Wód* oraz *Zakładu Hydrobiologii* za życzliwość, pomoc oraz możliwość nauki i rozwoju. Specjalne podziękowania składam panu *prof. dr hab. Mariuszowi Pełechatemu* za okazaną pomoc w kryzysowych chwilach.

Bardzo dziękuje wszystkim doktorantom z Zakładu Ochrony Wód – *Joanno, Anno, Natalio, Agnieszko, Małgorzato, Agnieszko* i *Kasprze*, dziękuje za kilka lat wspólnej pracy, wzajemnego przeżywania rozterek, porażek, radości i obron. Dziękuje za nieocenioną pomoc, sympatyczną atmosferę, wsparcie oraz mnóstwo chwil śmiechu.

Dziękuje również doktorantom z Zakładu Hydrobiologii – *Marcie, Łukaszowi* oraz *Marcinowi*, za wspólną organizację Konferencji, dyskusje oraz wsparcie.

Całej *administracji Collegium Biologicum* na czele z panią *dr Karoliną Cerbin* za pomoc w odnalezieniu się w administracyjnym labiryncie.

Bardzo dziękuje mojej *żonie Dorocie* oraz *Rodzicom* i *Siostrze* za ogromne i nieustające wsparcie; specjalne podziękowania kieruje mojemu Tacie, bez którego pomocy nie byłbym wstanie wykonać eksperymentów terenowych. Dziękuje, że pomogliście mi zacząć doktorat i doprowadzić go do końca.

Natura jest zawsze mądrzejsza od ludzkich pomysłów.

Antoni Kępiński

SPIS TREŚCI

PUBLIKACJE SKŁADAJĄCE SIĘ NA CYKL ROZPRAWY DOKTORSKIEJ

A1 **Experimental investigation into disturbance of Ca-Mg equilibrium and consequences for charophytes after iron and aluminium coagulants application**

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DOI: 10.15244/pjoes/90095

A2 **The inhibition of growth and oospores production in *Chara hispida* L. as an effect of iron sulphate addition: Conclusions for the use of iron coagulants in lake restoration**

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STRESZCZENIE

Postęp eutrofizacji wód powierzchniowych wymaga podjęcia działań naprawczych mających na celu zatrzymanie, a najlepiej odwrócenie trendu negatywnych zmian jakości wód. Najpowszechniej stosowaną metodą rekultywacji zbiorników wodnych, która może stymulować naturalne procesy odnowy ekosystemów, jest chemiczna inaktywacja fosforanów przy użyciu koagulantów na bazie żelaza lub glinu. Niestety cechy chemiczne koagulantów, zwłaszcza silnie kwaśne pH, stwarzają ryzyko powstania zaburzeń w biocenozie i środowisku abiotycznym. Wpływ koagulantów na makrofity, w tym bardzo cenne ekologicznie ramienice, jest słabo poznany. Celem badań było określenie reakcji ramienicy *Chara hispida* L. na zastosowanie koagulantów chemicznych. Gatunek ten często występuje w wodach eutroficznych, dlatego jest potencjalnie narażony na zabiegi rekultywacyjne. Skutkiem aplikacji koagulantów było zakwaszenie wody, które determinowało zmiany jakościowe wód i oddziaływało negatywnie na ramienice. Zakwaszenie zmieniało preferowane przez ramienice alkaliczne warunki środowiska i powodowało rozpuszczanie węglanowej inkrustacji, co w konsekwencji powodowało zburzenia równowagi wapniowo–magnezowej w wodzie. Zakwaszenie wody indukowało również rozpuszczalność i toksyczność glinu, zwiększając jego biodostępność. Wykazano, że *C. hispida* akumulowała glin wewnątrz komórek (stężenie 2,0 mg · g^{-1} suchej masy), co prowadziło do uszkodzenia struktur wewnątrzkomórkowych (rozpad chloroplastów) i odrywania komórek okorowania. Efektem wprowadzenia koagulantów były też zmiany fizykochemiczne wód (wzrost barwy i mętności wody), co wpłynęło na zaburzenia dostępności światła. Skutkiem były zmiany wzorca wzrostu kosztem rozmnażania generatywnego. Badania wykazały, że stosowanie koagulantów w tak wysokich dawkach stanowi zagrożenie dla funkcjonowania *Chara hispida.*

ABSTRACT

Various methods of water restoration are used to counter the negative effects of eutrophication. Restoration treatments stimulate processes that retrieve the natural diversity of the environment. One of the most commonly used method of water restoration is the chemical inactivation of phosphates using coagulants based on iron or aluminium. Regrettably, the chemical characteristics of coagulants, mainly the strongly acidic pH, pose a risk of disturbances in biocenosis and abiotic environment. The influence of coagulants on macrophytes and environmentally valuable charophytes is poorly understood. Thus, the aim of the study was to determine the reaction of *Chara hispida* L. under the influence of chemical coagulants. The selected species frequently inhabits eutrophic waters, what increase its exposure to restoration treatments. Direct and the strongest influence on charophytes resulted from water acidification. This determined further disturbances in water quality and caused negative consequences in *C. hispida* functioning. Acidification transformed alkalic environmental conditions, which are preferred by charophytes and caused encrustation dissolution. This process led to calcium-magnesium equilibrium disturbances in the water. Acidification induced increasing solubility and toxicity of aluminium followed by easier bioavailability. It was demonstrated that aluminium was accumulated in charophyte cells (up to 2.0 $mg \cdot g^{-1}$ dry mass) which resulted in damage of intercellular structures (chloroplasts disintegration) and detachment of corticating cells. Simultaneously coagulants transformed physicochemical properties of water (turbidity and colour increased) followed by light availability reduction for charophytes. The growth pattern modification and generative reproduction reduction were the effects of water properties changes under influence of iron and aluminium coagulants. The studies clearly indicated that high concentration of phosphates coagulants application is dangerous for *C. hispida* functioning.

WSTĘP

Ramienice (Characeae, Charophyta) mimo, że powszechnie uważane za element fitocenoz charakterystycznych dla wód czystych, alkalicznych i ubogich w związki biogenne, występują również w wodach eutroficznych (Gąbka, 2009; Pukacz i in., 2013). Pełnią rolę siedliskotwórczą (są uważane za organizmy pionierskie), przyczyniają się do stabilizacji osadów dennych (ograniczając resuspensję) oraz stanowią refugium i bazę pokarmową dla bezkręgowców i ryb. W wyniku zdolności do szybkiego wytwarzanie dużej biomasy są wydajną pułapką dla nutrientów. Dodatkowo wiążą bioprzyswajalne jony fosforanowe w trakcie wytrącania węglanu wapnia tworzącego inkrustację. Wytwarzane związki allelopatyczne podobnie, jak konkurencja o zasoby, hamują rozwój fitoplanktonu, co czyni zbiorowiska ramienic pożądanymi w procesach re-oligotrofizacyjnych wód (Kufel i Kufel, 2002; Van Donk i Van de Bund, 2002).

Postęp eutrofizacji wód powierzchniowych wymaga podjęcia działań naprawczych mających na celu powstrzymanie, a najlepiej odwrócenie trendu negatywnych zmian jakości wód. Stymulatorem przyspieszenia działań odnowy wód (najczęściej poprzez rekultywację) są wymogi Ramowej Dyrektywy Wodnej obligującej nasz kraj do przywrócenia dobrego stanu ekologicznego wód do roku 2027. Wśród wielu metod rekultywacji, chemiczna inaktywacja fosforanów jest jedną z najczęściej stosowanych w zbiornikach wodnych. Powszechność tej metody wynika z dużej skuteczności działania, szybkości uzyskiwania widocznych efektów oraz niskich kosztów stosowania (Jančula i Maršálek, 2011; Zamparas i Zacharias, 2014). Mechanizm działania polega na zastosowaniu nieorganicznych, kwaśnych soli żelaza lub glinu, które wiążą fosforany w postaci związków kompleksowych (Sobczyński i in., 2012; Dunalska i Wiśniewski, 2016). Dodatkowym pozytywnym efektem ich oddziaływania (koagulacji) jest tworzenie „kłaczków" o dużej powierzchni absorpcyjnej, sprzyjającej eliminacji zawiesiny, w tym planktonu. Tak powstałe kompleksy sedymentują grawitacyjnie do osadów dennych (Pizarro i in., 1995; Sobczyński i Joniak, 2009). Niemniej skutki biologiczne stosowania zabiegów, z uwagi na właściwości chemiczne koagulantów, nie zawsze dają się przewidzieć a skala ryzyka niekorzystnych zmian nie jest w pełni poznana. Niestety, często metody chemiczne są wykorzystywane w celu uzyskania poprawy jakości wody w trakcie okresowych wahań trofii (Joniak i in., 2013), które objawiają się zakwitami fitoplanktonu (zwłaszcza sinic)

i niekorzystnymi transformacjami właściwości fizykochemicznych wód. Ich konsekwencją są zaburzenia funkcji rekreacyjnej i gospodarczej, co zmniejsza możliwość wykorzystania wód i przynosi straty ekonomiczne (Pretty i in., 2003). W takich okolicznościach zabiegi chemiczne przynoszą tylko krótkotrwały efekt i przyczyniają się do dalszego rozchwiania homeostazy zbiornika (Joniak i in., 2013).

Dotychczasowe badania nad rekultywacją wód skupiały się na określeniu efektywności stosowanych metod bądź ich kombinacji (Søndergaard i in., 2007; Rosińska i in., 2018). Poszukiwano najwydajniejszych sposobów rekultywacji oraz nowych (ekonomicznych) metod, na przykład łączenia metod chemicznych z fizycznymi (natlenianie) i biologicznymi (biomanipulacja). W badaniach tych pomijany był element bezpośredniego wpływu substancji chemicznych na fitocenozy obecne w ekosystemie. Dotychczasowe prace skupiały się na przebudowie lub odnowie poszczególnych zbiorowisk roślinnych w następstwie zmian warunków środowiskowych (Hilt i in., 2006; Rosińska i in., 2017). Stosowanie metod chemicznych wiąże się z dużym ryzykiem, gdyż może wywołać zaburzenia w funkcjonowaniu makrofitów. Konsekwencją zabiegów chemicznej inaktywacji fosforanów jest szereg zmian środowiska, które w dużej mierze pozostają nie przewidywalne (np. eliminacja biocenoz), co w efekcie może zniweczyć wysiłek włożony w procesy re-oligotrofizacyjne.

CELE PRACY I HIPOTEZY BADAWCZE

Celem rozprawy było określenie mechanizmów reakcji *Chara hispida* L. na zabiegi rekultywacyjne z wykorzystaniem koagulantów fosforanów opartych na bazie żelaza lub glinu. Wybrany gatunek potraktowano, jako modelowy dla ramienic zasiedlających wody eutroficzne. Wśród celów szczegółowych znalazły się:

1. określenie mechanizmów oddziaływania koagulantów na ramienice,
2. identyfikacja zmian morfologicznych i zmian w wydajności reprodukcyjnej gatunku,
3. charakterystyka skutków toksycznego oddziaływania glinu i wielkości bioakumulacji w plechach.

Postawiono następujące hipotezy:

H1. Koagulanty zmieniają właściwości abiotyczne wód w sposób zakłócający funkcjonowanie ramienic (artykuł A1, A2).

H2. Koagulanty powodują zahamowanie wzrostu i rozmnażania ramienic (artykuł A2, A3).

H3. Glin wywołuje uszkodzenia plech i jest aktywnie akumulowany w komórkach (artykuł A4).

OBIEKT BADAŃ I METODYKA

Gatunkiem modelowym była ramienica kosmata *Chara hispida* L. (1753), powszechnie występująca w Europie, Azji i północnej Afryce. Jest to jeden z największych przedstawicieli rodzaju *Chara* – osiąga długość nibyłodygi od 30 do 200 cm; średnica do 4 mm; długość komórek międzywęźli od 10 do 15 cm; długość nibyliści do 8 cm (7-11 nibyliści w okółku). Jest to gatunek jednopienny, wytwarzający pojedyncze oogonia. *C. hispida* zasiedla osady organiczne, rzadziej torfowe i gytie. Występuje na głębokości od 1 do3 m i charakteryzuje się dużą tolerancją na zmiany natężenia promieniowania słonecznego (Andrews i in., 1984). Preferuje wody o odczynie neutralnym i alkalicznym z wysoką zawartością wapnia (Gąbka, 2009; Urbaniak i Gąbka, 2014). Z racji wymagań siedliskowych często występuje w wodach mezotroficznych i eutroficznych, gliniankach oraz torfiankach. Z uwagi na występowanie w wodach eutroficznych istnieje ryzyko jej narażenia na wpływ zabiegów rekultywacyjnych.

W trakcie badań analizowano oddziaływanie koagulantów: siarczanu (VI) żelaza (III) – $Fe_2(SO_4)_3$ oraz polichlorku glinu – $[Al_n(OH)_mCl_{(3n-m)}]_x$. Cechą chemizmu koagulantów jest niskie pH (<1.0) oraz barwa, ciemnobrązowa (koagulant żelazowy) lub jasnożółta (koagulant glinowy).

Badania przeprowadzono w oparciu o dwa rodzaje eksperymentów:

- laboratoryjny mikrokosmowy
- terenowy mezokosmowy.

Koagulanty dawkowano jednorazowo w stężeniach: 50,0 $cm^3 \cdot m^{-3}$ (dawka niska), 100,0 $cm^3 \cdot m^{-3}$ (dawka średnia) i 200,0 $cm^3 \cdot m^{-3}$ (dawka wysoka) Wybór stężeń i liczba aplikacji zostały określone na podstawie badań wyjściowych, gdzie poszukiwano najniższego stężenia koagulantu dającego efekt zupełnego strącenia

fosforanów i eliminacji z toni wodnej barwy i zawiesin (cechy tzw. „rekultywacji agresywnej"). Kolejne, dwie wyższe dawki były zwielokrotnieniem dawki najniższej. Wszystkie wybrane dawki mieściły się w zakresie stężeń stosowanych w rekultywacji zdegradowanych wód naturalnych (Bakker i in., 2016; Orihel i in., 2016).

Eksperyment laboratoryjny trwał 30 dni. Miał on na celu zdefiniowanie zmian morfologicznych i reprodukcyjnych ramienic oraz charakterystyk fizyczno-chemicznych wody w poszczególnych dawkach koagulantu. W eksperymencie 4 nibyłodygi (o długości 20 cm) z wcześniej określoną morfologią i liczbą oospor umieszczano w mikrokosmach wypełnionych przefiltrowaną wodą jeziorną (filtry z włókna szklanego, typ GF/C). Naczynia umieszczono w pokoju termostatycznym, gdzie panowała stała temperatura 25,5 °C i oświetlenie – 14h dzień (160,6 $\mu mol \cdot m^{-2} \cdot s^{-1}$) i 10h noc. Czas stabilizacji warunków i aklimatyzacji ramienic wynosił 72 godziny. Po tym czasie zaaplikowano siarczan żelaza, a następnie od razu określono stężenie fosforanów rozpuszczonych i siarczanów oraz wartości parametrów fizykochemicznych (temperatura, tlen rozpuszczony, pH, przewodnictwo właściwe, barwa, mętność). Pomiary morfologiczne ramienicy wykonano przed i po zakończeniu eksperymentu. Stężenia fosforanów analizowano przed eksperymentem, 72h po aplikacji i po zakończeniu eksperymentu, a pozostałe parametry mierzono co 72h.

Eksperymenty terenowe przeprowadzono z wykorzystaniem koagulantu żelazowego (w 2014 roku) oraz koagulantu glinowego (w 2015 roku). Miejscem realizacji było Jezioro Wielkowiejskie w Wielkopolskim Parku Narodowym (koordynaty geograficzne: N 52°17'43", E 16°40'5"). Jest to zbiornik rynnowy, o niewielkiej powierzchni (13,3 ha) i głębokości (maksymalna 4,2 m). Cechą charakterystyczną jeziora jest szeroki, okalający całe lustro wody pas szuwaru i duża różnorodność makrofitów z grup ekologicznych elodeidów i nymfeidów. W strefie fitolitoralu, przy wschodnim krańcu jeziora funkcjonuje duża, monogatunkowa łąka *Chara hispida*. Jej duża powierzchnia umożliwiła szeregowe rozmieszczenie 8 mezokosmów (po 2 na każdą dawkę oraz kontrola). Mezokosmy były konstrukcjami stalowymi (zabezpieczone antykorozyjnie) o wymiarach 1m×1m×2m, których ściany wykonane zostały z przezroczystej foli polietylenowej. Komory osadzone były na głębokości 30 cm w osadzie, co uniemożliwiało infiltrację wody z jeziora. Objętość komór w roku 2014 wynosiła około 1 m^3, a w 2015 0.8 m^3, co wynikało z poziomu wody w jeziorze. Okres stabilizacji warunków przed rozpoczęciem eksperymentu trwał

1 miesiąc. Wyjściowo określone zostały właściwości chemiczne wody i zmierzone parametry fizykochemiczne. Koagulanty dawkowano jednorazowo w stężeniach tożsamych z zastosowanymi w eksperymencie laboratoryjnym. Podczas aplikowania koagulant rozprowadzano na całej powierzchni wody w komorze i turbulentnie mieszano wodę by rozprowadzić koagulant.

W przedmiocie badań nad akumulacją glinu i sprawdzeniu efektu toksyczności wykonano laserową skaningową konfokalną analizę mikroskopową plech ramienic. Obrazy powierzchni i wnętrza komórek wykonane zostały za pomocą laserowego skaningowego mikroskopu konfokalnego Zeiss LSM 510 ze światłem wzbudzenia 543 nm emitowanym przez zielony laser helowo neonowy. Zdjęcia mikroskopowe analizowano programie Zeiss LSM software.

Osobniki *C. hispida* przeznaczone do pomiarów morfologicznych i analiz pierwiastkowych pobierano przed eksperymentem i w jego trakcie z 7-dniowym interwałem. Ramienice po pobraniu umieszczano w szczelnych plastikowych workach i przewożono do laboratorium, gdzie były niezwłocznie analizowane. Badania morfologii ramienicy obejmowały: długość osi głównej, długość i liczbę rozgałęzień I, II i III rzędu, długość najdłuższego nibyliścia w okółku i długość komórek międzywęźli w węzłach 1-3 licząc od części apikalnej. Określano również liczbę oospor na każdej nibyłodydze. Suchą masę ramienic określano po dokładnym przepłukaniu ramienic wodą kranową i wysuszeniu w suszarce w 80°C (ważenie z dokładnością do 0,0001 g).

Pomiary *in situ* wykonywano za pomocą następujących przyrządów: HI 98129, Hanna Instruments (temperatura, pH, przewodnictwo), Pro Plus YSI (tlen rozpuszczony), TN-100 Eutech (mętność). Natężenie promieniowania fotosyntetycznie czynnego mierzono za pomocą miernika kwantowego z sensorem sferycznych LI-COR, Biosciences. Barwę wody określano metodą wizualną w skali platynowo-kobaltowej. Analizy chemiczne wody obejmowały: fosforany rozpuszczone metodą z kwasem molibdenowym po filtracji przez filtry 0,45 μm (PN-EN ISO 6878), siarczany metodą grawimetryczną z chlorkiem baru (PN-ISO 9280), wapń i magnez metodą miareczkowania kompleksometrycznego (PN-ISO 6059), chlorki metodą miareczkowania azotanem srebra w obecności chromianu jako wskaźnika (PN-EN ISO 10304-4:2002P), żelazo ogólne i glin w wodzie oraz stężenia glinu w biomasie ramienic analizowano metodą atomowej spektrometrii masowej (spektrometr seria AA 200, Agilent Technologies) po mineralizacji mikrofalowej (Mars Xpress CEM).

WYNIKI I DYSKUSJA

W pierwszej części rozprawy (artykuły A1-A2) przedstawiono skutki zakwaszenia, jakie powstają w wyniku aplikacji koagulantu żelazowego i glinowego oraz konsekwencje z tego wynikające dla *C. hispida*. Skupiono się na jonach, których stężenia zmieniają się pod wpływem aplikacji koagulantu oraz są wprowadzane do środowiska wraz z nim, ale nie biorą udziału w procesie koagulacji fosforanów tj. wapnia, magnezu, siarczanowych i chlorkowych.

Spadek pH ściśle korelował z zastosowanym stężeniem koagulantu, jednakże przy zastosowaniu tych samych dawek, koagulant glinowy wywołał znacznie większe zakwaszenie. Najniższa dawka koagulantu żelazowego wywołała obniżenie pH o 0,9 jednostki, nie wywołując efektu zakwaszenia (pH 7,0). W komorach ze średnią dawką odczyn spadł do pH 6,7 a w przypadku aplikacji dawki najwyższej do pH 6,5. Najniższa dawka koagulantu glinowego wywołała obniżenie pH do 6,5, średnia do pH 5,7, a najwyższa do pH 4,3 (spadek o 5,4 jednostki). W tym przypadku nawet najmniejsze obniżenie pH, poniżej wartości obojętnej, powodowało zmiany w środowisku abiotycznym. Zakwaszenie istotnie zmieniało preferowane przez ramienice alkaliczne warunki środowiskowe i powodowało rozpuszczanie węglanowej inkrustacji oraz uwalnianie jej głównych składników – jonów wapnia (Ca^{2+}) i magnezu (Mg^{2+}).

Mechanizm powstawania inkrustacji wynika z pobierania przez ramienice jonów wodorowęglanowych (HCO_3^-) jako alternatywy dla dwutlenku węgla w procesie fotosyntezy. Na skutek tego procesu łatwo rozpuszczalny wodorowęglan wapnia jest transformowany w trudno rozpuszczalny węglan wapnia i wytrąca się w formie krystalicznej na powierzchni plech. Analogiczny proces występuje w przemianie wodorowęglanu magnezu (Raven i in., 1986; Cicerone i in., 1999; Kufel i in., 2016). Utrata inkrustacji zwiększa wrażliwość ramienic na czynniki środowiskowe, gdyż jej obecność wzmacnia komórki, zabezpiecza przed urazami mechanicznymi i chroni przed promieniowaniem UV (Raven i in., 1986; Coletta i in., 2001).

Analiza zmian stężeń wapnia w wodzie, w wyniku aplikacji koagulantu żelazowego, wykazała najwyższy wzrost stężenia w komorach ze średnią dawką – wzrost o 15% w porównaniu do prób kontrolnych. W mezokosmach z najwyższą dawką, mimo większego zakwaszenia, stężenie jonów Ca^{2+} było zbliżone do występującego w komorach z dawką niską i kontrolną. Analogiczny schemat zmian

stężeń dotyczył jonów siarczanowych (SO_4^{2-}). Ich koncentracja wzrosła pod wpływem aplikacji koagulantu w komorach ze stężeniem średnim o 70% w porównaniu do kontroli i była o 40% wyższa niż w komorach z dawką najwyższą. Pośrednie oddziaływanie siarczanów na ramienice wiąże się z intensyfikacją zasilania wewnętrznego i wzrostem trofii wody. Bezpośredni wpływ wiąże się z ich toksycznością, która jest implikowana stężeniem tlenu – w warunkach beztlenowych siarczany są redukowane do toksycznych siarczków (Van Der Welle i in., 2006; Cirkel i in., 2014). Dla ramienic nie stanowi to jednak zagrożenia, ponieważ ich siedliska charakteryzują się dobrym natlenieniem wody (Kufel i Kufel, 2002). Nie mniej jednak, stwierdzony w komorach z najwyższym stężeniem koagulantu żelazowego tożsamy wzorzec zmian stężeń jonów Ca^{2+} i SO_4^{2-} sugerował przekroczenie iloczynu rozpuszczalności siarczanu wapnia. W wyniku tego procesu wytrąciła się trudno rozpuszczalna sól, co spowodowało nieodwracalne zubożenie puli wapnia w środowisku. Taki rodzaj dekalcyfikacji prowadzi do zachwiania równowagi wapniowej w wodach, a w konsekwencji zaburzenia funkcjonowania zbiorowiska ramienic.

Aplikacja koagulantu glinowego i efekt większego zakwaszenia wody spowodowały uwolnienie z inkrustacji ramienic znacznie większych ilości jonów Ca^{2+} (proporcjonalnie do stężenia koagulantu). Dawka niska spowodowała wzrost o 30%, średnia o 40%, a wysoka o 70% w stosunku do kontroli. Jony wapnia są niezbędne dla wielu procesów życiowych zarówno u roślin, jak i u glonów. Wapń utrzymuje polaryzację błony komórkowej i reguluje aktywność metaboliczną (Edel i in., 2017) oraz jest kluczowym składnikiem wpływających na syntezę chlorofilu. Wysokie stężenie wapnia powoduje zaburzenia wydajności procesu fotosyntezy poprzez redukcję stężeń chlorofilu *b* i zmianę proporcji chlorofilu *a:b* (Gomes i Asaeda, 2010; Siddiqui i in., 2012; Asaeda i in., 2014). Porównywalnie do wapnia kształtowała się zmiana stężeń jonów chlorkowych (Cl^-). W komorach z niską i średnią dawką stwierdzono wzrost stężenia chlorków o 60%, natomiast w mezokosmach z dawką najwyższą o ponad 200% w porównaniu do komór kontrolnych. W tym przypadku nie obserwowano wytrącenia chlorku wapnia, co wynika z wysokiej rozpuszczalności tej soli. Chlorki są słabo reaktywne i całkowicie rozpuszczalne w wodzie. Jednak zmiany ich koncentracji wraz z wahaniami pH mogą powodować zaburzenia w ich transporcie przez błonę komórkową ramienic (Sanders, 1981; Parihar i in., 2015).

Pod wpływem koagulantu żelazowego nie nastąpiły zmiany stężeń jonów magnezowych. Wynikało to z faktu znacznie większej odporności węglanu magnezu na rozpuszczanie (Müller i in., 1972), a zakwaszenie uzyskane pod wpływem siarczanu żelaza było zbyt słabe by spowodować rozpad tej soli. Efekt ten uzyskano w eksperymencie z polichlorkiem glinu, ale tylko po zastosowaniu najwyższej dawki (wzrost o 8% w stosunku do kontroli). Wskazywało to na rozpuszczanie inkrustacji magnezowej przy pH poniżej 4,5. Wahania stężeń jonów magnezowych rejestrowane w czasie eksperymentu dowodziły niskiej efektywności jego wytrącania. Niemniej jednak, podwyższone stężenia Mg^{2+} hamują proces kalcyfikacji ramienic przez węglan wapnia. Wynika to z wiązania jonów Mg^{2+} z jonami HCO_3^- lub zajęciem miejsca wapnia w strukturze krystalicznej, co blokuje dalsze wytrącanie węglanu wapnia. W znaczeniu biologicznym jony magnezu są podstawowym elementem chlorofilu, stymulują elongację ramienic oraz rozwój rozgałęzień I, II i III rzędu (Gomes i Asaeda, 2010). Ponadto, wzrost stężenia magnezu powoduje wzrost stężenia chlorofilu *b* i zmianę stosunku chlorofilu *a*:*b* w przeciwną stronę niż jony wapnia. Zatem, obecność obu jonów w równowadze nie wywołuje zmian w stężeniu barwników fotosyntetycznych, co wynika z ich przeciwstawnego oddziaływania neutralizującego negatywny efekt jednego pierwiastka w obecności drugiego (Asaeda i in., 2014). W przeprowadzonych badaniach efekt równoczesnego wzrostu stężeń jonów wapnia i magnezu odnotowano tylko w wariancie z najwyższą dawką koagulantu glinowego. Reasumując, oznacza to, że zabiegi rekultywacyjne oparte na koagulancie żelazowym lub glinowym w większym stopniu powodują zmiany stężeń wapnia niż magnezu.

W tej części dysertacji zweryfikowano hipotezę H1 stwierdzając, że aplikacja koagulantów istotnie zmienia preferowane przez ramienice warunki środowiska. Pierwszą i jednocześnie najbardziej znaczącą zmianą jest zakwaszenie środowiska. Determinuje to dalsze, negatywne dla ramienic, przemiany środowiska abiotycznego. W badaniach wykazano, że przy takich samych stężeniach koagulantów, polichlorek glinu wywołał większy spadek pH niż siarczan żelaza. Niemniej jednak, już obniżenie odczynu poniżej wartości obojętnej zmieniło preferowane przez ramienice warunki środowiska oraz spowodowało rozpuszczenie inkrustacji *C. hispida*. Skutkowało to uwolnieniem do środowiska jonów wapnia w stężeniu zależnym od dawki koagulantu. Jony magnezu były uwalniane tylko podczas silnego zakwaszenia, a ich stężenie było znacznie mniejsze niż jonów wapnia. Wysokie stężenie koagulantu żelazowego powodowało zubożenie zasobów wapnia poprzez jego trwałe wytrącenie z

jonami siarczanowymi w postaci siarczanu wapnia. Tymczasem wysoka dawka koagulantu glinowego zwiększyła mobilność magnezu. Powyższe procesy spowodowały zaburzenie równowagi wapniowo–magnezowej, co skutkuje zakłóceniem kalcyfikacji oraz ma wpływ na metabolizm i rozwój ramienic.

W drugiej części rozprawy (artykuły A2-A3) przedstawiono zmiany wzorca wzrostu i rozmnażania *C. hispida* pod wpływem koagulantu żelazowego i koagulantu glinowego. W wyniku aplikacji koagulantów jednakowo zmieniły się warunki abiotyczne środowiska. Pierwsze zmiany (trwające 72h) polegały na zakwaszeniu, wzroście mętności i barwy wody. Towarzysząca temu eliminacja fosforanów rozpuszczonych była stanem trwałym. W późniejszym czasie w wyniku koagulacji i flokulacji powstawała zawiesina, która grawitacyjnie sedymentując pokrywała plechy ramienic. Ilość zawiesiny (ściśle oblepiającej ramienice) i intensywność barwy wzrastały wprost proporcjonalnie do dawki koagulantu. Barwa osadu powstałego po aplikacji koagulantu żelazowego była ciemnobrązowa, a koagulatu glinowego kremowo-żółta, opalizująca. Transformacja warunków środowiskowych doprowadziła do zmian morfologicznych *C. hispida*, lecz reakcje były zróżnicowane i zależne od rodzaju koagulantu.

W eksperymencie z siarczanem żelaza zmniejszyło się relatywne tempo wzrostu (RGR – ang. relative growth rate), co ujawniło się również poprzez zmniejszenie długości całkowitej ramienic. Było to widoczne zwłaszcza w najwyższej dawce koagulantu. Analiza komórek międzywęźli nie wykazała istotnych zmian długości, aczkolwiek w średniej i wysokiej dawce koagulantu obserwowano ich słabą elongację. Zahamowaniu uległ wzrost nibyliści, ale zmiany te nie były istotne statystycznie. Czynnikiem różnicującym była długość odgałęzień I-rzędu, która w średnim i najwyższym stężeniu koagulantu wzrosła ponad dwukrotnie. Analiza liczby oospor wykazała ich bardzo duży spadek w najwyższej dawce koagulantu (30% w stosunku do próby kontrolnej).

Po aplikacji koagulantu glinowego również zaobserwowano zahamowanie wzrostu osi głównej, lecz nie stwierdzono statystycznej istotności różnic między dawkami koagulantu a próbą kontrolną. Dodatkowo zahamowaniu uległ wzrost nibyliści. W wysokiej dawce koagulantu wykazano natomiast intensywny wzrost komórek drugiego i trzeciego międzywęźla. Analogicznie do eksperymentu z koagulantem żelazowym odnotowano zwiększenie długości rozgałęzień I-rzędu. Liczba rozgałęzień pozostała bez zmian.

Zarówno w eksperymencie z koagulantem żelazowym, jak i z glinowym zmiany w morfologii *C. hispida* były spowodowane ograniczeniem dostępności światła. Wynikało to początkowo ze wzrostu mętności i barwy wody, a następnie pokrycia plech opadłą zawiesiną. Obserwowane reakcje ramienic były mechanizmami obronnymi, które w przypadku koagulantu żelazowego polegały na zwiększeniu powierzchni asymilacyjnej poprzez rozwój rozgałęzień bocznych, a glinowego na szybkim dorastaniu do powierzchni wody. Należy zaznaczyć, że zwiększanie powierzchni asymilacyjnej nie było wcześniej opisanym mechanizmem obronnym i jest alternatywą dla typowej odpowiedzi na zacienienie, polegającej na szybkiej elongacji i dorastaniu do powierzchni wody (Blindow i Schütte, 2006). W obydwu eksperymentach zmiana wzorca wzrostu i szybki rozwój poszczególnych części plech następował mimo braku biodostępnych fosforanów w wodzie, co sugeruje alokację zasobów na przetrwanie niekorzystnych warunków środowiska (Kozłowski, 1992). Potwierdzało to znaczne ograniczenie produkcji oospor w najwyższym stężeniu koagulantu żelazowego, gdzie nastąpiło przekierowanie energii z kosztownego procesu reprodukcji na rozwój powierzchni asymilacyjnej.

Reasumując, w tej części rozprawy częściowo sfalsyfikowano hipotezę H2, ponieważ wzrost *C. hispida* nie został ograniczony, a jedynie zmienił się jego wzorzec. Ponadto potwierdzono, że zmiana warunków fizykochemicznych pod wpływem koagulantu żelazowego i koagulantu glinowego wpływa na rozwój ramienicy (H1). Zmiany te w głównej mierze polegały na pogorszeniu warunków świetlnych. W początkowym etapie spowodowane to było właściwościami chemicznymi koagulantów, a w dalszej kolejności przez pokrycie plech ramienic opadłą zawiesiną. W sytuacji aplikacji koagulantu w środowisku naturalnych oznacza to, że mimo uzyskania lepszych warunków świetlnych w toni wodnej ramienice są poddawane stresowi zacienienia. Uruchamia to u nich mechanizmy obronne powiązane z intensywnym zwiększeniem powierzchni asymilacyjnej i dorastaniem do powierzchni wody. W związku z brakiem biodostępnych fosforanów ramienice alokują energię z procesów rozmnażania na procesy przetrwania w niekorzystnych warunkach środowiska.

W trzeciej części rozprawy (artykuł A4) przedstawiono efekty oddziaływania polichlorku glinu na *C. hispida* oraz określono jej zdolności bioakumulacji glinu. Metal ten w neutralnym pH nie wykazuje właściwości toksycznych, które znacząco wzrastają wraz z zakwaszeniem (Gensemer i Playle, 1999). Zakwaszenie następujące po aplikacji

koagulantu jest zatem czynnikiem kluczowym dla regulacji toksyczności tego metalu. Badania wykazały, że toksyczne oddziaływanie glinu objawia się uszkodzeniami struktur wewnątrzkomórkowych (redukcja chloroplastów), odrywaniem komórek okorowania oraz ogólnym osłabieniem i zwiotczeniem nibyłodygi. Pierwsze symptomy toksyczności były widoczne po 24h nawet w niskiej dawce koagulantu. Po 72h od aplikacji we wszystkich mezokosmach stwierdzono dużą skalę uszkodzeń strukturalnych ramienicy. Aktywna asymilacja glinu przez komórki *C. hispida* była wypadkową zakwaszenia i czasu ekspozycji ramienic. W badaniach mikroskopowych zaobserwowano cząsteczki glinu wewnątrz komórek, co świadczyło o jego aktywnym transporcie.

Mechanizm toksycznego oddziaływania glinu na makroglony nie został jeszcze dokładnie sprecyzowany. Prace podejmujące tematykę wpływu tego metalu na glony jednokomórkowe wymieniają przede wszystkim inhibicję wzrostu połączoną z zaburzeniem asymilacji azotu i dwutlenku węgla (Gensemer i Playle, 1999; Kinross i in., 2000). Glin ogranicza napływ jonów wapnia i zajmuje ich miejsce w ścianie komórkowej, co ogranicza wzrost komórki (Reid i in., 1995). Jednakże nadrzędnym mechanizmem leżącym u podstaw toksyczności glinu wydają się być zaburzenia właściwości i spójności błon komórkowych (Ahn i in., 2002), a proces ten został potwierdzony również u ramienic (Takabatake i Shimmen, 1997; Takano i Shimmen, 1999). Poprzez zdolność glinu do wiązania grup karboksylowej i fosforanowej w ścianie i błonach komórkowych następuje dezintegracja błon. Bezpośrednim efektem tego procesu był rozpad chloroplastów, co skutkowało chlorozami i nekrozami na plechach. Powodowało to również spadek stężeń barwników fotosyntetycznych (chlorofil *a*, chlorofil *b*, karotenoidy), które ulegają degradacji (Rybak i in., 2016). Większość uszkodzeń była widoczna na nibyliściach, co często wiązało się z redukcją ich długości. Uszkodzenia komórek prowadzą do osłabienia pobierania jonów wodorowęglanowych, co zaburza metabolizm i wytwarzanie inkrustacji węglanowej. Skutki te są nieodwracalne, a ponowna alkalizacja środowiska nie pobudza odnawiania inkrustacji (Takano i Shimmen, 1999).

Najwyższe stężenie glinu w plechach odnotowano po 48h od aplikacji koagulantu. W późniejszym czasie, wskutek uszkodzenia komórek i uwalniania ich zawartości do środowiska, jego stężenie malało. Zauważono, że w mezokosmach ze średnią i wysoką dawką (wysoka była dwukrotnie wyższa od średniej) nie było różnic stężeń glinu w plechach (w obu przypadkach 2,0 mg · g^{-1} suchej masy).

Uzasadnieniem powyższego stanu były różne czynniki decydujące o asymilacji. W mezokosmach z wysokim stężeniem odczyn wody był optymalny dla tempa przenikania i bioakumulacji glinu (pH 4,3). Tymczasem w komorach ze średnim stężeniem koagulnatu, czasu dostępności jonów glinu rekompensował mniej wydajne warunki asymilacji. Wynika to z faktu znaczenia odczynu wody, którego zmiana o 0,3 jednostki powoduje 3-krotną redukcję tempa asymilacji glinu (Taylor i in., 2000). Czynnikiem zmniejszającym akumulację metalu w komórkach były fizyczne ich uszkodzenia. Wskaźnik akumulacji (BCF), który jest użytecznym narzędziem w ocenie zdolności akumulacyjnych, wyniósł u *C. hispida* maksymalnie 208. Oznaczało to zahamowanie absorpcji glinu na poziomie wskazującym na słabe zdolności gatunku do akumulacji i fitoremediacji. Generalnie, stwierdzone stężenie glinu w komórkach *C. hispida* było wysokie, w porównaniu do innych makrofitów zanurzonych (Albers i Camardese, 1993; Abu Bakar i in., 2013). Niski stopień bioakumulacji przypisać należy wysokiej koncentracji początkowej glinu w wodzie i stosunkowo krótkiej jego dostępności, wobec szybkiej alkalizacji środowiska wodnego, która blokuje biodostępność i redukuje toksyczność W ostatniej części rozprawy zweryfikowano hipotezę H3. Toksyczny wpływ glinu na *C. hispida* przejawiał się uszkodzeniami plech, takimi jak chlorozy, martwice, oderwanie komórek okorowania i zwiotczenie nibyłodygi. Uszkodzenia uwidaczniały się po 24 godzinach nawet w najniższym stężeniu koagulantu i intensyfikowały się w ciągu kolejnych 48h. Zdolność gatunku do bioakumulacji glinu była ograniczona do 2,0 mg Al $\cdot$ g^{-1} suchej masy, co stanowiło ilość znaczną, ale w odniesieniu do całkowitego stężenia metalu w wodzie musi być uznane za niskie. Uzyskane wyniki sugerują, że stosując koagulanty oparte na glinie w jeziorach ze zbiorowiskami ramienic należy zachować szczególną ostrożność.

PODSUMOWANIE

Odrębność środowiskowa każdego ekosystemu wodnego nie pozwala na odgórne wyznaczenie jednolitych metod rekultywacyjnych wraz z założoną *a priori* częstotliwością zabiegów. Dotyczy to głównie metod chemicznych, których efektywność ściśle zależy od chemizmu wód poddanych rekultywacji. Dlatego stosowanie koagulantów powinno być zawsze poprzedzone badaniami pozwalającymi określić bezpieczne dla danego ekosystemu stężenie koagulantu przy jednoczesnym

spełnieniu postawionych celów. Należy mieć na uwadze, że specyficzne zbiorowiska roślin podwodnych oraz ramienic występują również w wodach, które mogą z różnych względów, zostać poddane rekultywacji. Badania jednoznacznie wykazały, że źle dobrane dawki koagulantów wywierają negatywny wpływ na rozwój i funkcjonowanie zbiorowisk *Chara hispida*. Aplikacja koagulantów powoduje zakwaszenie środowiska, co jest jednym z głównych czynników indukujących kolejne negatywne zmiany. Zakwaszenie zaburza preferowaną przez gatunek równowagę wapniowo–magnezową oraz reguluje toksyczność glinu. Metal ten w kwaśnym odczynie wody jest aktywnie bioakumulowany do wnętrza komórek, co skutkuje szeregiem uszkodzeń takich jak rozpad chloroplastów, odrywanie komórek okorowania i ogólne zwiotczenie nibyłodygi. Poprzez zmianę warunków abiotycznych środowiska wodnego koagulanty wymuszają na ramienicy alokację zasobów i modyfikację wzorca wzrostu kosztem rozmnażania generatywnego. Powoduje to zubożenie banku oospor i jest zagrożeniem dla odnowienia się zbiorowiska w sytuacji jego eliminacji. Wszystkie te oddziaływania negatywnie wpływają na funkcjonowanie zbiorowiska. Efekt zaniku podwodnych łąk ramienic oznacza duże prawdopodobieństwo zniweczenia wysiłków włożonych w rekultywację.

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Pol. J. Environ. Stud. Vol. 28, No. 4 (2019), 1-9
DOI: *10.15244/pjoes/90095* ONLINE PUBLICATION DATE:

Original Research

Experimental Investigation into Disturbance of Ca-Mg Equilibrium and Consequences for Charophytes after Iron and Aluminium Coagulants Application

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Received: 13 Febrary 2018
Accepted: 18 April 2018

Abstract

Iron sulphate and polyaluminum chloride are commonly used in water restoration to eliminate cyanobacteria bloom and improve water quality. Nevertheless, the influence of coagulants on water organisms remains insufficiently studied. The study involves the analysis of phosphate coagulants' impact on calcium and magnesium concentrations in the *Chara hispida* community. The experiments were carried out in field mesocosms. Both coagulants were applied once in three different doses: 50.0, 100.0, and 200.0 $cm^3 \cdot m^{-3}$. The application of coagulants caused a decrease of pH and calcium carbonate and magnesium carbonate dissolution. Although the changes were proportional to the coagulant concentrations, the aluminum coagulant triggered more considerable disturbances. The highest dose of iron sulphate caused the precipitation of hardly soluble calcium sulphate and the elimination of part of calcium from biological circulation. The concentrations of magnesium in water increased only at pH <4.5 following the application of the highest dose of polyaluminum chloride. Shifts in the Ca-Mg equilibrium, which result in the disturbance of biogenic calcification, may affect charophyte metabolism and lead to the elimination of charophyte communities. Therefore, inactivation treatments using acidic coagulants in lakes with charophyte communities ought to be preceded by preliminary studies in order to determine the least harmful dosage for the ecosystem.

Keywords: charophyte encrustation, Ca-Mg equilibrium, chemical phosphate coagulants, acidification, lake restoration

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Introduction

Charophytes contribute to maintaining a clear water state by producing large quantities of biomass and accumulating large concentrations of nutrients on the one hand, and competing with phytoplankton in resources on the other [1-2]. They stabilize bottom sediments, limit the migration of nutrients, and create refugium and food base for animals [3]. Moreover, the water decalcification process is followed by co-precipitation of phosphates with calcite, resulting in reduced phosphorus bioavailability in water [4-5].

Despite the fact that charophytes are supposed to be a typical component of clear, alkaline, and nutrient-poor lakes, some species also inhabit meso-eutrophic and eutrophic water bodies [6-7]. These types of lakes are more vulnerable to restoration treatments when taking into account an urgent need to implement the guidelines of the Water Framework Directive. Furthermore, occasionally some restoration treatments are carried out in lakes with temporary trophic disturbances, which is most often correlated with phytoplankton blooms and shifts in physicochemical features of water [8-9]. One of the most common methods of restoration is the chemical inactivation of phosphates. Usually this is undertaken prior to or complementary to active biological and physical methods, e.g., wind aeration [10-11]. This method is based on the precipitation of phosphates to the bottom sediments after binding with iron or aluminium acid coagulant [12]. These metals, added to water, create with mineral phosphates salts, which undergo precipitation and sedimentation. Finally, aggregate-flocs are formed with a large absorptive surface as a result of coagulation and flocculation processes [13]. Although this process is very effective, acidification poses a threat to hydrobiota [14].

The hardness of water mainly depends on the presence of bivalent cations that are naturally present in water and follows features of the catchment as well the chemical composition of watercourses in the case of lakes [15]. Calcium (Ca^{2+}) and magnesium (Mg^{2+}) are the main elements that influence water hardness as well as affect the buffer capacity of lakes and regulate their biological productivity. A higher concentration of calcium and magnesium cations is vital for many organisms, especially the algae from the Chlorophyta, Rhodophyta, Phaeophyceae, and Chrysophyceae [16]. Thalli of these organisms is covered by calcium and/or magnesium carbonate, which is related to photosynthetic activity. This is a consequence of changes in pH caused by the use of hydrogencarbonate (HCO_3^-) as an alternative to carbon dioxide (CO_2) during photosynthesis. As a result, insoluble calcium and/or magnesium carbonate is deposited on the surface thalli, producing encrustation [16]. In this way, easily soluble calcium hydrocarbonate is transformed into hardly soluble calcium carbonate (1). An analogous process occurs during the transformation of magnesium hydrocarbonate into magnesium carbonate [17-18]. Encrustation is affected by various factors, such as pH of water, temperature, calcium, magnesium, and carbonate ions concentration as well as by plant age and photosynthetic activity [19].

$$Ca(HCO_3)_2 \leftrightarrow CaCO_3\downarrow + H_2CO_3 \quad (1)$$

Charophytes (*Characeae*, *Chlorophyta*) as the most encrusted group of algae, produce internal incrustation and external encrustations on the surfaces of cell walls and, in some species, organic-matrix-mediated calcification within the walls of the oogonium [5, 20]. Due to tight adherence to the thalli (unlike vascular plants), the encrustation fortifies the cell structure, increasing the resistance to mechanical injuries and protecting it from UV radiation [16, 21]. In the case of the most common genus, *Chara*, it is supposed that the increase of photosynthetic activity results in an increase in water decalcification [22].

Due to the chemical features of coagulants, their introduction to a lake causes changes in the physical and chemical properties of water. These disturbances may directly and indirectly influence the macrophyte and charophyte communities [23-24]. It has been hypothesized that chemical coagulants cause water acidification, which changes alkaline conditions preferred by charophytes and dissolves charophyte encrustation. The aim of the study was an analysis of the scale and direction of changes in calcium and magnesium concentrations under the influence of different doses of iron and aluminium coagulants in charophyte meadows, which was tested during mesocosm field experiments.

Methods

The field experiment was conducted in a shallow lake (area 13.3 ha, max depth 4.0 m) located in Wielkopolska National Park (N 52°17'43", E 16°40'5"). This lake is characterized by a dominance of charophyte meadows and a wide belt of helophytes. Eight steel constructions (open to the sediments and atmosphere; dimensions 1×1×2 m) with walls made of polyethylene foil, transparent to sunlight, used as mesocosms. The mesocosms were placed in the gyttja bottom sediments in the littoral zone (Fig. 1) and each of them had about 1 m^3 volume. This site was densely inhabited by only *Chara hispida* L. (approx. 50 shoots per m^2). The embedding of mesocosm in the lake sediments (to a depth of 30 cm) limited the infiltration of water to the inside and walls, reaching 20 cm above the water level excluding water inflow by waves. After inserting the chambers in the habitat, they were left for 1 month to allow to stabilize. Subsequently, baseline conditions were determined (day 0). The study was carried out in the peak of vegetation season (July) separately for aluminium coagulant – polyaluminium chloride (year 2014) and iron coagulant – iron sulphate (2015).

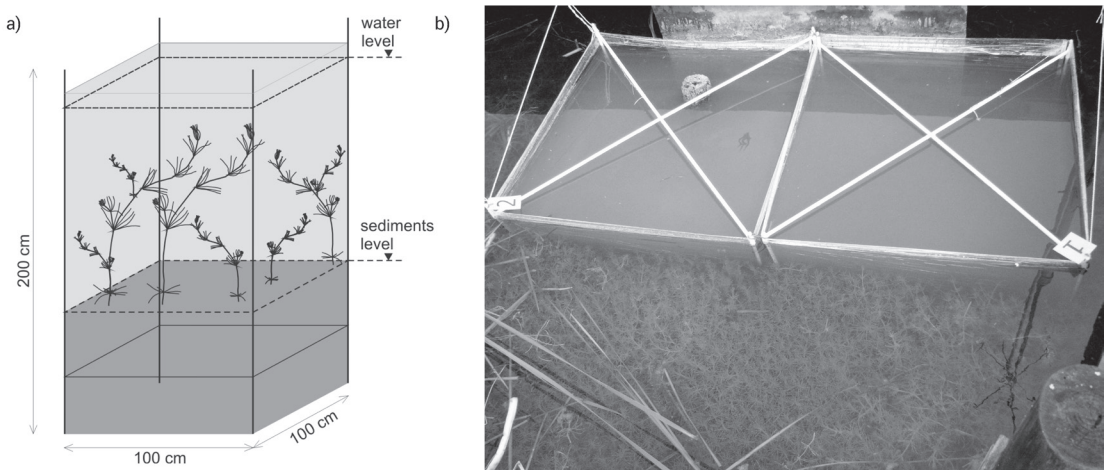

Fig. 1. Scheme of mesocosm construction a) and mesocosms in the field, immediately after coagulant application b).

The chemical characteristics of the iron coagulant, $Fe_2(SO_4)_3$ (trade name PIX 113) is: pH<1.0, density 1500-1570 kg m^{-3}, base substance: sulphuric acid (H_2SO_4), colour dark brown. In the case of aluminium coagulant $[Al_n(OH)_mCl_{(3n-m)}]_x$ (trade name PAX 18), pH = 1.0, density 1350-1370 kg m^{-3}, base substance hydrochloric acid (HCl), colour light yellow.

The charophyte species involved in the experiment was *C. hispida* L. The species is widely distributed in Europe (frequent in Poland) as well as in North Africa and Asia. This is one of the largest representatives of the genus – its stem-like length can reach up to 200 cm [6]. In Central and Eastern Europe, it inhabits shallow eutrophic lakes and waters with slightly acidic pH, such as peatland exploitation ponds or humic waterbodies [25-26]. *C. hispida* prefers alkaline and neutral waters with the calcium content between 17.0 and 167.0 mg Ca^{2+} dm^{-3} [6], and proves capable of growing with a wide range of available light radiation [27]. Due to habitat requirements, which are characteristic for eutrophic lakes, *C. hispida* communities are strongly vulnerable to restoration treatments.

The coagulants were applied one-off at the beginning of the experiment (time T_0) in three doses: low (L), medium (M), and high (H), in turn 50.0, 100.0, and 200.0 $cm^3 \cdot m^{-3}$, respectively. Concentrations of coagulants were determined in the laboratory experiment, where it was assumed that the lowest dose should lead to the total precipitation of suspension. Higher concentrations were a multiple of the lowest dose. To mesocosms chemicals were slowly added by a pipette (semiautomatic pipettor) and gently stirred by a hand mixer. Six of the mesocosms were used for the treatments (two for each concentration) and two of them were the control (C) trial.

The measurements and samples collection for chemical analysis were performed every day (for 3 days) and repeated after 1, 2, and 3 weeks. Measurements were done consistently at the same time of day (11:00). Dissolved oxygen (Pro Plus, Yellow Spring Instruments) and pH (HI 98129, Hanna Instruments) were measured in the field. The water samples for chemical analyses were preserved with sulphuric acid or nitric acid, depending on the method, directly after the collection. The following were analysed in a laboratory: total calcium and total magnesium (EDTA titrimetric method, ISO 6058:1984), sulphate (SO_4^{2-}, gravimetric method using barium chloride, ISO 9280:1990), and chloride (Cl^-, silver nitrate titration with chromate indicator, ISO 9297:1989).

Dry mass of charophytes was investigated to determine a potential loss of encrustation. For this purpose, 4 individuals of *C. hispida* from each mesocosm were collected at the same time as chemical samples. After transportation to the laboratory (in closed plastic bags) they were analyzed after drying in a dryer (80ºC) and weighted with accuracy to 0.0001 g.

The Shapiro-Wilk test was used to assess the normal distribution. The Levene test was applied to assess the equality of variances for groups. A two-way analysis of variance with dose and time as fixed factors (followed by Tukey multiple comparisons test) was used to check the differences between the examined elements and dry mass. All the statistical analyses were performed using Statistica 12.5 software.

Results

In both experiments, at the start the pH of water in mesocosms was slightly alkaline. The application of coagulants caused a temporary decrease in pH appropriate to the dose. The addition of iron coagulant caused a small pH decrease in the L dose (0.88±0.01, mean±SD), and a large one in the H dose (1.45±0.18). Meanwhile, the aluminum coagulant caused much higher reductions of pH – about 2.61±0.57 in L dose and 4.89±0.18 in H dose. Irrespective of the dose, the negative effect of acidity was reduced to the initial values 24 h after coagulant application (Fig. 2a-b).

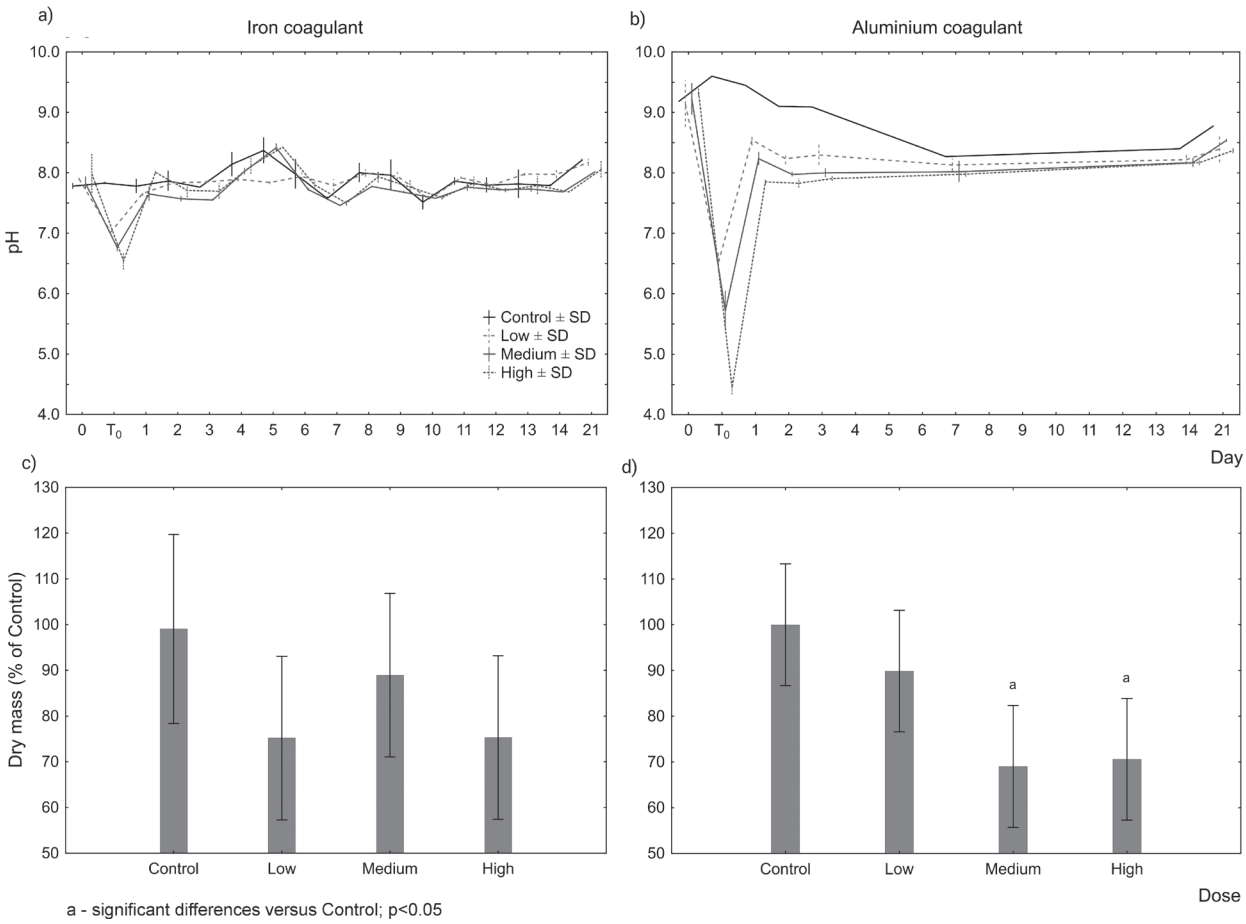

Fig. 2. Changeability of pH and dry mass of charophytes (as percent of control concentration) before and after application of iron coagulant (a, c) and aluminium coagulant (b, d).

The dry biomass of the charophytes in mesocosms with iron coagulant was lower than in control mesocosms (Fig. 2c). Values varied between 75% in L and H dose and 90% in M dose compared to C. There were no significant differences between particular treatments ($F_{3,2} = 1.75$; $p = 0.21$). In the case of aluminum coagulant the lowest decrease was observed in L dose (90% compare to control). In treatments with M and H coagulant concertation dry mass was reduced to 70%. Statistical analyses revealed differences ($F_{3,2} = 6.11$; $p<0.01$) between C chambers and M as well as H dose of coagulant (Fig. 2d). The changes were accompanied by bubbles of gases, which were tightly adhered to the charophytes thalli. In the case of M and H doses of both coagulants the amount of bubbles was so high that charophytes were even elevated above water level. As a result of bubble release, the water surface was covered by foam for 5 days.

At the beginning of each experiment the oxygen saturation of water in mesocosms was 100% O_2. After the addition of iron coagulant the oxygenation increased to max. 120% O_2 in L dose, min. 102% O_2 in H dose. In the experiment with aluminum coagulant there were no differences in oxygen saturation of water between the doses and control mesocosms.

The analysis of Ca^{2+} concentrations under the influence of iron coagulant showed the greatest increase in the case of M dose, where an increase by 15% was observed, in comparison with C (Fig. 3a). In H dose, despite higher water acidification, the value of calcium concentration was comparable to C and L. Hence statistical differences were shown between M dose and the other doses. The application of aluminum coagulant caused an increase in calcium concentration – linear in relation to the dose – by 40% in M dose and by 70% in H dose in comparison to control (Fig. 3b). A two-way ANOVA revealed that both the coagulant dose and time had a significant effect on Ca^{2+} concentration differences (Table 1). In the case of Mg^{2+} there were no statistically significant differences indicating the relationship between the dose and concentration of the element resulting from the application of iron coagulant (Fig. 3c). However, time was the key factor of this experiment (Table 1). Following the application of aluminum coagulant, magnesium concentrations increased significantly only in H dose (Fig. 3d). The dose as well as time were the differentiating factors, and to a lesser extent the interaction of both (Table 1).

The application of coagulants also caused an increase in concentration of ions constituting the base substance

of sulphates and chlorides. An interesting fact is that the highest concentrations of SO_4^{2-} were found in M dose, where they were 70% higher compared with control and 40% higher than in H dose (Fig. 3e). In the case of aluminum coagulant, the increase in the concentrations of chlorides in L and M doses was similar (60% in comparison to control), and noticeably higher in H dose (over 200%; Fig. 3f). A statistical analysis revealed that the dose of coagulant and time were the sources of variability of SO_4^{2-}concentrations, while the variability of Cl^- concentrations was due to the dose size, time, and interaction of both (Table 1).

Discussion

The coagulants applied in the experiment belong to the most common ones used in restoration treatments. Doses applied to mesocosms corresponded with the so-called 'aggressive restoration.' This type of restoration treatment results in the total elimination of phosphates, total suspended solids, and water colour after single, high-dose coagulant application [24]. This is the opposite method to 'sustainable restoration' consisting of low concentrations of coagulant application several times a year [28]. Although

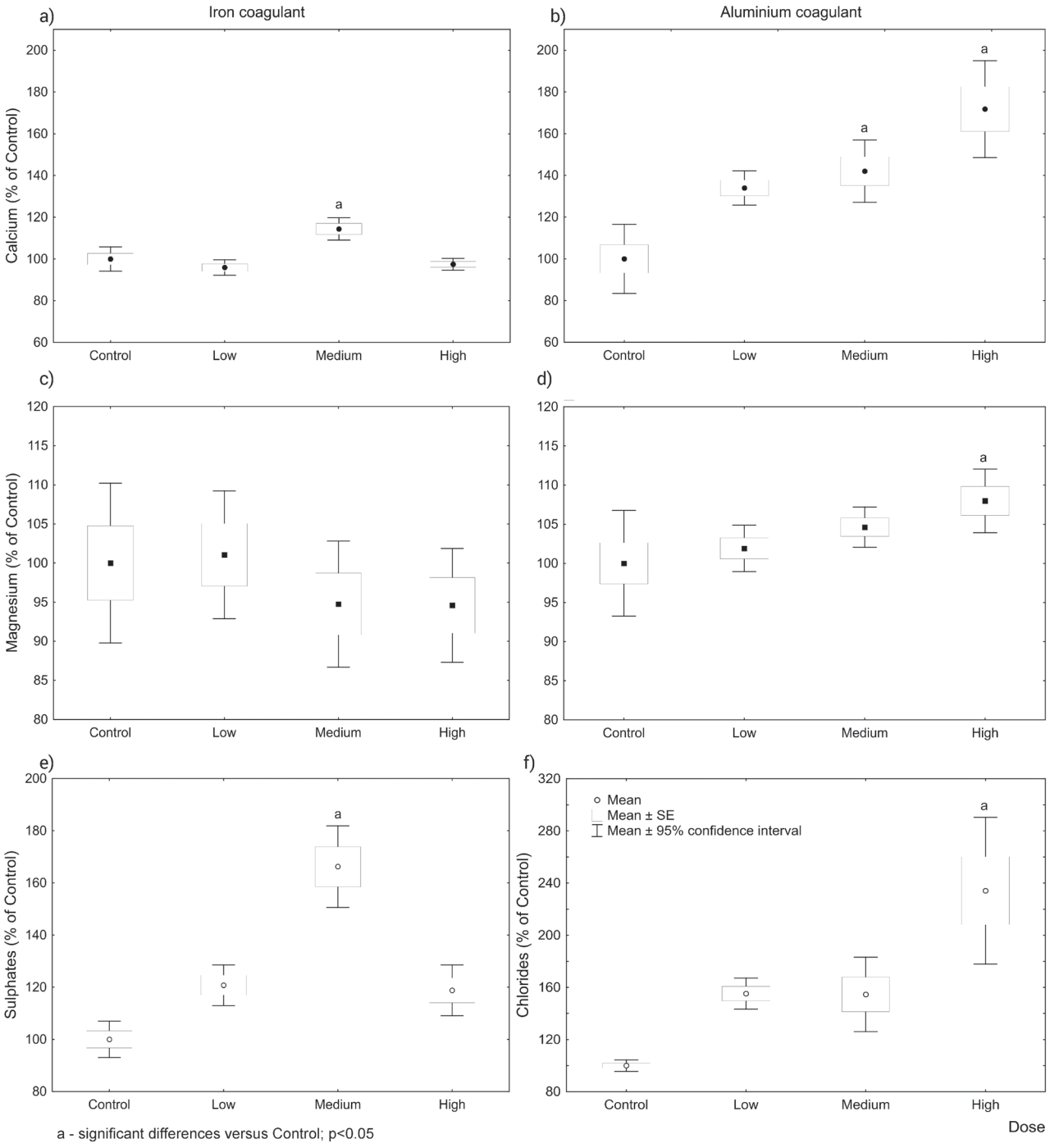

Fig. 3. Differences of ions concentrations between iron (a, c, e) and aluminium coagulant (b, d, f) doses.

Table 1. Results of the two-way ANOVA (*F*-ratios and *p* values) to test for the effects of coagulant dose, experiment time, and their interaction on elements concentration.

Iron coagulant						
Variable	Ca^{2+}		Mg^{2+}		SO_4^{2-}	
	F	p	F	p	F	p
Dose	42.97	<0.000	1.20	0.322	56.80	<0.000
Time	7.55	<0.000	4.57	<0.000	5.13	<0.000
Dose × Time	1.84	<0.03	1.37	0.154	2.41	<0.002
Aluminium coagulant						
	Ca^{2+}		Mg^{2+}		Cl^-	
Dose	110.94	<0.000	4.48	<0.02	37.01	<0.000
Time	20.34	<0.000	4.35	<0.02	7.95	<0.000
Dose × Time	7.71	<0.000	2.91	<0.03	2.23	0.053

concentrations used in the experiment seem to be high, all could be classified as commonly used in lake restoration [29, 30].

The phosphorus coagulants influenced calcium and magnesium concentrations, which affected *Chara hispida* communities. The main factor causing the changes was the decrease in pH determined by the coagulant concentration applied to the mesocosms. Lower pH caused a dissolution of charophyte encrustation, therefore releasing calcium and magnesium ions into the water. This was confirmed by decreasing the dry mass of charophytes, since encrustation can exceed even 80% of their dry weight [31]. An additional aspect causing changes in the abiotic environment was the increased concentration of ions constituting the coagulant. In the conducted experiments, acidification was neutralized within the first 24 h due to the reaction of hydrogen ions with calcium carbonate. Neutralization was supported by the photosynthesis process due to the increase in concentration of hydrogen carbonate ions originating from the dissolved encrustation. This was indicated by water oversaturation when pH changes were highest [32]. The slower pace of neutralization observed in the experiment involving polyaluminum chloride resulted from the more intensive acidification of the environment by hydrochloric acid. In natural conditions the encrustation is created as a result of CO_2 uptake from bicarbonates during photosynthesis [33]. The acidification caused the dissolution of $CaCO_3$ (2) and/or $MgCO_3$ (3), and a Ca^{2+} and Mg^{2+} ions were released into the water. In this process CO_2 is also released, which was visible as bubbles on charophytes thalli and was the reason of foam generation on water surface.

$$CaCO_3 + H^+ \rightarrow HCO_3^- + Ca^{2+} \quad (2)$$

$$MgCO_3 + H^+ \rightarrow HCO_3^- + Mg^{2+} \quad (3)$$

The difference in the concentration of released calcium ions, observed in the case of iron coagulant application between M and H dose, was contrary to expectations. Higher acidification was obtained in H dose, and therefore the highest calcium concentrations were also expected in this dose (such an effect was obtained in the case of aluminum coagulant). An analogous situation with the concentration of SO_4^{2-} suggests that the solubility equilibrium of calcium sulphate, $CaSO_4$ ($K_{sp} = 6.3 \cdot 10^{-5}$), was exceeded in H dose of coagulant. As a consequence, the precipitation of excess calcium ions occurred due to a high supply of sulphate ions. The product of solubility calculated on the basis of concentrations of Ca^{2+} and SO_4^{2-} ions occurring in Medium dose after 24 h was lower by one order of magnitude ($K_{sp} = 2.15 \cdot 10^{-6}$). It should be underlined that a 2-fold lower dose of coagulant was used here and, consequently, lower acidification of water affecting the dissolution of encrustation occurred. As a result of weak solubility of calcium sulphate ($CaSO_4$), a part of the calcium budget is permanently removed from biological circulation. This type of decalcification may result in a negative calcium balance in the lake ecosystem and, as a consequence, in the disturbance or elimination of charophyte communities. In the case of aluminum coagulant we observed the increase in calcium concentration proportional to acidification. The precipitation of calcium chloride was not found due to the high solubility of the compound. Calcium ions are vital for regulating various levels of functions in plants and algae, such as electric balance between the extracellular and intracellular spaces as well as metabolic activities [34]. Apart from these functions, calcium is an essential cation, which affects chlorophyll accumulation. However, extremely increased calcium content in water (when it does not precipitate) may be a disturbance factor in the photosynthesis process for charophytes [35]. As Pełechaty et al. indicated [31],

an average $CaCO_3$ precipitation can be estimated as 167 g m^{-2}. This implies that in a hypothetical situation when only 60% of encrustation is dissolved, an additional 40 g of Ca^{2+} is released into the water. However, the data indicate that the values for precipitated $CaCO_3$ may actually be higher than the above mean and exceed 1500 g $CaCO_3$ m^2 [36]. A larger than required amount of Ca^{2+} results in chlorophyll *b* reduction and changes the chlorophyll *a*:*b* ratio together with decreasing photosynthesis efficiency [2, 37]. Moreover, thalli damage that occurred under coagulant influence decreased photosynthetic pigment concentration and impaired recovery significantly. This is particularly dangerous in the case of aluminium coagulant. Acidification leads to increased toxicity of aluminium ions, which penetrated to charophyte cells and caused severe impairment [38-39].

In the case of magnesium, the application of iron coagulant did not cause noticeable changes in its concentration since $MgCO_3$ is more resistant to dissolution [40]. The acidification of water in the experiment was too weak to trigger the effect of the disintegration of $MgCO_3$. Therefore, the concentration changes in time were the result of natural and relatively small fluctuations. Much higher and statistically significant changes of Mg^{2+} ions concentrations were observed following the application of polyaluminum chloride, particularly in high doses. This indicated the existence of high potential dissolution of $MgCO_3$ at pH<4.5. The fluctuations of the element concentration observed during the experiment indicated small effectiveness of the process of its precipitation. High concentrations of magnesium inhibit the process of charophyte calcification by calcium carbonate [18]. This is the result of Mg^{2+} binding with hydrogen carbonate ions or getting onto the growing surface of the crystal, essentially preventing further precipitation of $CaCO_3$ [41]. Magnesium is an essential constituent of chlorophylls and therefore is regarded as an absolute requirement for green algae [42]. Increased magnesium concentration aid shoot elongation and the development of primary, secondary, and tertiary branchlets in charophytes [35]. Moreover, with the increasing Mg^{2+} ions content in the water, Chl *b* might increase as well, changing the Chl *a*:*b* ratio in an opposite way from Ca^{2+} [2]. Thus, the presence of both Ca^{2+} and Mg^{2+} resulted in no change in the Chl *a*:*b* ratio. This is caused by antagonistic interactions neutralizing the negative effect of one element in the presence of another, hence Ca^{2+} effects are reduced by Mg^{2+} and vice versa [2]. However, in conducted experiments the situation with increasing concentrations of both Ca^{2+} and Mg^{2+} occurred only in the one particular treatment with the highest dose of aluminum coagulant. This may imply considerably greater calcium effect on charophytes under chemical restoration treatments.

Apart from iron and aluminum, SO_4^{2-} and Cl^- are also introduced to water with coagulants. In the experiment with iron coagulant, the highest concentration of SO_4^{2-} was observed in M dose, and not in H dose, where $CaSO_4$ was precipitated (cf. above). In the oxygenic conditions sulphates do not have a negative influence on biota [43-44], but they intensify the secondary supply of water in phosphates from bottom sediments, and at the same time its eutrophication [45]. The content of Cl^- changed adequately to the dose of aluminum coagulant, reaching the maximum in H dose. Similar average concentration in L and M doses demonstrated in Fig. 3e) was a result of faster elimination of Cl^- in M than in L dose during the first week of the experiment. In the final phase, their concentrations in all mesocosms were similar to control. Chlorides, as weakly reactive, remain fully dissolved in water and only their small part is subject to sorption by bottom sediments. Meanwhile, they are the main mineral anion for plants [46]. Changes in their concentrations in conjunction with pH fluctuations may cause disturbances in Cl^- transport at the plasma membrane in charophyte cells [47].

Conclusion

The coagulants based on strong acids, which are used in lake restoration, influence Ca^{2+} and Mg^{2+} ion concentrations. These changes have an impact on *Chara hispida* communities, which inhabit eutrophic lakes. The main reason for changes is the pH drop, which significantly changes the abiotic environment preferred by charophytes and causes disturbances in the chemical equilibrium. It was shown that polyaluminum chloride causes stronger acidification of water than iron sulphate. A high dose of iron coagulant results in the elimination of a certain amount of calcium from biological circulation, while aluminum coagulant increases the mobility of magnesium. Both processes disturb the Ca-Mg equilibrium, which results in the disturbance of biogenic calcification and may affect charophyte metabolism and development. Treatments repeated in an amount that exceeds the environmental tolerance may disturb the whole ecosystem and cause profound changes in the abiotic environment. Therefore, applications of acid coagulants of phosphates for the purpose of improving a lake's ecological state may be dangerous for charophytes. In such a situation, the restoration effort may be diminished by a negative effect following the disappearance of underwater plant communities.

Conflict of Interest

The authors declare no conflict of interest.

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Ecological Engineering 105 (2017) 1–6

Contents lists available at ScienceDirect

Ecological Engineering

journal homepage: www.elsevier.com/locate/ecoleng

The inhibition of growth and oospores production in *Chara hispida* L. as an effect of iron sulphate addition: Conclusions for the use of iron coagulants in lake restoration

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ARTICLE INFO

Article history:
Received 16 December 2016
Received in revised form 22 March 2017
Accepted 27 April 2017

Keywords:
Lake restoration
Iron coagulants
Charophytes
Growth inhibition
Water management

ABSTRACT

Charophytes, as a group of algae inhabiting waters characterized by varied fertility levels, represent highly critical attributes which are important for ecosystem services. One of the popular methods adopted for lake restoration is chemical inactivation of phosphates using iron coagulants. This paper presents the findings of laboratory experiments on the effects of iron sulphates on the growth and production of oospores in the charophyte *Chara hispida* L. In the course of the investigations, responses of this species to three dosages (Low, Medium, High) of iron (III) sulphate corresponding to the Fe concentrations of: 5.4, 10.8 and 21.6 g m^{-3} Fe were analysed. The results demonstrated there was a decrease in the speed of growth of the major axis and reduced production of oospores on the one hand (differences were statistically significant only between High vs. Control), and stimulation of the development and growth of side branches of the first order on the other. Several factors that cause disorders in oospore growth and production were encountered, with a wide spectrum of physico-chemical changes in the water, which resulted in the charophyte thallus being coated by a brown colloid film (limiting access of light) and lowered pH level. The study showed that the use of iron coagulants for lake restoration poses a threat to the development (growth) of charophytes.

1. Introduction

The pollution of freshwaters with biogenic compounds, despite remedial treatments, is still a major threat to the ecological integrity and biodiversity of aquatic ecosystems (Hilt et al., 2006; Xu et al., 2014). Chemical restoration treatments consisting of the inactivation of phosphates using iron coagulants are usually undertaken prior to or complementary to active biological and physical methods (e.g. wind aeration) (Sobczyński et al., 2012; Gołdyn et al., 2014). Ad hoc use of treatments aiming at precipitation of phosphates produces only a short-term improvement in water transparency and penetration depth of photosynthetically active radiation (Joniak et al., 2013; Sobczyński and Joniak, 2013).

Phosphate coagulants are acidic solutions of inorganic salts of iron or aluminium in oxidized forms. Combined with phosphates, the ions of these metals create sparing salts which undergo precipitation and sedimentation. At the same time aluminium and iron salts hydrolyse rapidly and in an uncontrolled manner, forming a range of metal hydrolysis species. Finally, as an effect of post-coagulation/flocculation processes, aggregate-flocs are formed with a large specific surface area. Due to the large absorptive area, the uptake of contaminants is very effective and the resulting large mass prompts gravity sedimentation to the bottom sediments. A major problem when using such chemical substances is their low pH (<1.0), which has proved to be dangerous for lake ecosystems, especially in weakly buffered waters (Persson, 2008). As a result of the high concentration of iron, coagulants have a dark brown colour and persist in the water until the precipitation of phosphates has terminated, after which sedimentation of new-created aggregate-flocs occur (Cooke et al., 2005).

The effects of iron impacts on aquatic plants are characterized by varying degrees of severity depending on particular species (Lucassen et al., 2000). Experimental studies have shown that iron causes changes in growth and reproduction, induces oxidative

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http://dx.doi.org/10.1016/j.ecoleng.2017.04.044
0925-8574/

stress on a cellular level and disrupts cell membranes, pigments and even DNA damages, leading to death of the organism. The main changes are chlorosis and necrosis, reduction of the leaf surface area, general flaccidity and reduction of tillering (Wheeler et al., 1985; Linton et al., 2007; Van der Welle et al., 2007; Bakker et al., 2016). A high concentration of iron in the water reduces the availability of many essential nutrients and deteriorates light conditions (Wheeler et al., 1985; Gerhardt and Westermann, 1995).

Charophytes are an important component of freshwater ecosystems due to their ability to maintain good water quality (Van den Berg and Coops, 1999). By forming dense underwater beds they provide ecosystem services that rank among the most valuable assets in all freshwater ecosystems (Blindow et al., 2002; Kufel and Kufel, 2002). Charophytes significantly contribute to the physical stabilization of bottom sediments (Søndergaard et al., 2007) and play a refugial role for zooplankton against predation (Kuczyńska-Kippen et al., 2009; Liu et al., 2014). Many authors have remarked on the potential role of charophyte vegetation as a nutrient sink in water bodies as a result of their incorporation in biomass and carbonates or co-precipitation with calcite (McConnaughey and Whelan, 1997). The allelopathic activity of charophytes leads to phytoplankton growth reduction at a rate comparable to that of competition for nutrients (van Donk and van de Bund, 2002).

The impact of lake restoration using the iron coagulant-based method is still poorly understood in terms of its effect on macrophytes. Moreover, in spite of research on the effects of iron on macrophytes (Wheeler et al., 1985; Bakker et al., 2016) no specific mechanism explaining its toxicity can be said to have been identified. Literature concerning the impact of coagulants on charophytes is very scarce, although limited growth and biomass under the influence of high doses of iron such as iron (III) chloride has been demonstrated (Immers et al., 2013). In this study, the authors made use of an annual charophyte species of smaller size, without a stem cortex (*Chara virgata*, *Chara globularis*). Coagulant doses were of atypically high concentration, also applied to sediments and they frequently exceeded the quantity commonly used in restoration (Gołdyn et al., 2014; Kozak et al., 2015). To fulfil this gap we developed a laboratory experiment in which we tested the effect of iron sulphate on the growth and reproduction of *Chara hispida* L. It was assumed that the application of iron coagulant will cause the deterioration of abiotic conditions at an intensity proportional to the dose. Accordingly, energy will be directed to surviving adverse changes, which will result in growth inhibition and/or reduction of reproductive ability expressed by the number of oospores.

2. Material and methods

Charophytes as well as the water used in the experiment were collected at the end of May from the natural charophyte Wielkowiejskie Lake (N 52°17′43”, E 16°40′05”), a mesotrophic lake of good ecological state (Joniak and Kuczyńska-Kippen, 2008). The Charophyte species, *Chara hispida* from the genus *Chara* (Characeae, Charophyta), a species widely distributed in Europe as well as in North Africa and Asia (Gąbka, 2009), was used in the study. This charophyte creates monospecific meadows in lakes up to a depth of 3 m, being one of the largest representatives of the genus – its stem-like length can reach up to 200 cm. It is a monoecious alga producing single oogonia, and prefers neutral and alkaline waters with a wide range of calcium concentrations (Urbaniak and Gąbka, 2014).

The experiments were performed in June 2015 in 12 glass cylinders (2.3 dm^3 volume) which were placed in a cultivation room. Temperature was kept constant at 25.5 °C, while the light regime was set at 14 h light and 10 h darkness with a light intensity at the water surface of 160.6 $\mu mol\ m^{-2}\ s^{-1}$. Four apical shoots of *C. hispida*, each 20 cm in length, were placed in sediment-free cylinders which were then filled with 2.0 dm^3 of lake water (height of water column 24 cm). After 72 h (stabilization of the environment) iron (III) sulphate at doses of: 5.4 $g\ m^{-3}$ Fe (Low), 10.8 $g\ m^{-3}$ Fe (Medium) and 21.6 $g\ m^{-3}$ Fe (High) were added to the cylinders once. Cylinders with no addition of iron sulphate served as controls. Although the applied doses were high such amounts are commonly used in restoration practise in so-called “aggressive restoration”, which involves precipitation of phosphates, total suspended solids (including phytoplankton) and water colour (caused by high concentration of dissolved organic matter). “Aggressive restoration” is used to generate immediate improvement in the visual features of the water (after the elimination of the phytoplankton blooms). Doses were determined experimentally with the assumption that the lowest dose should be sufficient for the complete precipitation of suspension. Higher doses were a multiplication of the lowest dose, although all doses could be classified as used in lake restoration (Orihel et al., 2016). During application, the water was subjected to a gentle swirling for better dispersion of the coagulant. All four combinations were tested with three replications (Fig. 1). The cylinders were covered with LDPE foil containing holes (gas exchange). Biometric measurements of each specimen were made prior to the experiment and upon its termination (at an interval of 30 days), according to the following scheme: lateral shoot lengths, longest leaf in the nodes (nodes 1–3, counted from the top) and internode lengths (up to the 3rd node). The number of oospores on each branch was also determined. Each branch was labelled, hence measurements always related to the same specimen. Water temperature, pH, electric conductivity (HI 98129, Hanna Instruments), turbidity (using the TN-100 nephelometer, Eutech), colour (visual method in platinum-cobalt scale) and sulphate concentrations (gravimetric method, with barium sulphate precipitating) were measured every 3 days in each sample. Phosphates were analysed spectrophotometrically before application, after 3 days and at the end of the experiment using the molybdate method with the detection limit of 0.002 mg $PO_4^{3-}\ dm^{-3}$ (APHA, 1998). Photosynthetically active radiation was measured at the water surface using a quantum meter with a spherical sensor (LI-COR Biosciences).

Statistical tests were used to analyse the differences between the control and experimental treatments with respect to the algae features, the different relative growth rate (RGR) mean values and environmental conditions: an ANOVA with repeated measures with time as a fixed factor or one-way ANOVA followed by Tukey post-hoc tests (when ANOVA with repeated measures proved unworkable). The Shapiro-Wilk test was used to assess normal distribution. The Levene test was applied in order to assess the equality of variances for groups. All analyses were performed using Statistica 12.0 software.

The relative growth rate was calculated at the end of the experiment by the formula: $RGR = (\ln X_2 - \ln X_1)/(t_2 - t_1)$, where X_1 and X_2 were the algae mean lengths, at time t_1 and t_2, (start and end of experiment, respectively) (Hunt, 1990). The difference between the growth curve and treatments was tested with ANCOVA with the sampling day as a covariate and iron concentrations as fixed factors.

3. Results

For all specimens an increase in overall length (mean ± SD; 9.0 ± 3.2 cm) was recorded. Individuals subjected to the influence of the highest dose of coagulant were characterised by a significantly lower RGR versus the control (Table 1, Appendix A in the Supplementary material). A regression curve revealed a significant overall decrease in the average length of *Chara hispida* along the gradient of concentration of iron (based on coagulant doses) and accounted for 76% of the variance (Fig. 2). There were no signif-

Fig. 1. The experimental system in cultivation room after coagulant application (from the left: Control, Low, Medium, High).

Table 1
Comparison of changes in architecture traits and oospore production of *Chara hispida* (% of Control) and results of ANOVA tests with varied iron dosage.

	Iron sulphate dose				F	P	df
Length	Control	Low	Medium	High			
Total	100	94.4	97.4	89.4*	3.4	0.02	3, 42
1st degree branches	100	90.5	192.9*,**	190.5*,**	7.62	0.000	3, 34
2nd degree branches	100	119.7	77.0	134.4	1.88	0.16	3, 42
branches in 1st internode	100	108.3	91.7	100.0	0.59	0.62	3, 42
branches in 2nd internode	100	91.3	87.0	87.0	0.20	0.89	3, 42
branches in 3rd internode	100	94.1	85.3	91.2	0.38	0.77	3, 42
1st internode	100	87.5	112.5	112.5	0.16	0.92	3, 42
2nd internode	100	90.9	104.5	104.5	0.09	0.97	3, 42
3rd internode	100	89.2	110.8	97.3	1.45	0.24	3, 42
RGR (week^{-1})	100	94.2	95.1	75.7*	4.10	0.01	3, 42
Oospore number	100	104.4	114.8	35.4*,**,***	8.37	0.000	3, 41

* $P \leq 0.01$ versus Control.
** $P < 0.01$ versus Low.
*** $P < 0.000$ versus Medium.

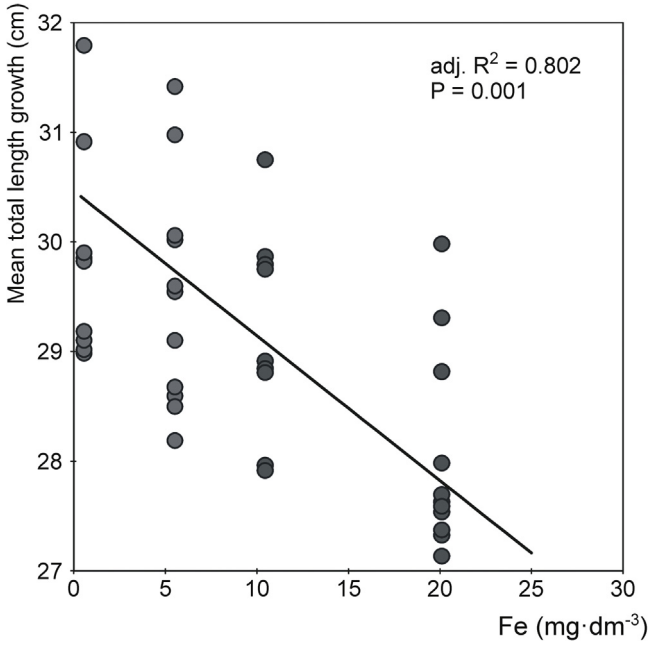

Fig. 2. Regression line of average total length growth of *Chara hispida* along the gradient of iron concentration in water.

icant differences in the length of branchlets or internodes. With regard to the architecture of specimens, the branch length was a parameter differentiating different variants of the experiment. Significant differences in the length of branchings of the first order were found in Medium and High doses (longer) versus Control and Low (shorter) (Table 1). No differences were determined for second-order branchings, and for the third-order ones they were sporadic.

The experiment demonstrated substantial differences in the abundance of oospores between Control, Low and Medium (where their devolvement was similar (142.0 ± 60.1)) and the High variant (46.8 ± 36.3) (Table 1, Appendix A Supplementary material).

Immediately after the addition of the coagulant, the pH in water decreased significantly from baseline values (8.48 ± 0.14), dropping to 6.99 ± 0.02 in the Low dose cylinder, 6.66 ± 0.04 in the Medium, and down to 6.25 ± 0.06 in the High dose one (Fig. 3). This occurred relatively quickly, because after 72 h the effect of acidification was reduced to the output level. However, throughout the experiment the pH level in the samples of Medium and High doses was lower than in the Control. Statistical analysis revealed that both coagulant dose and time as well as their interaction had a significant effect on pH ($F_{24,64} = 13.6$; $P < 0.000$). Coagulant application caused very strong but temporary (24 h) increase in water colour from 180.0 ± 0.0 mg Pt dm^{-3} in Low (5-times increased compared to control) to extremely high in High – 530.0 ± 0.0 mg Pt dm^{-3} (16-times increased). Similar changes were observed in turbidity which showed an almost sixfold increase in Low dose (8.6 ± 0.4 NTU) to over twenty twofold in High (33.4 ± 1.3 NTU). Statistical analyses demonstrated that dose as well as time had a significant impact on turbidity ($F_{24,64} = 568.77$; $P < 0.000$). After 24 h values of both

Table 2
Average values of physico-chemical parameters of water at start (T_0) and end (T_{30}) of experiment correlated with different doses of iron and results of ANOVA tests.

Parameter, unit		Iron sulphate dose				F	P	df
		Control	Low	Medium	High			
Temp., °C		25.7 ± 1.1	25.6 ± 2.1	26.0 ± 0.9	25.7 ± 0.8	0.54	0.665	3, 8
pH	T_0	7.9 ± 0.04	7.0 ± 0.01	6.7 ± 0.03	6.2 ± 0.04	13.6	0.000	24,64
	T_{30}	9.4 ± 0.14	9.1 ± 0.14*	9.1 ± 0.03*,**	9.0 ± 0.03*,**,***			
Colour, mg Pt dm^{-3}	T_0	33 ± 0.0	180 ± 0.0	320 ± 0.0	530 ± 0.0	Too low variability of the data		
	T_{30}	33 ± 0.0	32 ± 0.0	32 ± 0.0	32 ± 0.0			
Turbidity, NTU	T_0	1.5 ± 0.1	8.6 ± 0.4	16.8 ± 1.1	33.4 ± 1.3	568.77	0.000	24,
	T_{30}	1.4 ± 0.2	0.7 ± 0.3	0.7 ± 0.3*,**	0.7 ± 0.4*,**,***			64
EC, µS cm^{-1}	T_0	442 ± 6.4	441 ± 3.1	453 ± 4.6	469 ± 6.6	9.61	0.000	24,
	T_{30}	271 ± 14.8	284 ± 5.0	302 ± 1.6*,**	364 ± 2.2*,**,***			64
Phosphates, mg PO_4^{3-} dm^{-3}	T_0	0.04 ± 0.02				3.69	0.02	6,
	T_{30}	0.04 ± 0.01	b.d.*					16
Sulphates, mg SO_4^{2-} dm^{-3}	T_0	81.7 ± 3.1	99.3 ± 6.5	119.0 ± 6.7	149.3 ± 9.3	118.0	0.000	3,
	T_{30}	83.7 ± 5.2	84.7 ± 3.9*	114.7 ± 7.4*,**	148.7 ± 5.3*,**,***			8

b.d. – below detection limit.
* P < 0.05 versus Control.
** P < 0.05 versus Low.
*** P < 0.05 versus Medium.

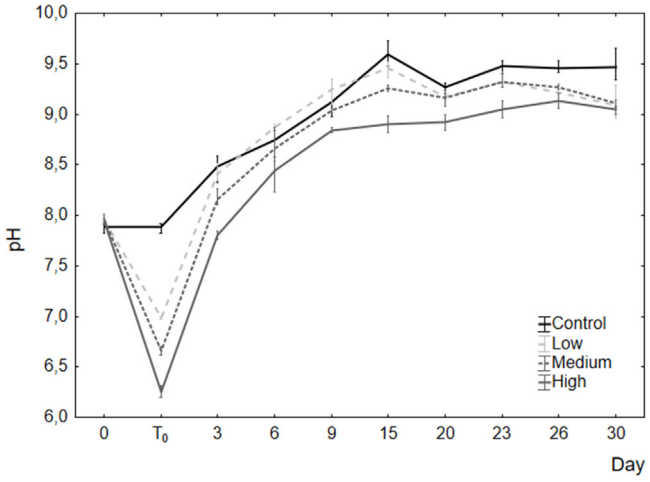

Fig. 3. Change in water pH during the experiment under different iron treatments (T_0–coagulant application and start of experiment; vertical bars – std. dev.).

parameters in all treatments decreased below Control values and remained constant to the end of the experiment. The reduction of colour and turbidity was associated with precipitation of sedimenting flocks, whose colloidal sediment covered charophyte thallus (the amount of sediment and its colour depended on coagulant concentration). Coagulant application caused the total elimination of phosphates in all treatments ($F_{24,64} = 3.37$; $P < 0.000$). In the case of electric conductivity (EC) changes were minor but significant ($F_{24,64} = 9.61$; $P < 0.000$). The addition of coagulant also resulted in an increase of sulphate concentrations whose levels continued to be elevated (compared to control) until the end of the entire experiment (Table 2). Statistical analysis revealed significant differences in sulphate concentrations for all coagulant doses ($F_{3,8} = 118.0$; $P = 0.000$).

4. Discussion

The results of the assay with *C. hispida* indicate that the addition of iron sulphate resulted in inhibiting growth and reproduction efficiency. Direct symptoms of Fe impact on aquatic plants include a reduction in the leaf surface, formation of local discolouration of leaves (necrotic spots) with the possibility of total necrosis, blackening of the roots, or failure to produce new branches (Van der Welle et al., 2006). Similarly to Immers et al. (2013), the changes listed above were not observed in the conducted experiment, which suggests that iron sulphate addition had rather an indirect impact on the studied charophytes.

In response to increasing doses of iron sulphate in charophytes two types of reactions were reported: (1) a reduction in the growth of the length of the major axis and (2) an increased growth rate of the lateral branches. The first reaction is at odds with the typical defence mechanism against limitation of light availability, namely rapid elongation towards the water surface (Blindow and Schütte, 2007). Application of iron coagulants causes disturbances in the availability of light owing to changes in water colour and turbidity. Although the change in the optical properties of the water is short-lived, the inactivation of phosphates and the co-precipitation of iron with other ions dissolved in water (Wheeler et al., 1985) produces a coloured colloidal suspension. The consistency of the suspended sediments leads to the smothering of the charophyte thallus, thereby altering their physical state. This smothering of charophytes by the strongly coloured colloid also creates a barrier to light. Therefore on the one hand the use of iron coagulant improves light penetration following the removal of water colour and turbidity, but on the other it limits assimilation capacity as a result of thallus smothering. Restricted movement of water over charophyte meadows (Bornette and Puijalon, 2011), high densities of specimens and specific shape of thallus can mean that removal of precipitated sludge may be a lengthy process.

As a consequence of the intensive growth of lateral branches, charophytes create a particular kind of “canopy”, which multiplies their assimilation surface. Any change in the shape of charophytes in a sterilized environment takes place at the expense of the use of their stockpiles, and in drastically adverse conditions leads to a decrease in fertility (Kozłowski, 1992). Increased investment in survival by shoot elongation was clearly seen in the high dose of coagulant which may explain the highest reduction in amount of oospores.

A decrease in pH, substantially altering the optimal environmental conditions, was attributed to the indirect influence of iron sulphate. The relatively rapid neutralisation of water acidity may have resulted from both the depletion of carbon dioxide due to increased photosynthesis (Smith and Bidwell, 1989), and from the reaction of the acid with calcium carbonate, forming encrustations (Urbaniak, 2010).

Charophytes are characteristic of waters with low concentrations of phosphorus (Blindow et al., 2002). In the experiment the concentration of phosphates fell below the threshold of detection in cylinders with a coagulant, suggesting its limitation to the growth

of stoneworts. Contrary to expectations the growth of stoneworts was not inhibited but its pattern was changed through the allocation of resources. This fact was probably due to the synergistic impacts of shading and a lack of an available form of phosphorus. However, as presented by Immers et al. (2013) who, in a similar experiment used *C. globularis* (growth significantly decreased) and *C. virgata* (growth was not affected), the mechanism of response to these two factors may be strongly determined by the individual physiology of the species.

On the basis of the conducted experiment it was found that an addition into the water of a single dose of 21.6 g m^{-3} Fe in the form of iron sulphate does not cause lethal effects, it does however, adversely affect the overall condition of stoneworts. This may lead in the long term to the rebuilding of the stonewort community (on more iron tolerant species) or in stonewort regression (Van der Welle et al., 2007; Immers et al., 2013). The supply of the diasporas bank becomes a problem in the case of limitation of oospore production. The buried diaspors bank plays an important role in the biodiversity of plant communities and in the rapid re-colonisation of vegetation after disturbances (De Winton and Clayton, 1996). In the water bodies that are subjected to restoration processes this is a matter for particular attention (Hilt et al., 2006). The use in the experiment of only a single application of coagulant should be emphasized. In the case of multiple-time dosing, the impact of coagulant would be cumulative, which would likely lead to the retreat of stoneworts.

In summary, charophytes under the influence of iron (III) sulphate grew more slowly and produced fewer oospores. They responded to the introduction of a stress factor into the water by expanding their assimilation area through the growth of new side branches, mainly of the first order; at the same time, a slowdown of their elongation towards the water surface occurred. The use of coagulants caused a number of environmental changes in the water, indirectly affecting charophytes, including a decrease in water pH and changes in light conditions.

The results obtained in the experiment indicate that concentration of iron in the form of iron (III) sulphate at 21.6 g m^{-3} Fe concentration (by way of one-off treatment) triggered the most negative consequence (inhibition of growth and oospore production). In lower concentration the maximisation of defence mechanisms was achieved without harm to sexual reproduction, which provides oospore inflow to the diaspors bank and in consequence recovery of charophyte communities after natural or anthropogenic disturbances. The study showed that use of iron coagulants for the purpose of lake restoration, if used in excessive amounts, poses a threat to the growth of charophytes.

Acknowledgments

Studies of protected species were conducted with the approval of the Wielkopolska National Park. The authors would like to thank Rob Kippen for proofreading.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ecoleng.2017.04.044.

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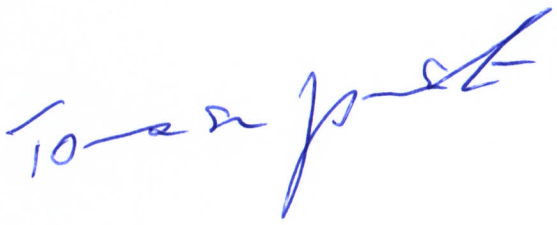
Rybak M., Joniak T., Gąbka M., Sobczyński T., 2017, *The inhibition of growth and oospores production in Chara hispida L. as an effect of iron sulphate addition: Conclusions for the use of iron coagulants in lake restoration*, Ecological Engineering 105: 1-6

polegał na: dyskusji koncepcji eksperymentu i wyników pracy oraz ostatecznej korekcie tekstu.

Mój całkowity wkład w pracę wynosi 10%.



Poznań, czerwiec 2018

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polegał na: wykonaniu 20% analiz statystycznych, dyskusji koncepcji eksperymentu oraz uzyskanych wyników.

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polegał na: wykonaniu 30% analiz chemicznych.

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T. Sobczyński

Limnol. Rev. (2018) 18, 1: 31–37
DOI 10.2478/limre-2018-0004

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Changes in *Chara hispida* L. morphology in response to phosphate aluminium coagulant application

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Abstract: Progressing eutrophication of waterbodies requires measures to be undertaken that aim at halting or reversing negative changes in the environment. Chemical restoration is one of the most common methods used for lake treatment, where iron or aluminium phosphate coagulants are applied. However, their chemical qualities pose the risk of acidification and aluminium ion release, which become toxic in acidic conditions. The influence of coagulants on aquatic plants, including charophytes that are very valuable from the ecological perspective, is little recognised. For this reason, the aim of the research was to define changes in the growth pattern of the charophyte *Chara hispida* under the influence of an aluminium coagulant. The research was carried out in mesocosms (0.8 m^3) located *in situ* in a lake. Polyaluminium chloride was applied once to each chamber in doses of 50.0, 100.0 and 200.0 ml m^{-3}. Coagulant concentrations reflected aggressive restoration aimed at precipitation of phosphates, suspension and water colour at the same time. It was proved that the coagulant had inhibited the growth and slightly reduced the length of branchlets, and simultaneously elongated internode cells. Changes in the total length as well as the length of branchlets were caused by a strong pH decrease of the environment which simultaneously induced higher aluminium solubility and toxicity. Elongation of internode cells was caused by reduced light availability, resulting from high water turbidity in the first stage of coagulant's application, and then from the charophytes' thallus being covered by a coagulated suspension precipitated from water.

Key words: lake restoration, aluminium coagulant, Charophytes, growth pattern, inland water management

Introduction

The widespread eutrophication of the environment has worsened the ecological conditions of inland waters in many parts of Europe and worldwide (Stoate 2009). In the case of lakes, the most negative effects comprise water blooming, including toxic cyanobacteria, a switch to a turbid phytoplankton-dominated state, as well as the loss of biodiversity, particularly elimination of underwater macrophyte communities (Hilt et al. 2006; Ławniczak 2016). Various methods of water restoration are used to counter the negative effects of eutrophication, and their aim is to stimulate processes that retrieve the natural diversity of the environment (Dokulil and Teubner 2000; Rosińska et al. 2017). Among all restoration methods, chemical inactivation of phosphates is one of the most common and widely used (Jiang and Graham 1998; Orihel et al. 2016). The method employs non-organic acidic aluminium or iron salts which bind phosphates into coordination complexes (Sobczyński et al. 2012; Dunalska and Wiśniewski 2016). Another positive effect of coagulation consists in the creation of aggregate-flocks with a large absorption area that foster elimination of the suspension. Such created complexes settle under their own weight on bottom sediments (Pizarro et al. 1995; Sobczyński and Joniak 2009). In restoration practices it is recommended to apply low doses several times, known as balanced restoration (Gołdyn et al. 2014), the opposite method is aggressive restoration when a high, one-time dose of a chemical coagulant is used to eliminate suspensions and water colour. So far, limnological studies have shown that phosphate coagulants may efficiently, though with a limit in time and space, reduce the concentration of biologically absorbent phosphates, thus curbing their initial production (Gibbs et al. 2011; Sobczyński et al. 2012). Nonetheless, the biological impact is not always predictable due to the diversity of particular water environments, and the scale of unfavourable effects remains largely unknown.

The main risk of using coagulants is their low pH (approx. 1.0). This is a serious threat for shallow water

bodies with small volume as well as for the stability of poorly buffered waters (Persson 2008). Another danger for plants and animals is constituted by the toxic qualities of base components of coagulants, especially aluminium in acidic conditions (Gensemer and Playle 1999). Attention is drawn to the fact that during field application water acidification and aluminum toxicity affect synergy, not to mention the release of other toxic metals from bottom sediments (Marschner 1991; Bakker et al. 2015). In Poland the aluminium coagulant is mostly used to restore deep water bodies with an anoxic bottom water zone (Grochowska et al. 2014), whereas in other European countries it is also used in shallow waters, interchangeably with iron coagulants (Cooke et al. 2005). The advantage of the aluminium coagulant is that it permanently binds phosphate ions, also in anoxic conditions (unlike the iron coagulant), hence eliminating them partially from the biological cycle (Rydin and Welch 1998).

What is important for the restoration of natural waterbodies is that chemical restoration with coagulants should be preceded by experimental research that would define optimal doses in view of the expected result and biocenosis safety. In fact, it is unacceptable to use coagulants without prior recognition of the causes of eutrophication or to use them as an interim method for enhancing water quality, for example to eliminate cyanobacteria bloom which is the result of intensive rainfalls that deliver nutrients from the catchment area (Sobczyński and Joniak 2013). Unfortunately, the use of coagulants to achieve short-term, provisional improvements in water quality is becoming more common (Joniak et al. 2013). The reason for this is mainly the disturbance of the recreational or economical function of a lake due to cyanobacteria blooms (Pretty et al. 2003; Brooks et al. 2016). Using aggressive restoration to revive a lake's previous functions is hazardous for the original physical and chemical character of the habitat. In consequence, the range of macrophyte communities and bottom macroalgae decreases or they disappear entirely. Any disorders of underwater macrophyte communities speed up eutrophication and deteriorate the quality of the water (Kufel and Kufel 2002; Hilt et al. 2006).

Charophytes (Characeae) are one of the most ecologically valuable submergent communities. By colonising bottoms, these organisms physically isolate and stabilise sediments, and create a refugium for hydrobionts, such as zooplankton and ichtiofauna. In addition, they efficiently compete for nutrients with phytoplankton due to their allelopathy (van Donk and van de Bund 2002; Blindow et al. 2002). Therefore, they are natural stabilisers of the trophic level and an element of the ecosystem that is indispensable for maintaining good water quality, especially in waterbodies endangered by eutrophication. It should be noted, however, that stonewort species also inhabit eutrophic waters (Urbaniak and Gąbka 2014), where they may be exposed to restoration treatments.

The charophyte's vulnerability to chemical restoration is little recognised. The aim of this study was to determine the growth pattern changes of *Chara hispida* (L.) under the influence of polyaluminium chloride application. The research commenced with an assumption that the coagulant would trigger changes in the charophyte architecture since it simultaneously affects pH, concentration of bioavailable phosphates, as well as the toxicity of the base element.

Material and methods

The research focused on the charophyte *Chara hispida* (Characeae, Charophyta), which is widely distributed in Europe, Asia, and North Africa (Gąbka 2009). In Central-Eastern Europe it occurs mainly in shallow eutrophic lakes and slightly acidic (pH = 6.4) waterbodies such as peatland exploitation ponds (Urbaniak and Gąbka 2014). It is a stem-like entity with a length ranging from 30 to 200 cm, diameter up to 4 mm, 10–15 internodes, and numerous branchlets (7–11) reaching up to 8 cm. *C. hispida* is a representative of the genus, since this species occurs in eutrophic waters and is more exposed to restoration treatments.

The research was carried out in summer in 2015 (July–August) as a field experiment with mesocosms (chambers) placed in the natural Lake Wielkowiejskie in the Greater Poland National Park (Poland). Eight chambers (1×1×2 m, water volume 0.8 m^3) were placed in sediments (no bottom water infiltration) of the littoral zone inhabited by *C. hispida*. The chamber walls were made of transparent material to allow easy light access.

The aluminium coagulant (polyaluminium chloride; $[Al_n(OH)_mCl_{(3n-m)}]_x$ trade name PAX 18) was applied once in each chamber in the following doses: low (L), medium (M) and high (H), i.e. 50.0, 100.0 and 200.0 ml m^{-3}, respectively. The doses (with repetition) were added to six mesocosms, while the two remaining ones were control chambers (C). The chemical substance used in the experiment is characterized by pH <1.0, density 1350–1370 kg m^{-3} and a light yellow colour.

Samples of *C. hispida* were collected after 1, 2, 4 and 8 weeks (4 individuals from each chamber). Next, the specimens were transported in sealed plastic bags to the laboratory where they were analysed on the same day. Each specimen was measured as follows: the main stem's total length, lateral shoot lengths, longest branchlet in a whorl (nodes 1–3) and internode lengths (up to the 3rd node) of both the main stem and its branches. *In*

situ water pH was measured (HI 98129, Hanna Instruments) and samples were taken to analyse phosphates according to the spectrophotometric method with ascorbic acid after filtration through membrane filters of pore size 0.45 μm (APHA 1998). The parameters were also analysed before the experiment, and afterwards, simultaneously with charophyte sampling.

An ANOVA with repeated measures with time as the fixed factor with the post-hoc Tukey test was used to analyse differences in the impact on plant characteristics. The Levene test was used to assess the equality of variance in comparable groups. All statistical analyses were performed using Statistica 12.5 software.

Results

The initial water pH was 9.4 ±0.3 (mean ±stand. dev.), and the concentration of dissolved phosphates was 0.03 ±0.01 mg PO_4 dm^{-3}. Having applied the polyaluminium chloride to the chambers, water pH decreased: it was lowest after L dose (pH 6.5 ±0.2), medium after M (pH 5.7 ±0.3), and highest after H (pH 4.4 ±0.1). When compared to the control group, the difference was statistically significant ($F_{3,3}$=5.15; $p < 0.01$). Within a week of the coagulant's application, water acidity was neutralized in all chambers (Fig. 1). By adding the coagulant, dissolved phosphates were entirely eliminated. This state prevailed only for a week. Later, the concentrations were observed to rise continually until they reached their initial level (Fig. 2).

Apart from sudden changes in chemical features of water after application of the polyaluminium chloride, there were also physical modifications, especially very strong turbidity and iridescence. Over the following

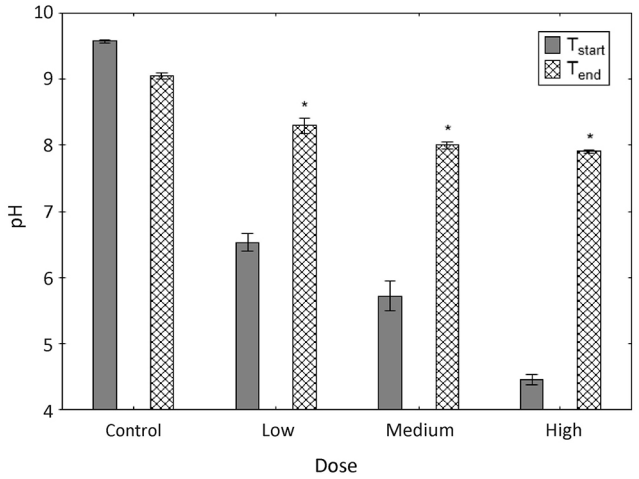

Fig. 1. Changeability of water pH at different doses of aluminium coagulant (whiskers – std. dev; T_{start} – short after application, T_{end} – the end of experiment, asterisks – statistical differences in particular doses between T_{start} and T_{end})

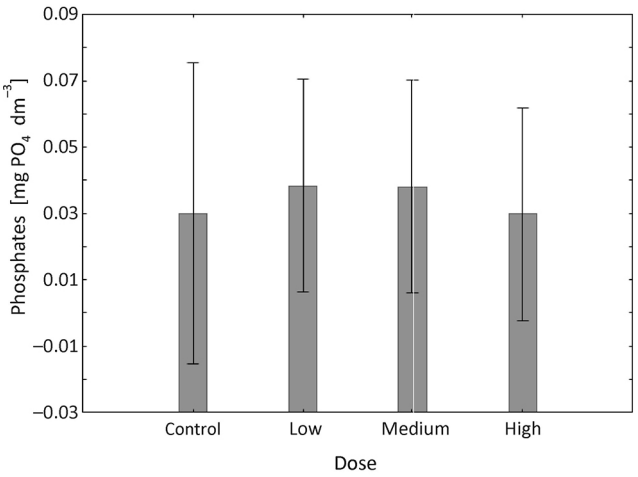

Fig. 2. Mean concentrations of phosphates dissolved at particular doses during the experiment (whiskers – standard deviation)

days both features ceased, but the so-called agregate-flocks appeared, and gradually started to sediment, covering the charophyte with a light-cream tight jelly like sticky layer. The amount of the sedimented suspension was proportional to the coagulant's dose.

Having performed biometric measurements of individual charophytes that had been treated with the polyaluminium chloride, the total length was found to be shorter by approx. 10 cm after L dose (82.8 ±19.6 cm) and H (80.9 ±18.5 cm), and 20 cm after M dose (72.5 ±16.4 cm). In C mesocosms their length amounted to 92.9 ±17.9 cm (Fig. 3a, 3b, 3c). High values of the standard variation resulted in the absence of statistical differences. Branchlet lengths in all nodes demonstrated a slight length reduction during the experiment. However, ANOVA results revealed that both the coagulant dose and time had a significant effect on branchlet length (Table 1). The surface of branchlets was covered by numerous chlorosis and necrosis, and after the H dose it often had no apical part.

The length of internode cells showed different biometric parameters. The length of internode 1 after every dose remained unchanged with slight statistically

Table 1. Results of an ANOVA with repeated measured tests for architecture traits vs. coagulant dose and time

Length	Dose		Dose × Time		df
	F	p	F	p	
Total	1.71	0.2	0.88	0.55	9.60
branchlets in 1st internode	2.64	0.06	**3.22**	**<0.001**	9.135
branchlets in 2nd internode	**3.09**	**<0.05**	**4.79**	**<0.000**	9.135
branchlets in 3rd internode	1.73	0.17	**6.60**	**<0.000**	9.135
1st internode	1.35	0.27	1.08	0.37	9.135
2nd internode	**4.48**	**<0.01**	**2.99**	**<0.01**	9.135
3rd internode	**11.01**	**<0.000**	**6.38**	**<0.000**	9.135
1st degree branches	**6.13**	**<0.01**	**2.43**	**<0.05**	9.51

insignificant fluctuations (Fig. 3d). The length of internode 2 significantly increased when compared to other doses (Fig. 3e) after dose H, while the effects of doses C, L and M were alike. The length of internode 3 (Fig. 3f) featured a growth proportionate to the coagulant dose, and it varied significantly from the others after dose H (Table 1).

The number of 1st degree branches did not change (4 branches on each specimen), but their length increased in chambers with the coagulant (23.4 ±4.8 cm) in comparison with the C chambers (21.7 ±6.7 cm). 2nd degree branches were rarely recorded, although their general number decreased from 6 in C chambers to 2 in L, M doses and 4 in H dose (Table 1).

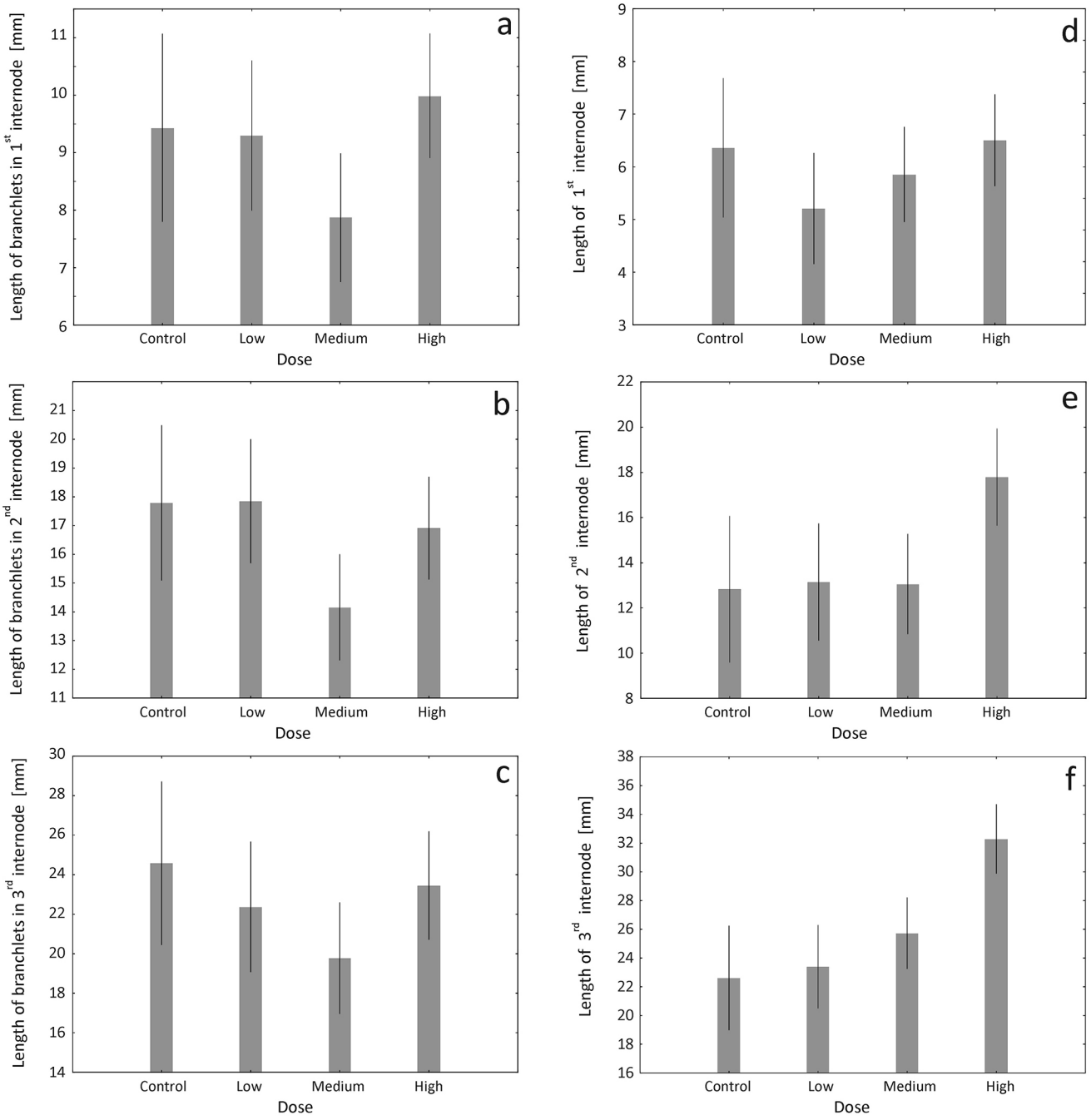

Fig. 3. Comparison of growth and architecture of *C. hispida* from experimental plots (column – mean, whiskers – 95% confidence interval; a, b, c – length of branchlets in internodes; d, e, f – length of internodes)

Discussion

The application of the aluminium coagulant significantly changed the abiotic features of the environment as well as the architecture of the specimens. There were two kinds of responses to the coagulant: 1) growth inhibition and slight reduction of branchlet length, and 2) elongation of internode cells. The first response stemmed from a change in abiotic conditions, particularly at the initial stage, that were preferred by the charophyte, such as high alkalinity and alkaline pH (Kufel and Kufel 2002). Immediately after application, environmental conditions favoured by charophytes are substantially altered. Increasing the frequency of treatments can lead to the transformation of a habitat and loss of communities. In addition, acidity initiated a rise in aluminium toxicity since pH was the most important factor responsible for toxicity and solubility (Goldbold et al. 1995). The most toxic forms are considered to be Al^{3+}, $Al(OH)^{2+}$, $Al(OH)_2^{+}$, $Al(OH)_4^{-}$ (Drabek et al. 2005) i.e. the ones that appear when aluminium polychloride is added to water (Jiang and Graham 1998). So far, the mechanism of the toxic influence of aluminium on plants and algae has not been precisely described. What is known is the fact that at pH 4.0 – as in the experiment – dissolved aluminium has the greatest toxic impact on plants (Kinraide 1991) and the charophyte (Takano and Shimmen 1999). Accumulation of metals in macrophytes is dependent on many factors, mainly: vegetation type (submerged species accumulate more Al than emergent), Al concentration in water and the time of exposure to contamination (Oberholster et al. 2012; Senze and Kowalska-Góralska 2014). Aluminum coagulant causes permanent damage to *C. hispida* thalli, such as chlorosis, necrosis, detachment of corticating cells and general softening of the thallus. Al ions are actively accumulated in the *C. hispida* biomass (to above 2.0 mg g^{-1} d.w.) immediately after application of coagulant, however, in comparison to other elodeids *C. hispida* is a poor bioaccumulator of aluminium (Rybak et al. 2017b). According to Marschner (1991) aluminium distorts the proportion of cations and anions in the ion exchange, and stops the absorption of Ca^{2+} and Mg^{2+} ions through cell walls, a process that is vital for the charophyte. Moreover, Ca^{2+} could be displaced in the cell wall by Al, leading to a disturbance in normal cell development and growth (Reid et al. 1995). Acidity neutralisation and simultaneous precipitation of aluminium from water in the process of coagulation gradually decreases the time of interaction and the negative effects of toxicity. As the charophyte incrustation is dissolved, calcium and magnesium, which are their basic components, are released into the water (Rybak et al. 2016b). Their structural damage results from degradation and changes in the proportion of photosynthetic pigments (chlorophyll *a* and b, carotenoids), thus intensifying the photosynthetic efficiency of those parts that have remained undamaged, and protecting itself against excessive exposure to light (Rybak et al. 2016a).

Greater elongation of internodes as well as the growth of 1st degree branches resulted from light availability. In the first stage of the experiment, limited penetration of radiation was caused by heavy water turbidity and iridescence. Once these optical conditions ceased and water clarity improved, flock coagulants settled on the charophyte and formed a colloid film limiting/reflecting light access (Immers et al. 2013). In such a situation the charophyte grew to reach the water surface, which was a typical defense reaction to shading (Blindow and Schütte 2007). The mechanism was triggered despite minute amounts of bioavailable phosphates. This can mean that available resources are allocated in order to survive in an unfavourably changed environment (Kozłowski 1992), in which apical parts are elongated towards the light at the expense of not developing branchlet rosettes. A similar defense mechanism against shading i.e. larger assimilation surface resulting from developing 1st degree branches at the expense of growth and reproduction was described in an article on the influence of the iron coagulant (Rybak et al. 2017a). Research on the influence of iron coagulants indicates that the effect depends on the physiology of the particular species (Immers et al. 2014). Thus, it can be expected that responses of different species would be various during application of aluminum coagulants as well. In the light of current knowledge, use of chemical coagulants in lakes with charophyte communities is hazardous.

Conclusions

The growth pattern of the charophyte *Chara hispida* is affected by the aluminium coagulant because the main stem's growth slows down and internode cells elongate in their apical parts. These changes are caused by a lowering of pH with the toxic influence of aluminium as well as shading. The charophyte becomes covered by coagulated suspension. The undertaken experiments have not defined a dose of coagulant that would be biologically safe for the charophyte. Owing to the great morphological diversity of the genus *Chara*, it is highly probable that each species can react in a different way. It is important for safe restoration of waterbodies to recognise the chemical features of water, original characteristics of the habitat, as well as the structure of plant communities. A thorough analysis and research on the influence of coagulants on charophytes and aquatic plants is indispensable.

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Poznań, czerwiec 2018

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polegał na: dyskusji koncepcji eksperymentu i wyników pracy, przeprowadzeniu eksperymentów terenowych oraz ostatecznej korekcie tekstu

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Ecotoxicology and Environmental Safety 145 (2017) 359–366

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Bioaccumulation and toxicity studies of macroalgae (Charophyceae) treated with aluminium: Experimental studies in the context of lake restoration

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ARTICLE INFO

Keywords:
Charophytes
Aluminium
Bioaccumulation
Lakes restoration
Phosphate coagulant
Mesocosm experiment

ABSTRACT

The objective of this study was to examine the impact of aluminium on the perennial macroalgae *Chara hispida* L. and its bioaccumulation capacities. Aluminium (Al) was introduced into the environment in the form of polyaluminium chloride, an agent utilized in the restoration of waterbodies. Research was conducted in an experimental setting using *meso*cosms (volume 0.8 m^3) placed in the littoral zone of a lake with *C. hispida*. Three doses of the coagulant were applied, each with a different volume: low – 6.1 g Al m^{-3}, medium – 12.2 g m^{-3} and high – 24.5 g Al m^{-3}. A significant acidification of environment was determined, which would imply the presence of toxic Al^{3+} ions. It has been demonstrated that aluminium penetrates and accumulates in the cells of the charophyte. This caused damage to the thalli, which manifested itself in chloroses, necroses, flaking of the cortex cells and softening of the thallus, whose severity was proportionate to the dose of the coagulant. The first negative signs were observed after 24 h. The study shows that *C. hispida* is a poor accumulator of aluminium (bioconcentration factor < 200), while bioaccumulation capacity was inhibited at the concentration of approx. 2.0 mg Al g^{-1} d.w. Accumulation in the thalli of the charophytes accounted for 58% of variation following removal of aluminium from the environment. The results of the experiment demonstrate a negative impact of aluminium on charophytes at concentrations used in aggressive restoration of lakes.

1. Introduction

Macroalgae are characterized by a capacity to accumulate toxic metals, and numerous species of this organisms are considered to be effective biomonitors and bioindicators (e.g. Murphy et al., 2007; Rybak et al., 2012; Clabeaux et al., 2013). Thus far, aluminium (Al) has not been taken into account in studies on metal accumulation, despite the fact that in acidic conditions it proves toxic to plant and animal life as reviewed by Gensemer and Playle (1999). One of the routes through which aluminium is introduced into an environment is application of chemical phosphorus coagulants in the course of surface water restoration (Lewandowski et al., 2003; Cooke et al., 2005; Sobczyński et al., 2012) and wastewater processing (Aguilar et al., 2002). Utilization of aluminium salts (e.g. alum – $Al_2[SO_4]_3{\cdot}14H_2O$) in lake restoration dates back to the early 1970s (Smeltzer, 1990). They have since been in widespread use as flocculation agents in Europe and United States (Cooke et al., 2005; Zamparas and Zacharias, 2014). At present, new generations coagulants are employed, e.g. pre-hydrated and polymerized products (containing polyaluminium chloride) which are more efficient than conventional aluminium coagulants (Grochowska et al., 2015). The principal advantage of aluminium-based coagulants is the durability of aluminium-phosphate bonds in anaerobic conditions, which inhibits release of bound phosphates in deep-water zones (Burley et al., 2001). Another major asset of polyaluminium chloride is that sulphates are not introduced into the environment. Sulphate contamination boosts eutrophication processes, leading to the formation of toxic sulphides in anoxic conditions and reduce iron availability to aquatic plants (Van Der Welle et al., 2007). Acidic pH is the parameter which restricts their application, as it can substantially affect abiotic conditions, especially in shallow and poorly buffered lakes (Łopata et al., 2013; Immers et al., 2015). Acidification of the environment causes the release of aluminium ions from mineral structures (e.g. aluminosilicates, alunite) and formation of aluminium hydroxide which then transitions into the active hexa-aqua-aluminium complex Al $(H_2O)_6{}^{3+}$, usually abbreviated as Al^{3+}. The resulting molecule is highly reactive, mobile, and easily absorbed by plants (Martin, 1986; Driscoll

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http://dx.doi.org/10.1016/j.ecoenv.2017.07.056
Received 3 April 2017; Received in revised form 21 July 2017; Accepted 24 July 2017

and Schecher, 1990). The mechanism of action of aluminium-based coagulants in aquatic environments owes to the synergistic effect of acidification and toxicity of aluminium in the first phase and, in the second phase, to the effect of Al ions in plant cells.

Application of coagulants without prior determination of the existing problem and mechanisms behind eutrophication in particular waterbodies is an ill-advised approach. Regrettably, coagulants are used increasingly often to achieve short-term, provisional improvements in water quality e.g. in case of seasonal fluctuation of trophic level or incidental inflow of biogens from the catchment area (Joniak et al., 2013). Temporary trophic disturbances result most often in phytoplanktonic blooms and shifts in physicochemical properties of water (Rosińska et al., 2017). Consequently, one observes increased turbidity combined with unpleasant odour of the water, as well as associated nocturnal oxygen deficiency which could lead to fish kills (Paerl and Huisman, 2008). Such procedures are also motivated by the need to restore hampered recreational functions of the affected waterbodies (Brooks et al., 2016). In either case, the changes which occur reduce their utilization capacity and lead to financial losses (Pretty et al., 2003). However, restoration treatments must only take place under strict supervision in lakes which constitute important habitats of plants, e.g. stonewort communities, which play an important role in maintaining good water quality (e.g. Van den Berg and Coops, 1999; Kufel and Kufel, 2002).

Charophytes (stoneworts, *Charophyta*) as "ecological engineers" are one of the major and most widespread groups of subaquatic plants (comprising around 400 species) found across a variety of waterbodies, from very shallow to deep. Nevertheless charophytes are threatened by direct anthropopressure in water ecosystems and increased pressure from the catchment area (Munsterhjelm, 2005; Torn et al., 2010). For this reason, hard oligo-mesotrophic waters with stonewort communities (code 3140) are encompassed by the European habitat conservation network Natura 2000 (Water Framework Directive, 2000). Stoneworts grow at substantial depths and form charophyte meadows which can cover extensive areas of the bottom. It should be noted, however, that stonewort species also inhabit eutrophic waters, where they may be exposed to pressure such as restoration treatments. Moreover, they are known for being pioneers of colonization, particularly in the waterbodies in which ecological balance has just been restored (Blindow, 1992; Van den Berg et al., 1998). Charophytes ensure shelter and source of nutrition to invertebrates, fish and waterfowl. They contribute to an improvement of water quality through physical stabilization and isolation of bottom sediment (Coops, 2002; Kufel and Kufel, 2002; John, 2003).

The administration of acid coagulants in natural waters may substantially and adversely affect the properties of the abiotic environment, rendering continued functioning of stoneworts and macrophytes impossible (Immers et al., 2013, 2014; Rybak et al., 2017). Previously, studies into bioaccumulation in charophytes have been undertaken sporadically, and even then they focused on toxic metals (Gomes and Asaeda, 2009). The literature concerning the impact of aluminium on stoneworts is equally scarce, and addresses only physiological response and the rates at which aluminium penetrates into isolated cells (Takano and Shimmen, 1999; Taylor et al., 2000). Thus, the aim of this study was to determine (1) the biological effect of polyaluminium chloride (types of lesions and their location) and (2) the accumulation capacity of evergreen *Chara hispida* L. for aluminium.

2. Materials and methods

The species selected for the experiment was *Chara hispida* L., a representative of the genus Chara (*Characeae*, *Charophyta*), widely distributed in Europe (frequent in Poland) and found in North Africa and Asia as well (Krause, 1997). The species prefers alkaline and neutral waters with the content of calcium compounds between 17.0 and 167.0 mg Ca dm^{-3} (Haas, 1994; Gąbka, 2009) and proves capable of growing with a wide range of available light radiation (Andrews et al., 1984; Menendez and Sanchez, 1998). In Central-Eastern Europe, it is encountered in shallow eutrophic lakes as well as in waters with slightly acidic pH, such as peatland exploitation ponds or humic waterbodies (Haas, 1994; Urbaniak and Gąbka, 2014). It is a monoecious macroalgae, one of the largest representatives of the genus: length of the stem ranges from 30 to 200 cm with axial diameters of 1–4 mm, internode length of 10–15 cm and 7–11 leaf-like structures, extending up to 8 cm in length (Krause, 1997; Urbaniak and Gąbka, 2014). It overgrows in highly hydrated, organic sediments, occurring less frequently on peat substrate and calcareous gyttja (Gąbka, 2009).

2.1. Experiment design

A *meso*cosm experiment was carried out over 3 days (beginning of August 2015), in the shallow Lake Wielkowiejskie (52°17′43″N; 16°40′5″E) where maximum depth reaches 4.0 m, located in Western Poland. The lake is characterized by the dominance of charophyte meadows and a wide belt of rushes. Eight open chambers (100 × 100 × 200 cm) were placed in the littoral zone, densely inhabited by *C. hispida* (approx. 50 shoots per m^2), separating the growing macroalgae into eight different treatment conditions. The chambers remained open both to the atmosphere and the sediments. Side walls were made of 2 layers of a transparent polyethylene foil, which allowed sunlight to infiltrate into the chambers from the sides as well. Water infiltration through sediments was limited by placing the chamber walls in semi-liquid bottom sediments at the depth of about 30 cm. Water depth in the chambers amounted to approximately 0.8 m. After securing the chambers in the studied habitat, they were left for 1 month to allow the separated, newly formed ecosystems to stabilize; subsequently, baseline conditions were determined.

2.2. Application and sampling

The chemical substance used in the experiment was polyaluminium chloride (pH < 1.0, density 1350–1370 kg m^{-3}), due to its strong flocculation/coagulation properties and widespread use in lake restoration treatments. Coagulant was added to the chambers in three dose volumes (two replicates per treatment): low at 6.1 g Al m^{-3} (680 kg per ha, referred to as Low), medium at 12.2 g Al m^{-3} (1360 kg per ha, referred to as Medium), and high at 24.5 g Al m^{-3} (2720 kg per ha, referred to as High). Two separate chambers served as controls (Control, 0.0 g Al m^{-3}). The doses administered in this study reflect the manner of "aggressive restoration" which would occur in a single application of the coagulant with resultant precipitation of phosphates, suspensions and reduction in water colour. Water and thalli samples were collected 24 h, 48 h and 72 h after Al application. Water samples (100 cm^3 each) were acquired by means of a pipette (semiautomatic pipettor by SwiftPet Pro) just above the community (without disturbing the sediment on the stoneworts), then transferred into glass bottles and fixed with concentrated nitric acid (Sigma-Aldrich). pH was measured each day at the same hour (Hanna Instruments HI 98129). Morphologically uniform thalli of *Chara hispida* (8–10 internodes, 40–50 cm length) were harvested, transferred into plastic bags (filled with a small amount of water) and transported to the cell biology laboratory at the Jagiellonian University in Cracow. Three replicates of each treatment were analyzed. Samples of fresh biomass of *C. hispida* were washed in distilled water.

2.3. Microscopic analysis and assessment of toxicity symptoms

To determine chloroplast reduction and metal bioaccumulation, the thinnest branches of the apical tips of thalli were mounted in a drop of water on microscope glass and closed with a cover slip (Martín-González et al., 2006). Images of the algal surfaces and internal parts of the thalli were obtained using a laser scanning confocal microscope

(Zeiss LSM 510) at an optical section thickness of 0.5–2.5 µm, with 543 nm excitation light of green helium-neon laser. The images were processed with Zeiss LSM software and edited for contrast in the ImageJ 1.44 g software (National Institutes of Health, USA).

Analysis of photographs and confocal imaging served to determine reduction of chloroplasts resulting in chlorosis, softening of the algal thalli and detachment of corticating cells in the cortex around central cells (designated as no effect, mild, moderate, severe). In order to analyse the post-treatment condition of the algae, visual observation and manual examination of the experimental samples were performed. Chloroplast reduction was determined by assessing the colour of the algae, whereby 100% green thalli were assumed to display no effect, ca 70% green denoted mild impact, ca 50% green moderate impact whereas thalli less than 30% green considered to be a severe effect. Softening of the thalli was determined by means of examination of morphological changes in the shape and hardness of the algae body, from branched hard thalli (no effect), through apical parts and branches soft and glued (mild), all branches soft and glued (moderate), all branches together with the central thallus soft and glued (severe). In order to determine the level of damage expressed in the detachment of corticating cells, the shape of the cilia and the amount of slime on the algae body were evaluated as follows: cilia covering the entire thallus and absence of slime (no effect), reduced cilia on the apical glued parts (mild), no cilia on the apical glued parts and reduced cilia on the central thallus (moderate), no cilia both on the glued apical and glued central parts of thalli (severe).

2.4. Chemical analysis

Prior to the analysis, stonewort samples were cleaned from surface contamination in tap water, and then left to dry at room temperature and in a dryer at 80 °C to obtain dry weight (d.w.). Al concentration in tap water amounted to 0.5 µg dm^{-3} and was not taken into account in further analyses as a secondary source of contamination. The dry mass was ground in a porcelain mortar. Aluminium concentration in the algae and the water was analyzed using Flame Atomic Absorption Spectroscopy (series AA 200, Agilent Technologies; abbreviation AAS). Representative samples of 0.5000 g of algae in the form of powder were mineralized in the Marsexpress CEM International microwave mineralization system (in teflon tubes with 8 cm^3 nitric acid – Sigma-Aldrich), acc. to the three-step program (400 W, 100 °C, 2 min; 600 W, 160 °C, 5 min; 1600 W, 200 °C, 10 min). After the mineralization process, the resulting solutions were filtered using a filter paper and diluted with deionized water to a volume of 50 cm^3. This procedure was repeated three times for each sample. After each step of treatment, samples were subjected to AAS analysis for aluminium content using a Spectra 280 AA spectrometer by Agilent. Final results consisted of median values of three simultaneous measurements. The same method was applied to analyse aluminium content in water samples. Before assay analytical curves were prepared on the basis of a series of freshly prepared standard obtained from standard solution of aluminium with the concentration of 1000 mg kg^{-3}. In order to confirm the reliability of the results obtained during the analyses, a validation was performed in every tenth sample using certified plant reference material NCS DC 73350 (Leaves of Poplar). A comparison of aluminium concentration in this study and certified reference material (certified value: 0.104 ± 0.006%)

The following parameters were employed to assess the type of environmental interactions (biotic – abiotic) which led to the removal of aluminium from water: bioconcentration factor (BCF) and percentage metal removal (*U-factor*). The former (BCF) was used to determine the ability of *C. hispida* to concentrate aluminium. The factor is defined as the ratio of metal concentration in dry mass to the initial metal concentration in the water (Raskin et al., 1994). The latter, i.e. percentage metal removal (in %) was calculated based on the uptake: $U = [C_0 - C_1)/C_0] \times 100\%$ where C_0 and C_1 (both in mg dm^{-3}) denote the initial and the remaining concentration of aluminium in the water, respectively (Abdel-Halim et al., 2003).

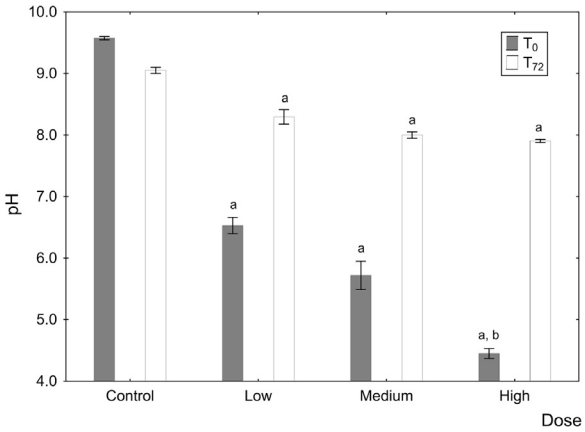

Fig. 1. pH shift of water following different doses of aluminium coagulant (the bars indicate mean values; error bars represent standard error; T_0: 0.5 h after application, T_{72}: 72 h after application; a: $P < 0.05$ vs. Control; b: $P < 0.05$ vs. Low and Medium).

2.5. Statistical analysis

The differences between the control and experimental treatments with respect to the analyzed parameters were assessed by means of one-way analysis of variance (followed by Tukey multiple comparisons test). The Shapiro-Wilk test was used to evaluate normal distribution, while the Levene test was applied for the assessment of the equality of variances for groups. All statistical procedures were performed using Statistica 12.5 (StatSoft, Poland).

3. Results

Application of the coagulant caused an instantaneous drop in water pH. In all chambers, pH decreased significantly compared with the control (pH 9.6), proportionally to the dose of the coagulant (time = 0.5 h; $F_{3,4} = 247.29$; $P = 0.000$). Shortly afterwards (24 h) pH rose to ≥ 8.0, but it remained markedly lower than the control (time = 72 h; $F_{3,4} = 57.15$; $P = 0.001$) throughout the duration of the experiment (Fig. 1).

3.1. Symptoms of aluminium toxicity

The toxic effect of aluminium on the alga *Chara hispida* manifested in softening of the tissues, detachment of cilia and external corticating cells as well as in the degradation of chloroplasts (Table 1; Fig. A.1). The changes became noticeable after 24 h, even in specimens exposed to the lowest dose. Maximum amount of damage was observed after 72 h, with the same level in all treatments. Over the subsequent days of incubation, toxicity symptoms increased in all chambers except for the control. Both Medium and High doses of coagulant caused acute lesions in algae, visible in global tissue damage.

Confocal scanning revealed high intensity of red autofluorescence in samples incubated with aluminium (Fig. 2). Although autofluorescence was also present in chloroplasts and corticating cells of control samples, the autofluorescence of samples treated with aluminium emitted much stronger light signal; consequently, tissue visualization in bright light proved impossible, since the microscope light was entirely absorbed on dense thalli. Fluorescent particles were mostly present inside the thalli, not on the surface, indicating that Al was actively transported into the algae. The most intense red and green light emissions were observed 48 h after incubation. On the third day, autofluorescence diminished due to severe cellular damage.

Table 1
Toxicity effects in *Chara hispida* following exposure to aluminium coagulant.

Dose	Time after application	Toxicity symptoms		
		Reduction of chloroplast	Softening of thallus	Detachment of corticating cells
Control	24 h	–	–	–
	48 h	–	–	–
	72 h	–	–	–
Low	24 h	+	–	+
	48 h	+ +	+	+ +
	72 h	+ + +	+ + +	+ +
Medium	24 h	+	+	–
	48 h	+ +	+ + +	+ +
	72 h	+ + +	+ + +	+ + +
High	24 h	+	+	+
	48 h	+ +	+ +	+ +
	72 h	+ + +	+ + +	+ + +

+ mild effect; + + moderate effect; + + + severe affect; – no effect.

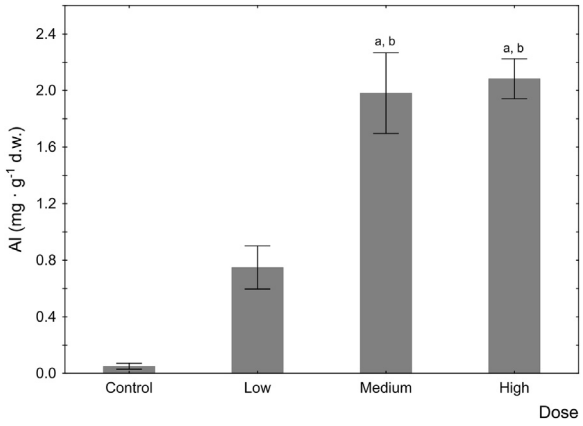

Fig. 3. Dose-dependent variation in aluminium concentration in the thalli after 72 h (the bars indicate mean values; error bars represent standard error; a: $P < 0.000$ vs. Control; b: $P < 0.001$ vs. Low).

3.2. Quantitative assessment of aluminium bioaccumulation

Accumulation in the thalli increased linearly with the Al concentration in water, reaching the maximum of 2.52 mg g^{-1} d.w. (for the Medium dose). Mean concentration of the metal in the thalli of specimens found in enclosures Medium and High was identical (approx. 2.0 mg g^{-1} d.w.), in spite of the 2-fold higher Al concentration in the High chambers. Statistical analysis demonstrated highly significant differences in the concentration of accumulated metal in the stoneworts from Medium and High enclosures compared with that of the Control and Low (Fig. 3).

BCF was highest in the specimens from the Medium enclosure (max. 208), and lowest in the High enclosure (max. 101) – the differences were statistically significant ($P > 0.05$). Intermediate concentrations were observed for the enclosures with the Low dose (Fig. 4).

The value of the *U-factor* suggested comparably high elimination of Al from water after introduction of Medium and High doses (mean 81% and 87%, respectively), with a significant difference with respect to the Low dose (mean 47%; $P < 0.005$). Regression analysis (Fig. 5) demonstrated that there was a close relationship between removal of Al from water and accumulation of Al in the thalli of the stoneworts ($R^2 = 0.5836$; $P < 0.000$).

4. Discussion

The study has demonstrated the following, that Al in the form of

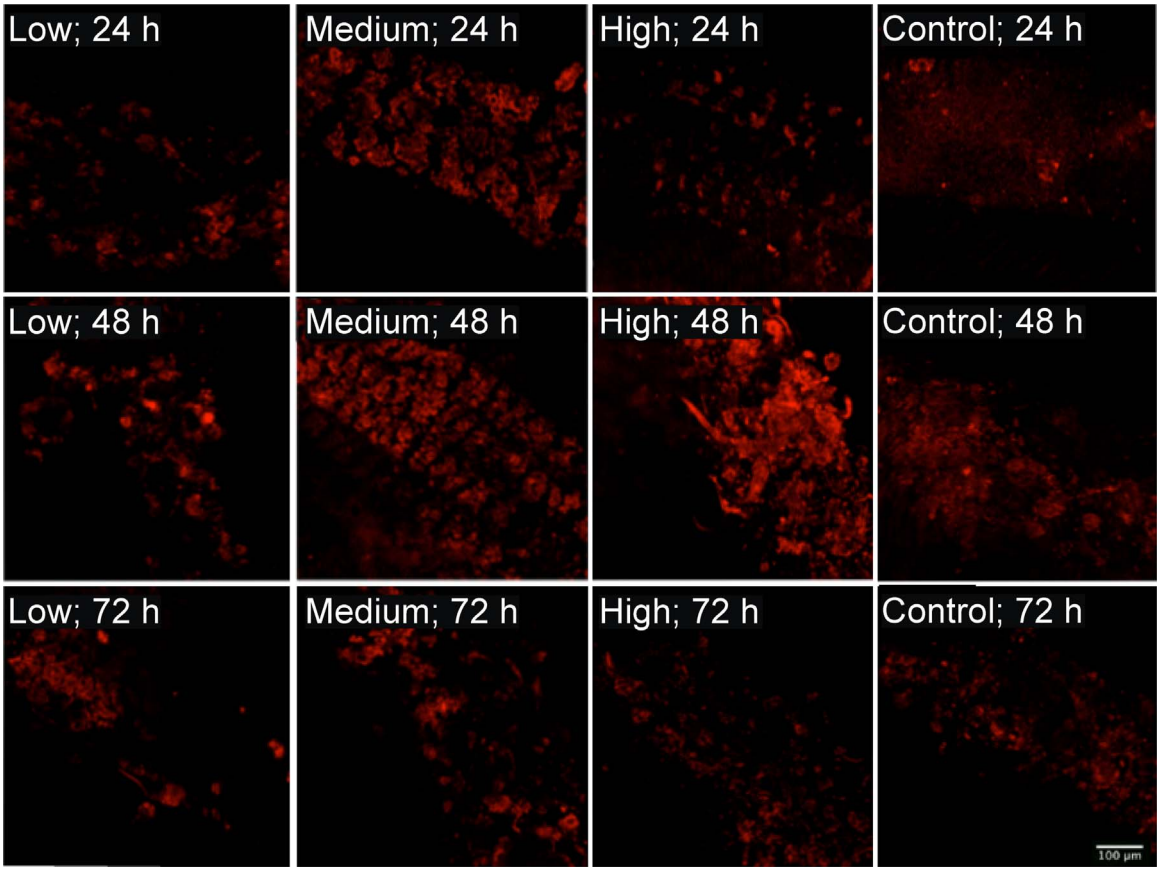

Fig. 2. Confocal images of the internal thalli of *Chara hispida* on 24 h, 48 h, and 72 h in low, medium and high concentration of Al compared to the control.

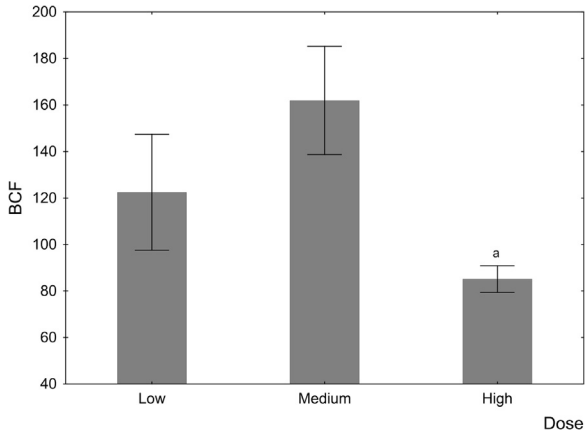

Fig. 4. Mean values of bioconcentration factor (BCF) following 72 h exposure to different doses of coagulant (the bars indicate mean values; error bars represent standard error; a: $P < 0.05$ vs. Medium dose).

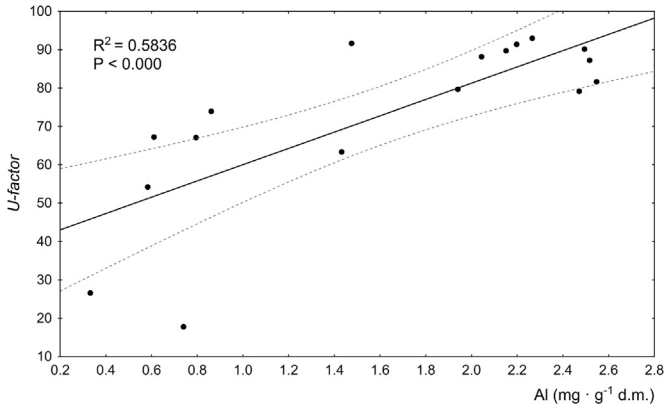

Fig. 5. Regression of *U-factor* and Al content in the dry mass of charophytes (dotted lines represent the 95% confidence interval around the regression line).

polyaluminium chloride: (1) has a toxic effect on *C. hispida*, penetrating into the cells and causing damage manifested as chloroses, softening of thallus and detachment of corticating cells; (2) is accumulated in the cells to the maximum amount of ca 2.0 mg g^{-1} d.w., while the degree of accumulation does not increase despite greater Al concentration in the solution. Consequently, this classifies *C. hispida* as a poor bioaccumulator of aluminium.

The volume of assimilated metal depended on its concentration in the *meso*cosm and water pH. Solution pH is widely known to be the main factor controlling toxicity, solubility and speciation of aluminium (Driscoll and Schecher, 1990; Gensemer and Playle, 1999). Solubility and toxicity of Al are directly proportional to acidity (Godbold et al., 1995). In this experiment, pH dropped below neutral irrespective of the dose of coagulant. High buffering capacity of waters in lakes where charophytes occur prevents sudden decline in pH, to a considerable extent. However, as demonstrated, application of a single large dose of coagulant eliminates the buffer, leading to increased phytotoxicity of Al. Acidification processes are dangerous for charophytes since they modify the preferred alkaline conditions of the habitat, and cause their eradication at pH lower than 5.2 (Yan et al., 1985). On the other hand, acidity increases solubility of toxic metals (e.g. release from bottom sediments) and a shift of dissolved inorganic carbon balance towards CO_2 (Nixdorf et al., 2001; Brouwer et al., 2002). The pH change in the High dose chamber approached the value at which Taylor et al. (2000), in a laboratory setting, showed the fastest rate of metal ion transport through the cellular membrane in *Chara corallina*. In that work fluctuations of pH by 0.3 unit resulted in a more than 3-fold reduction of the speed of metal penetration. With sufficiently long exposure, the amount of assimilated Al may reach a similar level, especially given lack of signs of saturation in the first 3 h. Lowering of the pH in the water-coagulant mixture slightly below neutral and reduced Al concentration in the environment account for the decreased concentration of the metal which accumulated in stonewort specimens in the Low-dose chamber. It should be noted that *Chara corallina* which Taylor et al. (2000) examined in their experiment (with $AlCl_3$) is a species devoid of corticating cells and poorly encrusted, and thus differs from *C. hispida* used in this experiment.

Our research demonstrates that aluminium accumulation in the thalli of *C. hispida,* albeit limited, caused numerous lasting lesions observed even at the lowest administered dose. The dosages widely employed in "aggressive restoration" of water bodies range from 5 to 30 g Al m^{-3} (Rydin and Welch, 1998; Reitzel et al., 2003). Meanwhile, the mechanism responsible for the toxic effect on plants and algae has not been conclusively established as yet. In studies concerning the impact of metals on unicellular algae, their complex response has not been precisely defined either (Trenfield et al., 2011). Growth inhibition as the main negative effect is often reported (Claesson and Tornqvist, 1988; Kinross et al., 2000) with isolated instances of inhibited assimilation of nitrogen or CO_2 (Gensemer and Playle, 1999). There is a number of works devoted to higher land plants (e.g. Delhaize and Ryan, 1995 and other works cited there), but the effects observed among such species (e.g. root changes, transport disorders) cannot be related to thallophytes from the genus *Chara*. Disturbed properties and integrity of cellular membranes appear to be the principal mechanism underlying the toxic effect (Ahn et al., 2002), a fact which has been confirmed for charophytes as well (Takabatake and Shimmen, 1997; Takano and Shimmen, 1999). The damage leads to impaired HCO_3^- ion influx, which involves CO_2 fixation, thus resulting in defective production of carbonate encrustation. In addition, Al inhibits the influx of calcium ions and replaces them in the cell wall, thus considerably reducing cellular growth (Reid et al., 1995).

Neutralization of pH and its increase above neutral, occurring ca 24 h from coagulant application, stops dissolution of encrustation and eliminates toxic Al^{3+} ions (Martin, 1986; Bertch, 1989). Even re-alkalization of the environment does not appear to boost renewed encrustation (Takano and Shimmen, 1999). Nevertheless, the period preceding the neutralization of acidity was sufficiently long to enable metal ions to penetrate through the cell wall and into the protoplast (Taylor et al., 2000). What is more, even when toxic Al^{3+} ions are eliminated from the external environment, they tend to be conveyed from the cell wall to the protoplast (Rengel and Reid, 1997). This is borne out by the occurrence of an energetically active bioaccumulation on top of physicochemical biosorption to the surface of the cell wall. It should be underlined that in its first stage bioaccumulation resembles biosorption, i.e. binding of metal ions to the cell wall. Ultimately, however, the ions are actively transported into the cell (Chojnacka, 2010). Nonetheless, a substantial amount of the accumulated aluminium is concentrated in charophyte cell wall (Rengel and Reid, 1997). Therefore, the observed thallus softening caused by reduced pH along with dissolved encrustation may be a lasting effect in cells damaged by aluminium, while recovery from aluminium inhibition may prove impossible even over a prolonged period. Penetration of Al into the protoplast exerted an impact on the inner cell membranes as well. This resulted from the capacity of Al to bind to the carboxyl and phosphate groups of the cell wall and membrane, respectively (Gunsé et al., 1997), leading to disintegration of chloroplasts as well as chloroses or necroses materializing on the surface of the thalli. Photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoids) undergo degradation as well, which is evinced by their rapidly decreasing concentrations (Rybak et al., 2016). Most lesions of that type were observed on the leaf-like structures with simultaneous reduction in length; in extreme cases these were dead altogether. Another conspicuous symptom of aluminium toxicity was detachment of cortical cells and elimination of septa. Physical damage to specimens affects the stability of bottom

ecosystems, which may have serious consequences for the functioning of communities which occur there (Torn et al., 2010; Matuszak et al., 2012).

4.1. Quantitative assessment of aluminium bioaccumulation

Analyses have shown that the amount of aluminium absorbed by the charophytes did not exceed 2.0 mg g^{-1} d.w. with Medium and High doses, despite water pH being optimal for the rate of ion penetration through cell membrane (Taylor et al., 2000) and double the concentration of Al. Given the data reported by other authors these values were high. Accumulation of aluminium in submerged macrophytes ranges from 0.28 mg g^{-1} d.w. in *Hydrilla verticillata* to 0.85 mg g^{-1} d.w. in *Cabomba piauhyensis* (Albers and Camardese, 1993; Abu Bakar et al., 2013). In the case of helophytes and nympheides, accumulation varies to an even greater extent – from 0.02 mg g^{-1} d.w. in *Nymphaea alba* and *Juncus bulbosus* to 3.1 mg g^{-1} d.w. in *Phalaris arundinacea* (Samecka-Cymerman and Kempers, 2001). In the aforesaid studies, aluminium originated from natural sources or anthropopressure (e.g. mining wastewater), but its concentrations were lower than in the conducted experiment (0.008 – 4.4 g Al m^{-3}).

Values of the BCF, which proves useful in the evaluation of plant potential with respect to accumulation of toxic metals, confirmed that absorption of aluminium was arrested. Overall, however, it needs to be noted that the amount of metal taken up by the cells was considerable. Low BCF (Zayed et al., 1998) indicate that the studied species is a poor accumulator and phytoremediator of aluminium. Macroalgae with high phytoremediation capacity should absorb metals at high absorption rates (*U-factor*) and BCF over 1000 (Sooksawat et at, 2013). The relatively high Al concentration in cells and low absorption rates should be attributed to the high concentration and brief availability of the metal in water. Al is subsequently polymerized following re-alkalization of the habitat, which in turn precludes bioaccumulation. The accumulation process was impaired by physical cell damage, though it did not lead to the death of entire specimens. Decreased bioaccumulation capacity may also be due to the activation of the protection system which occurs in some freshwater algae (Chojnacka, 2010). The latter mechanism has not been confirmed in charophytes and requires further investigations.

Bioaccumulation of aluminium in the thalli of *C. hispida* accounted for 58% variation of metal concentrations in water. Bearing that in mind, in order to determine the mechanism of aluminium elimination from the environment, one should consider chemical characteristics of polyaluminium chloride and reactions taking place once the coagulant has been applied. The substance is a combination of strong hydrochloric acid and dissolved metal (9.0 ± 0.3%). Low pH (< 1.0) increases affinity of metal ions to phosphates (Alberti et al., 2005). Following addition of water, aluminium reacts with mineral phosphates forming insoluble complex compounds which, precipitated as aggregate (flocs), are gravity-sedimented in the bottom sediments. Ions also bind to dissolved organic matter, sulphates and silicates (Doucet et al., 2001; Auvray et al., 2006). This meant that from the moment of application, aluminium was eliminated through complexation, precipitation and sedimentation from the aquatic environment (and biological circulation). The process was simultaneous to the assimilation of the metal by the charophytes, which occurs within the first minutes from exposure (Taylor et al., 2000). However, one should take into account that this study was concerned only with *C. hispida* as a representative of charophytes found in mesotrophic and eutrophic waters, while studies using iron coagulants (Immers et al., 2014; Rybak et al., 2017) warrant the presumption that the effect of aluminium ions may vary depending of the physiology of species.

5. Conclusions

Aggressive restoration in lakes which provide habitats to charophytes entails acidification of the environment and introduction of a toxic form of aluminium (Al^{3+}), which is then easily assimilated by the thalli of the Chara specimens. The toxic effect is subsequently manifested in cellular damage, such as chloroses, necroses, cortex detachment and thallus softening. The damage becomes perceptible at the dose of 6.1 g m^{-3} Al, occurring after 24 h (or, in extreme instances after 72 h). The capacity of *Chara hispida* to take up aluminium from water is limited to approximately 2.0 mg g^{-1} d.w., which represents a substantial amount in dry biomass, but it may be considered low with relation to overall concentration present in the environment upon application of the coagulant. The results obtained in the course of this study imply that extreme caution should be exercised when applying aluminium-based agents in the restoration of Chara-dominated lakes. It would be legitimate to administer doses which do not exceed 5.0 g Al m^{-3} of lake water, but further research is required to determine the dose of aluminium-containing coagulants which would be safe for charophytes.

Appendix 1

See Appendix Fig. A1

Fig. A.1. Visible effects of toxicity effects in *Chara hispida* following exposure to aluminium coagulant after 72 h in low, medium and high concentration of Al compared to the control.

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Poznań, czerwiec 2018

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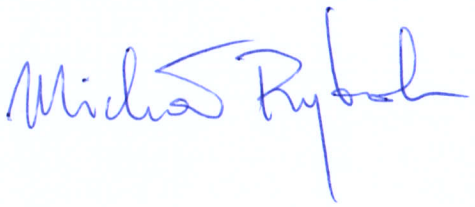
Oświadczenie określające wkład w powstanie artykułu

Niniejszym oświadczam, że mój wkład w powstanie poniższego artykułu:
Rybak M., Kołodziejczyk A., Joniak T., Ratajczak I., Gąbka M., 2017, *Bioaccumulation and toxicity studies of macroalgae (Charophyceae) treated with aluminium: Experimental studies in the context of lake restoration*, Ecotoxicology and Environmental Safety 145: 359-366 polegał na: wypracowaniu koncepcji i hipotez, przeprowadzenie eksperymentów terenowych, wykonaniu analiz statystycznych, napisaniu pierwszej wersji pracy, wprowadzeniu uwag współautorów i przygotowaniu wersji ostatecznej, przygotowaniu rycin i tabel, pracach redakcyjnych wg. wymagań czasopisma oraz wysłaniu publikacji.
Mój całkowity wkład w pracę wynosi 70%.



Kraków, listopad 2018

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Oświadczenie współautora określające wkład w powstanie artykułu

Niniejszym oświadczam, że mój wkład w powstanie poniższego artykułu:

Rybak M., Kołodziejczyk A., Joniak T., Ratajczak I., Gąbka M., 2017, *Bioaccumulation and toxicity studies of macroalgae (Charophyceae) treated with aluminium: Experimental studies in the context of lake restoration*, Ecotoxicology and Environmental Safety 145: 359-366 polegał na: wykonaniu i opisaniu analiz mikroskopowych.

Mój całkowity wkład w pracę wynosi 5%.

Poznań, czerwiec 2018

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Oświadczenie współautora określające wkład w powstanie artykułu

Niniejszym oświadczam, że mój wkład w powstanie poniższego artykułu:

Rybak M., Kołodziejczyk A., Joniak T., Ratajczak I., Gąbka M., 2017, *Bioaccumulation and toxicity studies of macroalgae (Charophyceae) treated with aluminium: Experimental studies in the context of lake restoration*, Ecotoxicology and Environmental Safety 145: 359-366

polegał na: dyskusji koncepcji eksperymentu i wyników pracy, przeprowadzeniu eksperymentów terenowych oraz ostatecznej korekcie tekstu

Mój całkowity wkład w pracę wynosi 10%.

Poznań, kwiecień 2017

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Oświadczenie współautora określające wkład w powstanie artykułu

Niniejszym oświadczam, że mój wkład w powstanie poniższego artykułu:

Rybak M., Kołodziejczyk A., Joniak T., Ratajczak I., Gąbka M., 2017, *Bioaccumulation and toxicity studies of macroalgae (Charophyceae) treated with aluminium: Experimental studies in the context of lake restoration*, Ecotoxicology and Environmental Safety 145: 359-366

polegał na: wykonaniu analiz chemicznych próbek ramienic i wody.

Mój całkowity wkład w pracę wynosi 5%.

Poznań, czerwiec 2018

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Oświadczenie współautora określające wkład w powstanie artykułu

Niniejszym oświadczam, że mój wkład w powstanie poniższego artykułu:

Rybak M., Kołodziejczyk A., Joniak T., Ratajczak I., Gąbka M., 2017, *Bioaccumulation and toxicity studies of macroalgae (Charophyceae) treated with aluminium: Experimental studies in the context of lake restoration*, Ecotoxicology and Environmental Safety 145: 359-366

polegał na: opracowaniu koncepcji pracy oraz dyskusji wyników.

Mój całkowity wkład w pracę wynosi 10%.

Maciej Gąbka