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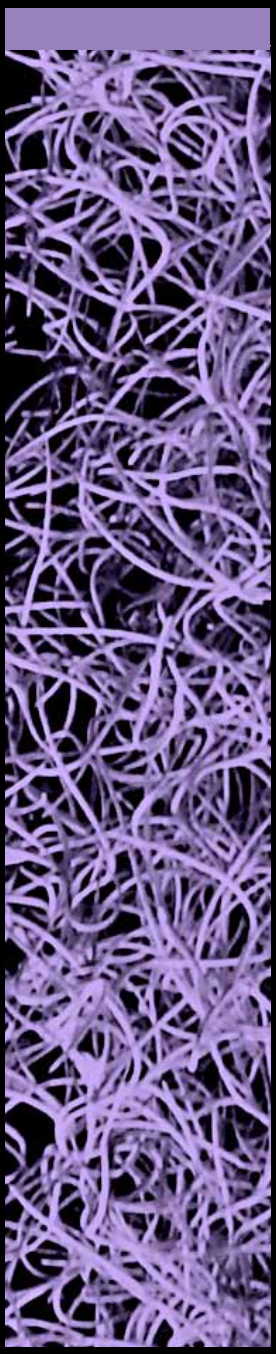

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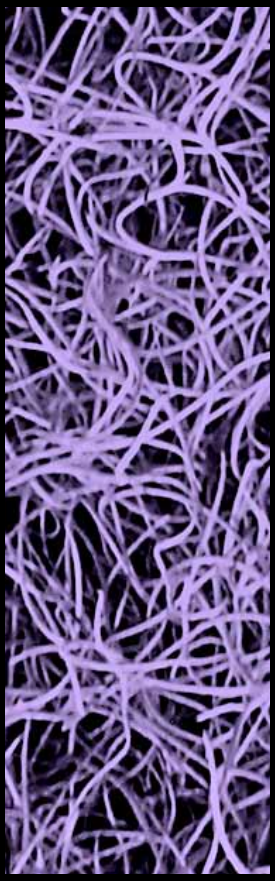

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In memoriam: Mihael Bricelj (1946–2016)

Mihael Bricelj oziroma Mišo za kolege in prijatelje ni bil zgolj mikrobiolog ampak zlasti vizionar, ki si je že dolgo pred časom zastavljal ključna raziskovalna vprašanja okoljske mikrobiologije. Šele današnje obdobje »ómike« in »post-ómike« bosta dali dokončne odgovore, ki jih je Mišo bežno razkril že pred desetletji.

Svoje raziskovalno in pedagoško delo je začel kot asistent na Katedri za molekularno biologijo na Oddelku za biologijo Biotehniške fakultete. Študentje se ga spominjajo kot pedagoga, ki je še dolgo po uradnem zaključku vaj debatiral in razlagal novosti s področja genetike (takrat je bila genetika nov predmet, drugačen od klasičnih bioloških predmetov). Potrebe po mikrobiologu (bakteriologu) so ga pripeljale v skupino za limnologijo, ki je delovala na Nacionalnem inštitutu za biologijo. Tu je sodeloval pri raziskavah in monitoringih jezer in rek. Vzporedno je razvijal mikrobiološke metode sledenja kraških voda z bakteriofagi in se pridružil mednarodnim projektom sledenja (Grčija, Avstrija) ter plodno sodeloval z Inštitutom za raziskovanje krasa Znanstvenoraziskovalnega centra SAZU.

Bricljev raziskovalni opus kaže na njegovo široko raziskovalno zanimanje. Velik odtis je pustil v raziskovanju kraške hidrologije. Lahko bi rekli, da je bil pravzaprav prvi slovenski mikrobiolog, ki se je zavedal kompleksnega pomena in vloge mikrobov v kraških procesih. Njegova doktorska disertacija (1994) »Sledenje podzemnih voda z bakteriofagi bakterije *Salmonella typhimurium*« pa tudi ostalo raziskovalno delo s tega pomembnega področja kraške hidrologije, npr. sledenje s sporami in umetnimi sledili, sta dobila pomemben odmev ne samo v domači, ampak tudi v mednarodni raziskovalni srenji. Predaval je tudi na Tehniški Univerzi v Gradcu o bakteriofagih in hidrobiologiji v okviru mednarodne šole »Post Graduate Training Course on Groundwater Tracing Techniques«.

Posebej ga je zanimalo stanje vodnih teles ter vpliv onesnaženja na mikrobioto in fitoplankton. Primarne produkcije ni mogoče razumeti brez dobrega poznavanja fototrofov, vključno mikroalg in cianobakterij. Prav zaradi tega je v soavtorstvu z Danijelom Vrhovškom, Gorazdom Kosijem, Brankom Vrešem in Tinetom Valentičičem objavil monografijo »Sladkovodne alge ali jih poznamo?« (1985). K mikroalgam se je z aplikativnega vidika ponovno vrnil v svojem zadnjem obdobju raziskovalnega ustvarjanja, ko je skušal v fotobioreaktorju z zeleno algo *Chlorella vulgaris* izboljšati biosintezo in akumulacijo lipidov, ki so osnova za t.i. »biodizel«.

Za časa svojega raziskovalnega dela na Nacionalnem inštitutu za biologijo v Ljubljani (od 1972 do 2012) je bil priča različnim politikam, ki so ustvarjale in usmerjale raziskave, kar mu velikokrat ni bilo všeč in je to tudi jasno in temperamentno povedal. Opozarjal je na potrebo po usmerjenih raziskavah, vezanih na algologijo, sicer deficitarne biološke discipline v Sloveniji. Za razvoj algologije zgolj monitoring vodnih teles, ki vključuje le najosnovnejše in zakonsko določene parametre, brez dobre raziskovalne osnove, prav gotovo ni dovolj. Sicer pa svojega znanja ni ljubosumno hranil samo zase in ga širil zgolj preko ustaljenih »znanstvenih kanalov« v obliki znanstvenih člankov, ampak ga je posredoval tudi preko številnih strokovnih in poljudnih objav, saj mu številni tuji jeziki sploh niso bili »tuji«. Mogoče pa je bil še najpomembnejši tisti neformalni način razširjenja njegovih znanj in informacij preko diskusij in t.i. »brain-storminga«. Rad je priskočil na pomoč zlasti mlajšim kolegom, ki so se šele začeli uveljavljati pri svojem raziskovalnem delu, saj je sam tudi iz svoje »pionirske« izkušnje vedel, da je pogosto pravi nasvet ob pravem času vreden več kot ducat prebranih znanstvenih člankov.

Čeprav je Mišo dobro poznal biološke zakonitosti, ki ustvarjajo in vodijo življenje, in nekatere tudi sam odkril, je sam prehitro podlegel enemu najpogostejših vzrokov, ki jemlje človeška življenja. Mišo bi, kljub upokojitvi, še lahko marsikaj dodal v naše mozaično in pomanjkljivo znanje o življenju. To pa je sedaj popolnoma odvisno od njegovih naslednikov in vseh tistih, ki bodo vestno prebrali njegove prispevke o bioti in okolju, ter si tako priskrbeli inspiracijo za nadaljevanje zgodbe o življenju.

Janez Mulec
Gorazd Kosi
Tina Eleršek

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Germination rate of stinkwort (*Dittrichia graveolens)* and false yellowhead *(D. viscosa)* in relation to salinity

Kaljivost smrdljive (*Dittrichia graveolens*) in lepljive ditrihovke (*D. viscosa*) v odvisnosti od slanosti

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Abstract: This study was conducted to investigate the effect of salinity on germination rate of stinkwort (*Dittrichia graveolens* (L.) Greuter) and false yellowhead (*D. viscosa* (L.) Greuter). Lettuce (*Lactuca sativa* L.) was used as a positive control. Seeds of all three test species were sown on agar plates with three different NaCl treatments (2.5 g NaCl/L ≈ 42 mM NaCl, 5 g NaCl/L ≈ 85 mM NaCl and 10 g NaCl/L ≈ 171 mM NaCl) and control treatment without NaCl. The three tested species germinated under all salinity conditions. However, they thrived best under control conditions and their germination rate was gradually declining with increasing salinity. Additionally, the start of their germination was delayed with increasing NaCl concentration. According to our findings, we can conclude that both *Dittrichia* species are very tolerant to salinity.

Keywords: salinity, germination rate, tolerance, *Dittrichia graveolens*, stinkwort, *Dittrichia viscosa*, false yellowhead, *Lactuca sativa*, lettuce

Izvleček: V raziskavi smo ugotavljali, kakšen je učinek slanosti na kaljivost smrdljive ditrihovke (*Dittrichia graveolens* (L.) Greuter) in lepljive ditrihovke (*D. viscosa* (L.) Greuter). Kot pozitivno kontrolo smo uporabili solato (*Lactuca sativa* L.). Semena vseh treh testnih vrst smo posejali na agarne plošče s tremi različnimi koncentracijami NaCl (2,5 g NaCl/L ≈ 42 mM NaCl, 5 g NaCl/L ≈ 85 mM NaCl and 10 g NaCl/L ≈ 171 mM NaCl) ter kontrolnim tretmajem brez NaCl. Vse tri testne vrste so kalile pri vseh tretmajih, vendar je bilo njihovo uspevanje najboljše v kontrolnih razmerah, kaljivost pa je z naraščajočo slanostjo postopoma upadala. Poleg tega se je z naraščajočo slanostjo zamaknil tudi pričetek procesa kalitve. Na podlagi rezultatov lahko zaključimo, da sta obe vrsti ditrihovk zelo tolerantni na slanost.

Ključne besede: slanost, stopnja kaljivosti, toleranca, *Dittrichia graveolens*, smrdljiva ditrihovka, *Dittrichia viscosa*, lepljiva ditrihovka, *Lactuca sativa*, solata

Introduction

Stinkwort (*Dittrichia graveolens* (L.) Greuter) and false yellowhead (*D. viscosa* (L.) Greuter) are the only known species belonging to the genus *Dittrichia*, which is classified under the *Asteraceae* family (Wraber 2010). They both originate in the Mediterranean region and can be found

in Slovenia. However, only false yellowhead is considered to be indigenous in Slovenia, whereas stinkwort was only first discovered in 2008 and is regarded as an invasive alien species in this area (Frajman and Kaligarič 2009). The occurrence of false yellowhead in Slovenia is limited solely to its coastal part, where it is quite common in ruderal habitats (Wraber 2010, Jogan et al. 2001). Stinkwort has an even more intriguing distribution pattern, spreading only in ruderal habitats along highway (Frajman and Kaligarič 2009) together with some regional roads. The coastal region is greatly affected by the sea salt, whereas sites along main roads are frequently exposed to excessive application of ice-melting salts in the winter. Thus, we hypothesized that the occurrence of both *Dittrichia* species in Slovenia could be limited to sites with seemingly elevated salinity level.

There exist various types of salts among which many are essential in terms of plant survival. Nonetheless, they quickly become detrimental in excessive amounts. One of the most fundamental and ubiquitous salts is NaCl. Soil salinity can be either primary, i.e. caused by natural processes, or secondary, that is human-induced by different human activities such as irrigation, fertilization and application of de-icing salts (Kotuby-Amacher et al. 2000, Parihar et al. 2015). Most plants cannot bear high salinity level as it causes water stress, ion toxicity, oxidative stress, nutritional disorders, alteration of metabolic processes, membrane disorganization, etc. (Hasegawa et al. 2000, Carillo et al. 2011). All these small-scale consequences reflect in their most vital processes, e.g. germination, growth and photosynthesis (Parihar et al. 2015) and can sometimes even lead to early senescence and death (Zhu 2007).

Seed germination is believed to be the most critical phase of the plant life cycle. Therefore, species that are able to germinate under elevated salinity level have a substantial competitive advantage over species whose germination is entirely suppressed or at least delayed (DiTommaso 2004).

Throughout evolution, some plant species have evolved different mechanisms to combat high salinity, for instance restriction of salt uptake, control of long distance transport of salt, extrusion of salt from the plants, compartmentalization of salt (Carillo et al. 2011, Parihar et al. 2015) and production of compatible solutes otherwise known as osmoprotectants (McNeil et al. 1999).

So far, not many studies have discussed the relationship between either of the two *Dittrichia* species and salinity. Only a handful of studies have already reported about stinkwort being more sensitive to elevated salinity level (Ghorbanali et al. 2013) and the ability of false yellowhead to thrive in such conditions (Curadi et al. 2005). False yellowhead had already been proven to be tolerant to elevated salinity level (Flowers et al. 2012–2016) and to be able to survive in drastic salinity conditions with electrical conductivity values reaching up to 52 000 μS/cm (Curadi et al. 2005). Two other studies have focused more on the capability of stinkwort and false yellowhead to cope with dry conditions, where similarly false yellowhead did better than stinkwort (Öztürk and Mert 1983, Pérez-Fernández et al. 2006).

Our major goal was to find out how increased salinity level affects germination of both *Dittrichia* species, as the existing distribution patterns of stinkwort and false yellowhead in Slovenia indicate to a potential competitive advantage of both species in habitats with elevated salinity level.

Materials and methods

We collected plant material from the following locations:

- Flowering shoots of *Dittrichia graveolens*: Slovenia, Ljubljana, Roje, Obvozna cesta, gravel road bank, 46°6'17.17" N 14°28'54.36" E. Leg. & det.: S. Strgulc Krajšek & S. Anžlovar, 24. 9. 2013.

- Flowering shoots of *Dittrichia viscosa*: Slovenia, Primorska, Koper, near the road exit from Istrska road to Ljubljanska street, along the fence enclosing a warehouse next to Planet Tuš commercial complex, ruderal site, 45°32'16.51" N 13°44'7.38" E. Leg. & det.: S. Strgulc Krajšek & S. Anžlovar, 10. 10. 2014.

Lettuce seeds were bought from a local seed producer Agrina.

Harvested plant material of both *Dittrichia* species was air-dried and stored separately in a dark and dry room until use. Only mature seeds were selected for this experiment.

Before sterilising the seeds, we removed all seed appendices excluding pappus hairs. Seeds of

all three test species were then surface sterilised by 15 min immersion in aqueous solution of sodium hypochlorite (16.5 g/l, Arekina, Šampionka Renče) and afterwards rinsed three times in distilled water for 5 min each time.

To set the germination test, we first prepared 2% water-agar solutions, to which we added appropriate quantity of NaCl to get the desired NaCl concentrations (0, 2.5, 5 and 10 g NaCl/L). All test solutions were thereafter autoclaved and poured into 9-cm sterile Petri dishes. Seeds were placed on agar plates in a 5 × 5 cm array the following day. We used 3 or 4 replicates with 25 seeds for each tested combination. Agar plates were wrapped with transparent foil to limit evaporation and were stored at room temperature in good light conditions until the end of the experiment on its 25th day.

Seeds were examined every day at roughly 24-hour intervals. A seed was considered germinated on the day of emergence of its radicle. We also monitored cotyledon opening as a marker of further seedling development.

Data analysis was done using survival analysis in programme GraphPad Prism 5.01, which automatically compared curves representing different data sets (treatments) using Log-rank (Mantel-Cox) test.

Along with germination tests, we also measured electrical conductivity of the soil from sampling locations of both *Dittrichia* species and electrical conductivity of the two test solutions with the highest NaCl concentrations. To avoid potential influence of any impurities while measuring electrical conductivity, ultrapure water (Milli-Q Plus 185 system) was used to prepare samples instead of distilled water. We repeated each measurement three times in 10-min intervals and ultimately expressed it as a mean value.

Results

Seeds of all three test species germinated under all salinity conditions (Figure 1: A, C, E). However, their germination rate was decreasing correspondingly with increasing NaCl concentration. Minor differences in this general trend could be due to numerous fungal infections in some Petri dishes. In addition to lowering final germination rate, increasing concentration of NaCl also delayed the sole process of germination in both *Dittrichia* species.

As expected, lettuce reached the highest final germination rate of the three tested species (Figure 1: E). With the exception of the highest NaCl concentration, increasing salinity seemed to have a very negligible negative effect on its final germination rate. Nevertheless, the differences between 2,5 g NaCl/L and 5 g NaCl/L treatments and the control were statistically significant ($P < 0.001$).

Final germination rate of stinkwort (Figure 1: A) and false yellowhead (Figure 1: C) in control treatment was very comparable. In general, ontogenesis was slow in both *Dittrichia* species, but considerably faster in false yellowhead compared to stinkwort. Although differences between treatments were a little less evident in false yellowhead than in stinkwort, they were still statistically significant in most cases. The alternation of dynamics of germination with increasing salinity was more pronounced in stinkwort than in false yellowhead.

Cotyledon opening revealed similar findings as the process of germination (Figure 1: B, D, F), as the increasing NaCl concentration was slowing down the process of seedling development. In the case of *D. graveolens* in 10 g NaCl/L treatment, none of the tested seedlings developed to the phase with opened cotyledons (Figure 1: B). The percentage of *D. viscosa* seedlings with opened cotyledons in 5 and 10 g NaCl/L treatments was very low as well (Figure 1: D).

Electrical conductivity of the soil from both sampling locations was relatively low and differed only slightly between both sampling locations (63.9 µS/cm and 80.5 µS/cm in Ljubljana and Koper, respectively). In contrast, high values of electrical conductivity were measured in our test NaCl solutions (9 000 and 18 000 µS/cm in 5 and 10 g NaCl/L solutions, respectively), with the exception of control treatment (1 µS/cm in 0 g NaCl/L solution).

Discussion

Overall, elevated salinity had a restraining effect on both germination of the seeds as well as cotyledon opening in seedlings, and was getting more and more intense with increasing NaCl

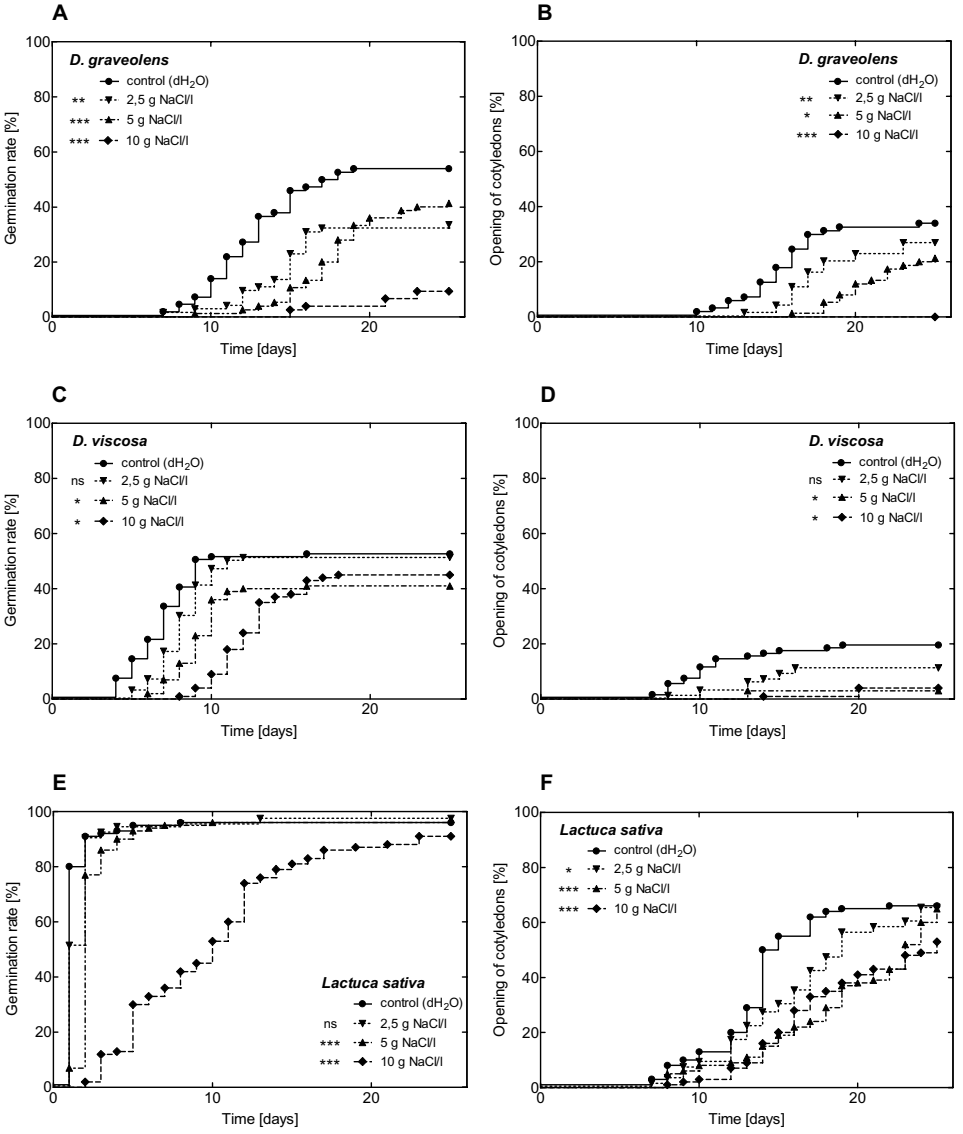

Figure 1: Germination rates and percentage of seedlings with opened cotyledons of *Dittrichia graveolens* (A, B), *Dittrichia viscosa* (C, D) and *Lactuca sativa* (E, F) in relation to salinity. The results of survival analysis done using Log-rank (Mantel-Cox) test, that compared each curve with corresponding control treatment, are shown in the legends of all graphs (***: $P < 0.001$, **: $0.001 < P < 0.01$, * $0.01 < P < 0.05$, ns: $P > 0.05$).

Slika 1: Kaljivost semen in odstotek kalic z razprtimi kličnimi listi vrst *Dittrichia graveolens* (A, B), *D. viscosa* (C, D) in *Lactuca sativa* (E, F) v odvisnosti od slanosti. Rezultati preživetvenih testov, narejenih z uporabo Log-rank (Mantel-Cox) testa, s katerim smo primerjali vsako od krivulj s kontrolo, so prikazani v legendah ob vsakem grafu (***: $P < 0{,}001$, **: $0{,}001 < P < 0{,}01$, * $0{,}01 < P < 0{,}05$, ns: $P > 0{,}05$).

concentration in all three test species, especially in stinkwort and false yellowhead. It was gradually slowing down their development and lowering the final percentage of germinated seeds and seedlings with opened cotyledons. Similar changes in patterns of germination dynamics under increasing salinity levels have also been observed by Läuchli and Grattan (2007). Along with having a direct negative impact on young plants, increasing NaCl concentration also hindered their development indirectly by affecting their roots. The roots in different NaCl treatments had dark root tips and less root hairs than the control seedlings. Many root tips have also turned upwards, as was already observed in a study by Levizou et al. (2002), where *Lactuca sativa* seeds were treated with *Dittrichia viscosa* extracts.

Considering smaller differences in final germination rates between treatments and less variable developmental dynamics in false yellowhead compared to stinkwort, false yellowhead appeared to be more successful in dealing with elevated salinity level than stinkwort, which is in accordance with related preceding studies (Öztürk and Mert 1983, Curadi et al. 2005, Pérez-Fernández et al. 2006, Ghorbanali et al. 2013).

In our case, final germination rate is not the best indicator of the sensibility of the tested plant species to increased salinity, as final germination rates of seeds treated with different NaCl concentrations did not differ much from the results of the control. The differences were much more visible in the following phases of seedling development, so the phase of cotyledon opening was more informative. It would be interesting to prolong the experiment to see further development, but for such purpose the experiment design should be different.

According to USDA Agricultural Research Service, the threshold value that defines saline soils is 4 dS/m (Criteria for Diagnosing Saline and Sodic Soils 2006), which equals 4 000 µS/cm. Therefore, soil salinity of samples from both sampling locations was well below values that delineate non-saline soils from saline soils, whereas salinity of all of the tested NaCl solutions, even our lowest NaCl concentration (2.5 g NaCl/L), turned out to be above the aforementioned limit (except distilled water as control treatment).

Regardless of the negative effect of elevated salinity level on the two *Dittrichia* species, our results have proven that both species, false yellowhead as well as stinkwort, are very tolerant regarding salinity. Their seeds were able to germinate and also mostly normally further develop in a remarkably wide range of NaCl concentrations, spanning from as low as 0 to the extreme 10 g NaCl/L (≈ 171 mM NaCl), while the majority of known plant species hardly grow or even cannot survive in such high salinity level as 100–200 mM NaCl (Carillo et al. 2011). Hence, according to our measurements of electrical conductivity, both *Dittrichia* species managed to handle even more than astonishing 200 times higher values of electrical conductivity compared to those that occur on their usual growing sites.

Conclusions

Apparently, elevated salinity level cannot be considered as the main factor in determining the occurrence of stinkwort and false yellowhead. It is presumably only one of many different factors that can also act in a hostile manner towards most plant species on growing sites with higher salinity level. For this reason, the distribution pattern of both *Dittrichia* species in Slovenia could be due to little competition on their growing sites and also their plasticity, which enables them to thrive on generally less favourable sites with many limitations.

Povzetek

Smrdljiva ditrihovka (*Dittrichia graveolens*) in lepljiva ditrihovka (*D. viscosa*) sta vrsti iz družine nebinovk (*Asteraceae*), ki imata v slovenskem prostoru različen status glede izvora. Lepljivo ditrihovko obravnavamo kot domorodno vrsto, ki pri nas uspeva le v primorski regiji (Jogan et al. 2001, Wraber 2010) v bližini morske obale oz., kjer je vpliv morja še zaznaven, medtem ko so pojavljanje smrdljive ditrihovke v Sloveniji prvič zabeležili šele leta 2008. Vse od takrat se hitro razrašča le vzdolž avtocestnega križa (Frajman in Kaligarič 2009) ter ob nekaterih večjih prometnicah. Glede na opisana vzorca razširjenosti obeh

vrst smo domnevali, da obravnavani vrsti zelo dobro prenašata povišano slanost. K povišani slanosti na rastiščih lepljive ditrihovke najverjetneje prispeva morje, v primeru smrdljive ditrihovke pa se slanost tal poviša predvsem na račun zimskega soljenja avtocest. S to raziskavo smo skušali ugotoviti, kako povišana slanost vpliva na kalijovst in zgodnji razvoj kalic obeh vrst ditrihovk.

Izvedli smo kalitvene teste na agarnih ploščah. Kot testno vrsto smo poleg obeh vrst ditrihovk uporabili še solato kot pozitivno kontrolo. Pripravili smo štiri različne koncentracije NaCl vključno s kontrolo, kamor smo dodali le destilirano vodo (0, 2,5, 5 in 10 g NaCl/L). Izmerili smo tudi elektroprevodnost vzorcev tal z nahajališč obeh vrst ditrihovk.

Obe vrsti sta najbolje kalili v kontrolnem tretmaju (0 g NaCl/L). Višanje koncentracije NaCl je vse bolj zaviralo kalitev ter nadaljnji razvoj kalic. Določena semena do konca poskusa sploh niso vzklila, preostala pa so glede na kontrolo vzklila bistveno kasneje. Zaviralen učinek soli je bil nekoliko bolj opazen na smrdljivi ditrihovki. Kljub vsemu se je izkazalo, da sta obe vrsti ditrihovk zmožni kaliti in uspevati pri povišanih koncentracijah soli, ki so tudi dvestokrat višje kot na njunih rastiščih v naravi. Prav zaradi tako širokega razpona uspevanja pri različnih koncentracijah soli ju v odnosu do slanosti lahko obravnavamo kot zelo tolerantni vrsti. Iz rezultatov lahko razberemo, da na vzorec razširjenosti smrdljive in lepljive ditrihovke v Sloveniji lahko vpliva tudi povišana slanost tal, saj prisotnost višje koncentracije soli na rastišču zmanjša konkurenčnost drugih vrst, ki nimajo tolikšne tolerantnosti na prisotnost soli kot obe vrsti ditrihovk.

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The effects of leaf extracts of crack willow (*Salix fragilis*) on the growth of Japanese knotweed (*Fallopia japonica*)

Vpliv listnih izvlečkov krhke vrbe (*Salix fragilis*) na rast japonskega dresnika (*Fallopia japonica*)

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Abstract: Japanese knotweed (*Fallopia japonica*) is one of the most invasive of species in Europe, and can substantially reduce local native biodiversity. In the present study, the allelopathic potential of crack willow (*Salix fragilis*) on growth of Japanese knotweed was investigated. Aqueous extracts of 0.1% and 1% (w/v) were prepared from liophilised willow leaves and used for watering of young knotweed plants. Their growth was monitored for 196 days. Shoot height and leaf number were not affected but the mass of leaves and especially roots was reduced (up to 32%). At the end of experiment, biochemical characteristics related to physiological state (photochemical efficiency of PSII, protein content, enzyme activity of guaiacol peroxidase, lipid peroxidation) were measured. Mostly, they were at control levels, but the activity of guaiacol peroxidase and lipid peroxidation in roots increased. The extracts of crack willow showed moderate inhibitory effect on roots of treated knotweeds while the growth of shoots was unaffected. Given the root reduction described here, further studies with willow extracts and field studies with crack willow and Japanese knotweed plants would be reasonable.

Keywords: *Fallopia japonica, Salix fragilis,* invasive species, leaf extract, allelopathy, growth

Izvleček: Japonski dresnik (*Fallopia japonica*) sodi med najbolj inavzivne tujerodne vrste v Evropi in pomembno vpliva na zmanjševanje lokalne biodiverzitete. V raziskavi smo preučili alelopatski potencial krhke vrbe (*Salix caprea*) na rast japonskega dresnika. Iz liofiliziranih listov vrbe smo pripravili 0,1 % in 1 % vodne izvlečke, s katerimi smo zalivali mlade rastline dresnika in spremljali njihovo rast 196 dni. Višina poganjkov in število listov sta bila podobna kot v kontrolnem tretmaju, medtem ko se je masa listov, predvsem pa korenin, močno zmanjšala (do 32 %). Ob koncu poskusa smo z biokemijskimi analizami (fotokemična učinkovitost FSII, vsebnost beljakovin, encimska aktivnost gvaiakol peroksidaze, lipidna peroksidacija) ocenili še fiziološko stanje rastlin. Večina izmerjenih lastnosti je bila na kontrolni ravni, razen peroksidazne aktivnosti in lipidne peroksidacije, ki sta se pri tretiranih rastlinah povečali. Izvlečki krhke vrbe kažejo zmeren alelopatski učinek na korenine tretiranih dresnikov, medtem ko so poganjki rasli neprizadeto. Glede na opisano zmanjšanje koreninskega sistema

predlagamo nadaljnje raziskave z izvlečki vrbe in terenske raziskave o vplivu krhke vrbe na rast japonskega dresnika.

Ključne besede: japonski dresnik, krhka vrba, invazivne vrste, listni izvleček, alelopatija, rast

Introduction

Japanese knotweed (*Fallopia japonica* (Houtt.) Ronse Decr., *Polygonaceae*) is among the 100 most invasive taxons in the world (Lowe et al. 2000) and is a well known and problematic invasive species in Slovenia, too. It grows in dense populations mainly in ruderal habitats and river banks. The main strategy for its spread in invaded regions is vegetative reproduction with rhizomes and stolons and high level of regeneration (Bailey et al. 2009). Sexual reproduction outside its natural range is limited but seed formation and germination were proved for Slovenian populations (Strgulc Krajšek and Dolenc Koce 2015). So far, the mechanical removal and frequent cutting of knotweed plants have been the most common and efficient ways to limit their growth and spread. For the biological control of invasive plant species, allelopathy could be a potential mean. Allelopathy refers to chemical interactions among plants, including those mediated by microorganisms (Weston and Duke 2003) and is defined as supression of the growth and/or establishment of neighbouring plants by chemicals released from a plant or plant parts (Inderjit et al. 2011). These allelochemicals could be synthesized in all plant tissues and released in environment by leakeage, root exudates, evaporation and degradation of organic material (e.g. decomposed leaf litter). Majority of allelochemicals are secondary compounds; phenolic substances, flavonoids, terpens, alcaloids, steroids and function also in antimicrobial protection. Many plant species, including weeds and crops, have allelopathic activity, the most known and well studied are *Juglans nigra, Ailanthus altissima, Alliaria petiolata, Centaurea maculosa* (Weston and Duke 2003). Allelopathy could also be the mechanism which enables invasive plant species successful colonisation of new habitats; a mechanism known as a novel weapons hypothesis (Bais et al. 2003, Callaway and Aschehough 2000, Callaway et al. 2005, Hierro and Callaway 2003, Inderjit et al. 2011). Some studies on the allelopathic potential of knotweeds have already been carried out and biologically active compounds have been defined in this context (Dommanget et al. 2014, Fan et al. 2010, Gerber et al. 2008, Murrell et al. 2011, Vrchotová et al. 2007).

As previously mentioned, river banks are often overgrown by Japanese knotweed and the same habitat type is characteristic for some species of willow (*Salix*). Willows have high physiological and ecological plasticity, they grow rapidly and can accumulate metals, and for these reasons they are commonly introduced in ecosystems for phytoremediation of degraded habitats (Alvarez et al. 2003, Kuzovkina and Quigley 2005, Prach and Pyšek 2001). However, negative effects of willows on neighbouring plants (inhibited growth) were also reported due to shading, competition for mineral nutrients and allelopathy. At least 12 phenolic allelochemicals were detected in leaves of goat willow (*Salix caprea*) (Ikonen et al. 2002, Hallgren et al. 2003, Moohammadnor et al. 2010). In some areas, crack willow (*Salix fragilis*) is even considered invasive (Cremer 2003). It affects river ecosystems by releasing secondary compunds and organic matter (leaves, cork, fruits), shading, which all changes nutrient cycling, food chains and biodiversity (Groninger and Bohanek 2000, Doody and Benyon 2011). It has high level of regeneration and small fragments of shoots are dispersed by water to new locations where they quickly regenerate and start a new population (Budde et al. 2011). Leaf composition can also account for allelopathic potential of crack willow; leaves are compact and degrade slowly relasing tannins and phenolic compounds for a longer time (Julkunen-Tiitto 1985, Haapala et al. 2001).

The aim of the present study was to evaluate the allelopathic potential of crack willow on invasive Japanese knotweed. Developmental

changes during growth of young knotweed plants were observed and their physiological state was determined by measuring photochemical efficiency of PSII, protein content, activity of antioxidative enzyme guaiacol peroxidase and lipid peroxidation.

Materials and methods

Plant materials

The mature leaves of crack willow (*Salix fragilis* L.) were collected in Ljubljana, Slovenia (46° 3′ 55.63″ N, 14° 27′ 42.53″ E) in October. Following their collection, the leaves were lyophilised and stored in dark at room temperature until the extracts were prepared.

Young shoots of Japanese knotweed (*Fallopia japonica* var. *japonica* (Houtt.) Ronse Decr.) were collected in Ljubljana, Slovenia (46° 2′ 59.84″ N, 14° 28′ 28.52″ E) in April of the following year. The collected shoots had approx. 3 cm high aboveground stem and 2 cm long underground rhyzome with at least one stem bud. Each shoot was planted in a separate pot filled with mineral substrat vermiculite. The pots were kept in a climate chamber under control conditions of temperature (25 ±1 °C), humidity (40%), and light/ dark cycle (16h/8h; light intensity 160 μM $m^{-2}s^{-1}$) for 2 months. After that, the plants were transplanted to pots with commercial garden soil for additional 4 months (196 days in total) and grown under laboratory conditions of ambient temperature, humidity and light intensity.

Preparation of willow leaf extracts

The lyophilised mature willow leaves were homogenised using a mortar and pestle. Following suspension of the ground leaves in distilled water (5 g leaves in 100 ml distilled water), the extraction was carried out for at least 24 h on a shaker (170 rpm; room temperature). The extracts were then vacuum filtered, once with filter paper for general use and twice with fine filter paper (388 Grade, 84 g/m^2) to provide the 5% (w/v) aqueous extract that was frozen at -20 °C until needed. Defrosted extract was diluted in distilled water to give the final concentration of 0.1% and 1% (w/v). The extracts of final concentration were prepared fresh prior to watering the knotweed plants.

Measuring of Japanese knotweed growth

The knotweed plants were watered with distilled water as the control (N=5), and with the 0.1% and 1% aqueous willow extracts (N=10 for each treatment). In the first two months, the plants were watered with willow extracts once a month and in the following 4 months twice a month because their growth increased substantially. In the interim period, all knotweed plants were watered with distilled water every 3-4 days to prevent dehydration. Every week, the shoot height was measured and leaf number was counted to evaluate the growth dynamics. After 196 days of observation, the photochemical efficiency of the photosystem II was measured, and roots and shoots were separated, weighed, frozen in liquid N_2, and stored at -20 °C prior to biochemical analyses.

Photochemical efficiency

The photochemical efficiency of PSII was measured on the 2nd or 3rd youngest leaf of the same size using modulated fluorometer PAM 2100 (Walz, Germany) according to Germ et al. (2005). After 15 min dark adaptation, leaves were illuminated with a saturating beam of white light (photosynthetic photon flux density = 8000 μmol m^{-2} s^{-1}, 0.8 s) to excite the fluorescence of chlorophyll *a* and the optimal quantum yield (F_v/F_m) was detected. The effective quantum yield of PSII was measured by providing a saturating pulse of white light (PPFD = 9000 μmol m^{-2} s^{-1}, 0.8 s) using a standard 60° angle clip.

Biochemical analyses

Samples of ~100 mg roots and shoots/ leaves were homogenised in 1.5 ml of potassium phosphate buffer (100 mM, pH 7), centrifuged (20 817 g, 20 min, 4 °C) and the resulting supernatants were used to spectrophotometrically (UV-1800 Shimadzu) determine the protein concentration (BCA Protein Assay Kits, Novagen), specific enzyme activity and MDA content (as the measure of lipid peroxidation), as previously described (Dolenc Koce et al. 2014) and as indicated briefly below.

The activity of guaiacol peroxidase (G-POD; EC 1.11.1.7) was measured at 470 nm (e = 26.6 $mM^{-1} \cdot cm^{-1}$). The reaction mixture contained 900 µl potassium phosphate buffer (50 mM, pH 7) with 1% guaiacol and 10 mM H_2O_2, with the addition of 100 µl of the supernatant samples described above.

Lipid peroxidation was evaluated in terms of the content of malondialdehyde (MDA). Here, 200 µl of the supernatant samples obtained for the protein extraction described above was added to 800 µl acetic reagent (0.5% (w/v) thiobarbituric acid in 20% (w/v) trichloroacetic acid). The mixture was incubated for 30 min at 95 °C, and then chilled to stop the reaction. The MDA content was measured spectrophotometrically at 532 nm and 600 nm (Dolenc Koce et al. 2014).

Statistical analysis

Means and standard errors were calculated, and the samples were compared by t-test and ANOVA (Microsoft Excel, GraphPad Prism 3.02). The level of significance was set at $p < 0.05$.

Results

During the experiment which lasted cca. 6 months, some knotweed plants did not survive and final number of plants for statistical analysis was 4 for control, 8 for 0.1% extract and 10 for 1% extract treated plants. At the end of experiment, the roots of knotweed plants that were treated with willow leaf extracts were more affected than the aboveground stems and leaves. Shoot height and leaf number were at all measuring points similar as in control plants (Fig. 1). In case of 1% extract, the shoots were on average even heigher but the difference was not statistically significant.

The root growth was evaluated at the end of the experiment by weighing the mass of the root system and was 21% and 32% lower in knotweed plants treated with 0.1% extract and 1% extract, respectively (Fig. 2). Despite the substantial decrease, the differences were not statistically significant ($p = 0.148$). Similar, but less inhibitory effect was observed with the total leaf mass which was approx. 20% lower in treated plants ($p = 0.402$).

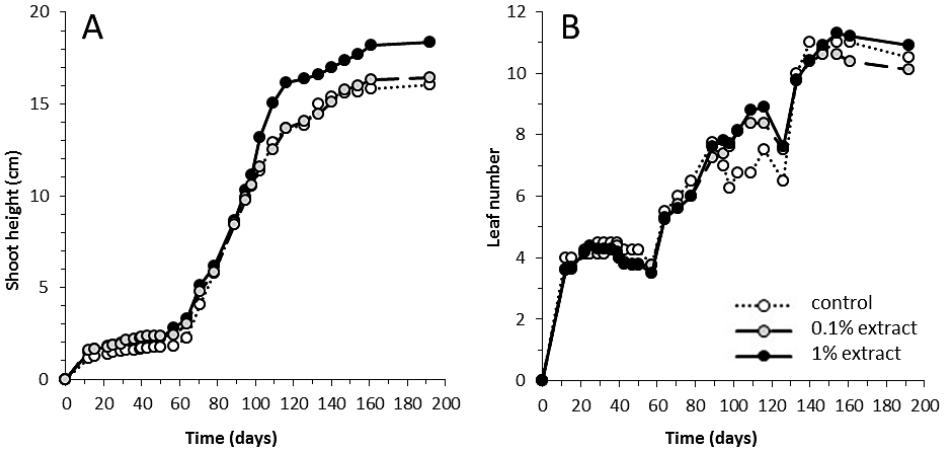

Figure 1: Growth of knotweed plants treated with willow leaf extracts. **A** – shoot height, **B** – leaf number. Data are means (n = 4 for control, 8 for 0.1% extract and 10 for 1% extract treatment).

Slika 1: Rast japonskega dresnika, tretiranega z listnimi izvlečki krhke vrbe. **A** – višina poganjka, **B** – število listov. Prikazane so povprečne vrednosti (n = 4 za kontrolo, 8 za tretma z 0,1 % izvlečkom in 10 za tretma z 1 % izvlečkom).

Long-term treatment with willow extracts showed diverse and mainly statistically insignificant effects at the biochemical level (Tab. 1). Protein content increased up to 23% in roots and decreased up to 21% in leaves, the specific enzyme activity of G-POD incresed in all investigated tissues with maximal (313%) increase in roots, treated with 0.1% extract (ANOVA for G-POD in roots; $p = 0.012$). Lipid peroxidation, estimated as MDA content, was up to 76% higher in roots (ANOVA; $p = 0.119$) and up to 15% lower in leaves (ANOVA; $p = 0.084$). The increase of lipid peroxidation in roots correlates with decreased root biomass caused by treatment with willow extracts (Fig. 3). The negative correlation between these two parameters was higher (Pearson correlation coefficient $r = -0.6353$ and -0.1669 for 0.1% and 1% treatment) than in roots and leaves of control plants (for all root data $r = -0.3562$ and for all leaf data $r = 0.0285$).

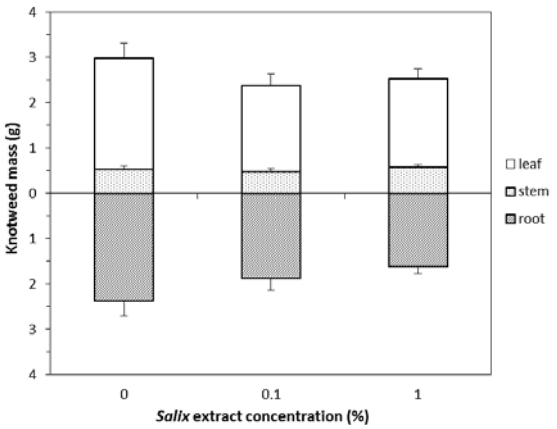

Figure 2: Mass of leaves, stem and roots of knotweed plants treated with willow leaf extracts. Data are means ± SE (n = 4 for control, 8 for 0.1% extract and 10 for 1% extract treatment).

Slika 2: Masa listov, stebla in korenin japonskega dresnika, tretiranega z listnimi izvlečki krhke vrbe. Prikazane so povprečne vrednosti ± SN (n = 4 za kontrolo, 8 za tretma z 0,1 % izvlečkom in 10 za tretma z 1 % izvlečkom).

Relatively good physiological state of leaves was confirmed also by results of photochemical efficiency of PS II, expressed as optimal quantum yield (F_v/F_m) which was not affected when willow extracts were applied (Tab. 1).

Discussion

Biological suppression of growth by allelopathic interactions is a possible mechanism to control and reduce spread of invasive plant species. Among most noxious species is Japanese knotweed which grows over river banks and anthropogenically degraded habitats and reduces biodiversity (Bailey et al. 2009). In the present study, leaf extracts of crack willow were used as a source of potential allelochemicals because both species grow in the same habitat type and share growing conditions. When young knotweed plants were watered with willow extracts of two concentrations (0.1 and 1%), the mass of root system decreased and its physiological state was disturbed which was confirmed by increased biochemical parameters associated with oxidative stress. The activity of guaiacol-peroxidase which is the enzyme involved in the hydrogen peroxide degradation increased for more than 3-fold in roots and the content of malondialdehyde which is a product of peroxidation of membrane lipids increased up to 76%. Nevertheless, the aboveground shoots were less affected; the shoot height, leaf number,

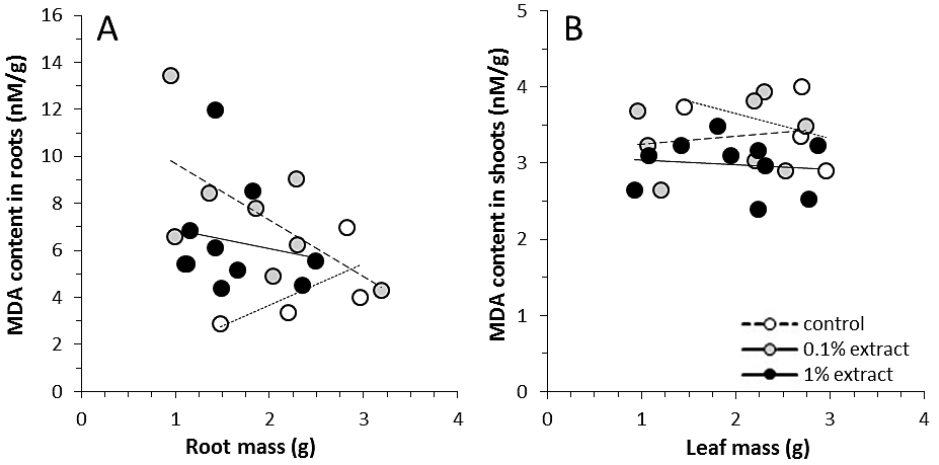

Figure 3: Correlation of malondialdehyde (MDA) content with mass of (A) roots and (B) leaves of knotweed plants treated with willow leaf extracts. Data are individual measurements per treatment (n = 4 for control, 8 for 0.1% extract and 10 for 1% extract treatment). Trendlines are linear regression lines.

Slika 3: Korelacija med vsebnostjo malondialdehida (MDA) in maso (A) korenin in (B) listov pri japonskem dresniku, tretiranem z listnimi izvlečki krhke vrbe. Prikazane so posamezne meritve za tretma (n = 4 za kontrolo, 8 za tretma z 0,1 % izvlečkom in 10 za tretma z 1 % izvlečkom). Trendne črte so linearne regresijske premice.

Table 1: Biochemical analysis and photochemical efficiency of knotweed plants treated with willow leaf extracts.

Tabela 1: Biokemijska analiza in fotokemična učinkovitost japonskega dresnika, tretiranega z listnimi izvlečki krhke vrbe.

	Control	0.1% extract	1% extract
Roots			
Protein concentration (µg/ml)	896.78 ± 168.87	982.94 ± 112.19	1102.68 ± 119.34
G-POD (µM/min*mg)	29.26 ± 3.22	**120.96 ± 22.58***	63.35 ± 10.35
MDA (nM/g)	4.31 ± 0.92	7.60 ± 1.01	6.39 ± 0.73
Leaves			
Protein concentration (µg/ml)	2086.26 ± 343.73	1739.17 ± 259.02	1639.57 ± 205.50
G-POD (µM/min*mg)	85.68 ± 19.67	151.35 ± 24.02	127.93 ± 14.00
MDA (nM/g)	3.50 ± 0.24	3.34 ± 0.16	2.98 ± 0.11
F_v/F_m	0.82 ± 0.01	0.81 ± 0.01	0.81 ± 0.00

Data are means ± SE (n = 4 for control, 8 for 0.1% extract and 10 for 1% extract)

G-POD, specific enzyme activity of guaiacol peroxidase; MDA, malondialdehyde content; F_v/F_m, optimal quantum yield
Bold *, statistically significant difference between treated and control plants (t-test; $p < 0.05$).

photochemical efficiency and lipid peroxidation were at control levels. On the other hand, the mass of leaves decreased and the activity of G-POD increased but the differences were not statistically significant. Different effects of *Salix caprea* leaf extracts were reported previously. The reduction of root mass was proved for seedlings of *Picea abies* treated with *Salix caprea* extracts (Schütt and Blaschke 1980). Also decreased germination of *Arrhenaterum elatius, Lotus corniculatus* and *Plantago lanceolata* was reported when seeds were directly treated with leaf litter of *S. caprea* but the effect was less inhibitory or even stimulatory when litter was added to substrate improving its quaility (Mudrak and Frouz 2012).

The inhibition of growth can be related to some methodological problems as well. It was shown that type of substrate can influence the plant growth (Mudrak and Frouz 2012); when seedlings of tested plant species grew in sand, the allelopathic effects of willow were more pronounced because the sand has lower absorption capability and chemical interactions between compounds in plant extracts and sand are less intense than in soil. The same effect was observed in our study; when knotweed plants grew in mineral substrate vermiculite first 2 months, their growth was slow. When they were transferred to soil, their growth increased but the change was also related to different temperature, humidity and light conditions. To prove allelopathic potential of an extract, activated carbon can be added to the substrate to absorb chemicals (Prati and Bossdorf 2004). Also the use of distilled water for control could have negative effects on plants. The practical experiences show that watering with distilled water results in weaker growth than watering with tap water which contains higher level of minerals.

The reduction of the underground tissues of Japanese knotweed could be important mechanism for limiting its growth and spread because rhyzome and stolons are the prime structures that enable knotweed successful vegetative reproduction (Bailey et al. 2009). To prove allelopathic potential of crack willow for biocontrol of Japanese knotweed further studies are necessary using willow extracts of higher concentrations, root extracts, more frequent watering with extracts and field studies with co-growth of knotweed and willow plants in the same substrate. The latter is of special importance because in natural conditions other factors can influence allelopathic potential of certain plant species. Soil biota and chemistry, abiotic factors related to seasonal differences and neighbouring organisms (Inderjit et al. 2011) affect interactions among organisms therefore appropriate methodolgy for integrating chemically mediated interaction into ecology is crucial (Inderjit and Callaway 2003). In case of interactions between willows and knotweeds the allolopathic potential of knotweed to reduce willow growth should also be considered and tested.

Conclusions

Long-term experiments are important way to study allelopathic effects because they simulate conditions in natural environment. Our study shows moderate allelopathic effect of leaf extracts of crack willow to roots of Japanese knotweed while shoots developed unaffected. In roots, biomass decreased over 6 months of growth in the presence of willow extracts and biochemical characteristics related to oxidative stress elevated.

Povzetek

Japonski dresnik (*Fallopia japonica*) sodi med 100 najhujših invazivk v svetovnem merilu (Lowe et al. 2000). Njegova hitra rast in vegetativno razmnoževanje s korenikami in stoloni mu omogočajo uspešno naseljevanje novih habitatov, kjer zaradi svoje invazivnosti izpodriva avtohtone vrste in spreminja ekosisteme ter povzroča gospodarsko škodo. Njegovo odstranjevanje je večinoma kemično, kar lahko povzroča negativne posledice na ostale rastline in okolje na splošno, in mehansko. Eden od možnih biotičnih načinov zatiranja bi lahko bila alelopatija, tj. negativen vpliv ene rastline na drugo preko delovanja alelopatskih spojin, ki jih rastlina izloča ali sprošča v okolje in vključuje tudi delovanje preko mikroorganizmov, povezanih z rastlinami.

V raziskavi smo želeli ugotoviti, ali vodni izvlečki iz listov krhke vrbe (*Salix fragilis*) vplivajo na rast in razvoj japonskega dresnika. Vrbe so vrste, ki se pogosto uporabljajo v poskusih stabilizacije in obnove uničenih ekosistemov. Poleg vlagoljubnih

vrst najdemo med njimi tudi hitro rastoče pionirske rastline zgodnjih sukcesijskih faz, ki naseljujejo nove, s pomočjo človeka nastale odprte površine, torej podobna rastišča kot japonski dresnik. Poleg tega je zanje značilen tudi alelopatski potencial, ki skupaj s senčenjem in tekmovanjem za hranila zavira rast podrasti.

Iz liofiliziranih listov krhke vrbe smo pripravili 0,1 in 1 % vodni izvleček, s katerim smo 1 do 2-krat mesečno zalivali mlade rastline japonskega dresnika, ki smo jih posadili v vermikulit in zemljo. Njihovo rast in razvoj smo spremljali 196 dni.

Ugotovili smo, da so izvlečki zavrli predvsem razvoj korenin, kar se je pokazalo z zmanjšanjem njihove mase, povečale pa so se značilnosti, povezane z oksidativnim stresom, tj. lipidna peroksidacija, ocenjena kot vsebnost malondialdehida, in aktivnost antioksidativnega encima gvajakol peroksidaze. Poganjki so bili manj prizadeti, saj so bili višina poganjka, število listov, fotokemična učinkovitost FS II in lipidna peroskidacija na kontrolnih vrednostih. Zmanjšala se je masa listov, povečala pa aktivnost gvajakol peroksidaze, vendar so bile razlike statistično neznačilne.

Slabša rast korenin zaradi delovanja izvlečkov iz listov krhke vrbe predstavlja temelj za nadaljnje raziskave, v katerih bi bilo potrebno preučiti delovanje koreninskih izvlečkov, izvlečkov z višjo koncentracijo, pogostejše zalivanje z izvlečki, ter ugotoviti kako vplivajo na rast japonskega dresnika vrbe, ki bi rastle skupaj z njimi v istem substratu. S tem bi lahko potrdili alelokemijsko delovanje krhke vrbe in njen potencial za biološko kontrolo invazivnih tujerodnih rastlin.

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Control of alpine dock (*Rumex alpinus*) by non-chemical methods

Trajnostno odstranjevanje alpske kislice (*Rumex alpinus*)

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Abstract: Alpine dock (*Rumex alpinus*) is a troublesome weed particularly in protected zones or Natura 2000 areas, in which only non-chemical control measures can be applied. The aim of our study was to investigate by means of a field experiment the effectiveness of various non-chemical methods: mowing, manual removal, heating, foil and grazing by cattle and pigs. Floristic changes, cover, number of shoots and biomass were monitored at 14-day intervals for three consecutive years. Manual removal and foil were most successful, with almost complete removal of the biomass and cover of alpine dock, and mowing, which reduced the cover to 50%. Other methods were not as efficient. Animals avoid grazing on *R. alpinus* and heat merely supresses dock growth for a short period.

Keywords: Slovenia, mountain pastures, agricultural management strategies, weed control, *Rumex alpinus*

Izvleček: Alpska kislica (*Rumex alpinus*) je problematičen plevel gorskih pašnikov, še posebej v zaščitenih območjih ali območjih Natura 2000, kjer lahko uporabimo le nekemične metode zatiranja. S poljskim poskusom smo testirali učinkovitost različnih nekemičnih metod odstranjevanja alpske kisice: košnjo, ročno odstranjevanje, toploto, folijo in pašo goveda oz. prašičev. V 14- dnevnih obdobjih smo tri vegetacijske sezone spremljali floristične spremembe, pokrovnost, število poganjkov in biomaso. Najbolj uspešni sta bili metodi ročnega odstranjevanja in folija, kjer smo biomaso in pokrovnost kislice skoraj popolnoma odstranili, s košnjo pa smo ju zmanšali za polovico. Ostale metode niso bile tako uspešne. Živali se izogibajo alpski kislici, z ožiganjem pa smo rast kislice zaustavili le za krajši čas.

Ključne besede: Slovenija, gorski pašniki, kmetijsko-upravljalske strategije, zatiranje plevelov, *Rumex alpinus*.

Introduction

Alpine dock (*Rumex alpinus* L.) is a perennial species consisting of a horizontal rhizome (and a root up to 300 cm long), above-ground vegetative shoots with three to five big leaves, and fertile stalks bearing smaller leaves and up to several thousand flowers and fruits (Kutschera and Lichtenegger 1992, St'astna et al. 2010). *R. alpinus* is a common plant species in all mountains of western, central

and eastern Europe, including the Apennines, the mountains of the Balkan Peninsula and the Caucasus (Meusel et al. 1965). It builds species-poor monodominant stands and the cover of *R. alpinus* is often close to 100% (Kliment and Jarolimek 1995). The species is strongly nitrophilous and grows on nutrient-rich soils frequently near farm buildings. A high content of plant available potassium and nitrogen in soil favours *R. alpinus* (Bohner 2005). It can be regarded as a pasture weed, dominating and reducing valuable pasture areas. *R. alpinus* (as many grassland weeeds) has several harmful characteristics: low nutritive value, noxious (high oxalic acid content), avoided by animals, not suitable for conservation and high competitors occupying large areas (Bohner 2005, Dietl 1982, Vasas et al. 2015)

Docks (*Rumex* spp.) are very troublesome weeds in agricultural land in many countries, in arable crops or permanent grasslands (Jeangros and Nosberger 1990), for short period only (Niggli et al. 1993).

Non-chemical control of docks (*Rumex* spp.) has become important in recent years, mainly because of an increase in organic farming, and several non-chemical methods are applied: biological, mechanical and cultural. These consist of: frequent cutting, mechanical removal, heating, use of predators and parasites, grazing (Hejcman et al. 2014, Van Eekeren et al. 2006, Zaller 2004 and references cited there). The main aim is to hinder the build-up of seeds and weaken regrowth capacity by destroying biomass (Zaller 2004).

In addition to organic farming, agriculture in Natura 2000 areas (in lowlands and mountains) requires management practices that do not rely on the use of herbicides and are beneficial for the conservation of species, habitats and the environment. In mountainous areas, agriculture is mainly Alpine dairy farming, with the animals grazed over the summer. Mountain pastures include a significant fraction of the plant species pool of Alpine regions (Bätzing 1991), and well-managed pastures can support species-rich plant communities of high conservation value (Spatz 1975).

Mountain pastures were often not properly managed in the middle of last century. One threat is abandonment or low management intensity, which leads to secondary succession and overgrowing of areas with shrubs and trees. The other is intensification by inappropriate grazing practices or use of fertilisers, which results in eutrophication and weed invasion (Galvánek and Janák 2008). The latter is responsible for the invasion of nitrophilous plant species (and *R. alpinus* as the most significant) and communities, which can spread over large areas and are not useful as pastures and farmers are not entitled to subsidies from agri-environmental-climate packages within the current Slovenia's Rural Development Programme (Dular et al. 2013). Another impact of alpine dock spreading in protected areas is a reduction of species diversity and the endangerment of rare plants (St'astna et al. 2010).

The aim of our study was to investigate the use of various non-chemical methods on the control of *R. alpinus* in a mountain pasture under field conditions. To the best of our knowledge, only two field experiments for the control of *R. alpinus* have been set up without the use of herbicides, but with fewer methods applied (Corradini and Artigianelli 1991) or monitoring was done only for one season (Tsarik 1987).

Methods

Study site

The plot experiment was established in stands with 100% cover of *R. alpinus* on the high mountain pasture Korošica (northern Slovenia) at an elevation between 1500 m – 1570 m (46.434348 N, 14.291490 E). Korošica is a part of the Karavanke Natura 2000 area (SI3000285). There are 56 ha of pasture land on Korošica and, according to the state prescribed pasture grazing order, 80 animals (cattle and horses) or 65 LU can graze from mid-June till mid-September. Alpine dock has spread abundantly, presumably due to excess stock or the use of mineral fertilizers during the last ca. 20 years, and now occupies 9% of the grazing area (Dular et al. 2013).

The climate is cool and humid (Ogrin 1996). Average annual precipitation is 1680 mm (Podljubelj meteorological station), and average annual temperature is 3.6 °C (Krvavec meteorological station) (Anonymous 2014).

Grassland vegetation on Korošica is classified as *Homogyno alpinae-Nardetum* Mráz 1956 – mat-grass acidophilous pastures of submontane to supramontane belts of mountain ranges and is a priority habitat type (Annex I habitat type 6230).

Soil analysis was done before the set-up of the experimental plots in 2012. The analysis showed a strongly acidic soil (pH 4.2) with a low content of phosphorus (P: 20.18 mg/kg^{-1}) and sufficient content of potassium (K: 283.79 mg/ kg^{-1}). Soil samples were analysed by Agrochemical laboratory of the Agricultural Institute of Slovenia.

Experimental design

Testing of sustainable removal of *R. alpinus* on Korošica lasted three vegetation seasons (2012–2014). The removal experiment was set up as a random block on 4 x 4 m test plots (in 4 replicates) and two 10 x 15 m plots (in single versions). Various methods were tested: mowing, flaming, foil cover, manual removal and grazing (cattle and pigs).

For *Mowing*, *Heat*, *Foil* and *Manual*, we used four replicates, while *Cattle* and *Pigs* were only tested in the first year, and using a larger fence, because of the difficulty of using the animals (we were unable to set larger plots and the number of animals was limited) and the remote location of the Alpine dairy farm for transport. Heat treatment was done with an open flame and plants were flamed until foliage was burned down. Plots were mowed every 14 days (during the vegetation season), starting mid-June (2012), and the biomass was removed. Docks were manually excavated in the first year and the roots were collected and removed from the plots. Black polyethylene foil was installed permanently for two seasons. Animals were enclosed in the two larger plots: cattle grazing – 6 cattle / 2h a day / through the whole season and pigs rooting – 2 pigs for 4 weeks. In the case of pigs, we used the only autochthonous breed in Slovenia (Krškopolje pig), which is adapted to be kept outdoor.

After treatment of the plots, we sowed a commercial grass seed mixture (*Trifolium repens* 5%, *Phleum pratense* 16%, *Lolium perenne* 79%, a product of Semenarna (Ljubljana). Commercial seed mixture was used because site-adapted mixtures were not available.

Data collection

Vegetation was sampled according to the Braun-Blanquet (1964) method on 9 m^2 plots. Relevés were made prior to the start of management and at the end of every vegetation season, after the different removal treatments.

All data were sampled prior to the start of the experiment, except for biomass, and repeatedly at the end of every vegetation season. Above ground biomass was sampled on two subplots (0.5 x 0.5 m) within every sampling plot and only *R. alpinus* plants were cut. The biomass was air dried and then kept at 104 °C for 24^h and weighed. Every 14 days, the height, cover estimation and number of shoots were sampled on two 1 m^2 subplots. Cover was visually estimated in percentages. An individual shoot was defined as a group of leaves that clearly formed a separate shoot, although not necessarily from a different root.

Data analysis

All data were tested for normality and homogeneity of variance prior to testing the differences. ANOVA and Non-parametric tests were used in STATISTICA (StatSoft 2007).

The floristic composition was compared in a matrix (plots by species) arranged in JUICE (Tichý 2002). De-trended correspondence analysis (DCA) was done in Canoco (ter Braak and Šmilauer 2002). Species cover values in percentages were square root transformed.

Results

The association *Rumicetum alpini* Beger 1922 was fully developed before the experiment, with characteristic species *R. alpinus* and *Stellaria nemorum* dominating and with 100% cover of the site. Stands were species poor, with a few nitrophilous species (*Urtica dioica*) and species indicating of periodically wet soil being present (*Deschampsia cespitosa*, *Ranunculus repens*).

Changes in floristic composition differed among treatments. The greatest changes in species composition were in plots covered by foil and those subjected to manual excavation (Fig.

1). Dock plants were completely removed there and replaced with grassland species (*Lolium perenne*) from seed mixture. Changes were gradual on mowed plots, where species turnover was slower and alpine dock covered half of the plot after three years, although grasses were already developed. Other plots had a similar position on the DCA ordination graph, indicating smaller changes after treatments (Fig. 1).

The species number per plot after three years (results not shown) increased in all treatments except for *Foil* but the highest increase was on mown plots.

Comparisons within treatments showed significant differences in biomass between the first year and the following two for *Manual*, *Foil* and *Mowing*, while *Heat*, *Cattle* and *Pigs* did not differ from *Control* (Fig. 2).

Differences in biomass calculated for the last sampling year 2014 (one-way ANOVA, Bonferroni test) showed that *Heat, Cattle* and *Pigs* did not significantly differ from *Control* plots. The treatments *Manual*, *Foil* and *Mowing* were significantly different from *Control* but were not significantly different from each other.

Changes in the cover of *R. alpinus* within each treatment show a significant reduction of cover for *Manual* and *Foil*, while *Mowing* significantly reduced cover only after the second year (Fig. 3). *Pigs* were very successful in cover reduction but *R. alpinus* recovered in the third year because the treatment lasted for only one year. There was a similar result with the *Cattle* treatment.

The height of the dock plants was already significantly lowered by the *Manual* and *Foil* treatments after the first year, while *Mowing* reduced it only in the last sampling year (Fig. 4). Other removal treatments were not successful in that.

The height of plants was also very variable among sampling years, since this plant trait is related to seasonal climatic variables that differed during the experiment (e.g., very dry 2013 and wet 2014 season).

The number of shoots was significantly lowered by *Manual, Foil* and *Mowing* (Fig. 4). The number also varied among years, since sampling of particular shoots is very subjective, although it was done by the same observer.

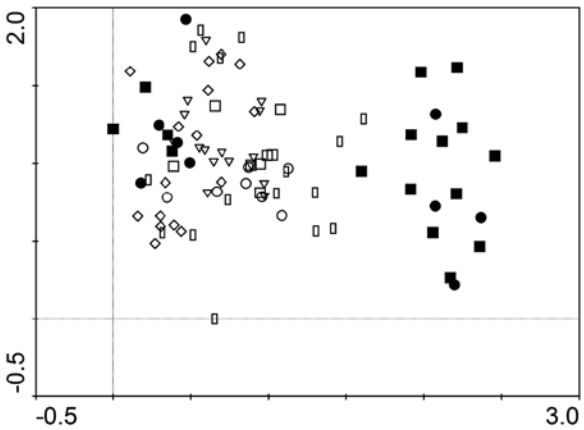

Figure 1: DCA analysis of vegetation plots. Eigenvalues for the first four DCA axes are 0.390, 0.171, 0.128 and 0.100, respectively. Foil-full circle, manual-full quadrat, mowing-rectangle, heat-triangle, control-diamond, pigs-empty circle, cattle-empty quadrat.

Slika 1: DCA analiza vegetacijskih ploskev. Lastne vrednosti prvih štirih osi so 0,390, 0,171, 0,128 in 0,100. Folija: poln krog, ročno odstranjevanje: poln kvadrat, košnja: pravokotnik, toplota: trikotnik, kontrola: diamant, prašiči: prazen krog, govedo: prazen kvadrat.

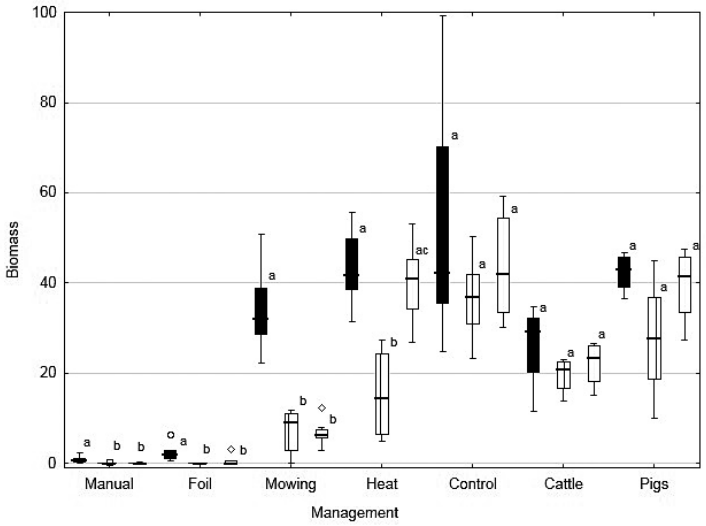

Figure 2: Box plot of biomass of *R. alpinus* on plots with different management in three consecutive years. Means with the same letter are not significantly different from each other (one-way ANOVA, P<0.05).

Slika 2: Škatla z brki biomase alpske kislice na ploskvah z različnim načinom zatiranja v treh zaporednih letih. Povprečja, označena z isto črko, med seboj niso statistično značilno različna (eno-fakorska ANOVA, P<0,05).

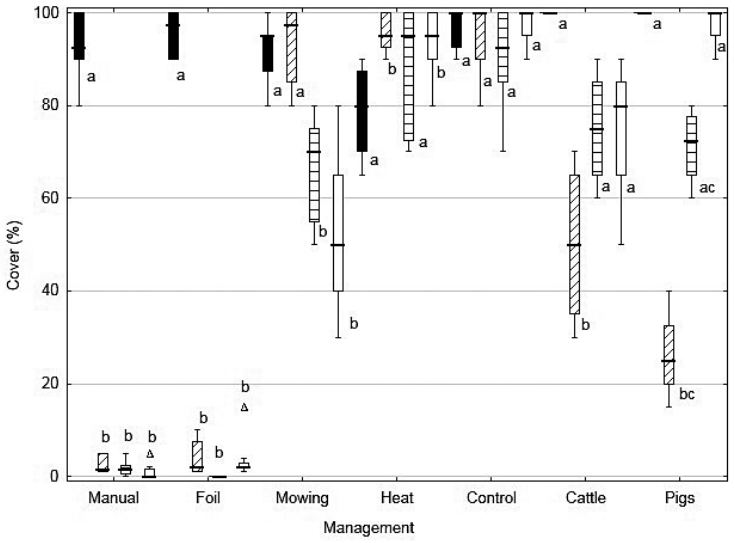

Figure 3: Box plot of cover of *R. alpinus* on plots with different management in three consecutive years. Means with the same letter are not significantly different from each other (one-way ANOVA, P<0.05).

Slika 3: Škatla z brki pokrovnosti alpske kislice na ploskvah z različnim načinom zatiranja v treh zaporednih letih. Povprečja, označena z isto črko, med seboj niso statistično značilno različna (eno-fakorska ANOVA, P<0,05).

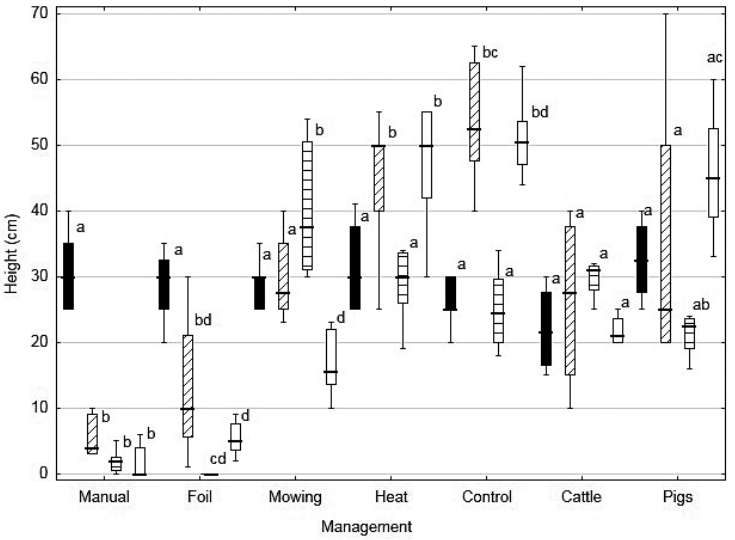

Figure 4: Box plot of height of *R. alpinus* plants in plots with different management in three consecutive years. Means with the same letter are not significantly different from each other (one-way ANOVA, P<0.05).

Slika 4: Škatla z brki višine alpske kislice na ploskvah z različnim načinom zatiranja v treh zaporednih letih. Povprečja, označena z isto črko, med seboj niso statistično značilno različna (eno-fakorska ANOVA, P<0,05).

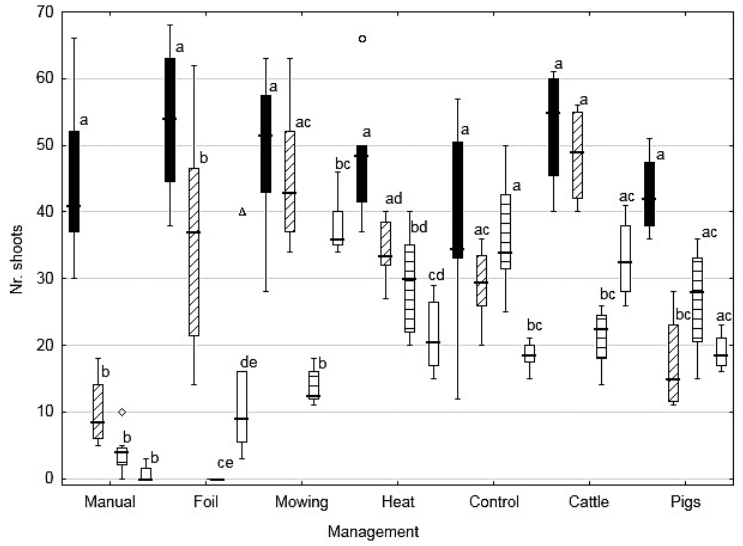

Figure 5: Box plot of number of shoots of *R. alpinus* in plots with different management in three consecutive years. Means with the same letter are not significantly different from each other (one-way ANOVA, P<0.05).

Slika 5: Škatla z brki števila poganjkov alpske kislice na ploskvah z različnim načinom zatiranja v treh zaporednih letih. Povprečja, označena z isto črko, med seboj niso statistično značilno različna (eno-fakorska ANOVA, P<0,05).

Discussion

Most research interest in floristic changes related to various management techniques has been oriented into changes of semi-natural grasslands in lowlands after changes in management or its cessation, and the impact of other *Rumex* species (*R. obtusifolius* and *R. crispus*). Studies dealing with *Rumex* spp. are in general dedicated to the removal of docks and less with the species composition of the plant community they invade. The only study monitoring floristic changes was made by Corradini and Artigianelli (1991).

Re-sowing of grasses was important to establish a new plant community and to prevent *R. alpinus* from growing again and to suppress its competitive ability. It is known that the seed bank has a limited role in the restoration of degraded sites (Handlova and Munzbergova 2006). The use of autochthonous seed mixtures of potential vegetation types or use of hay from an identical plant community would facilitate the succession but they were not available.

Although docks are troublesome weeds in grasslands, most control studies have been done on "lowland" species, e.g., *R. obtusifolius* and *R. crispus* (Van Eekeren et al. 2006, Zaller 2004). In addition, studies about alpine dock have often been partial, using particular methods (also chemical), or short term (see St'astna et al. 2010).

The largest reduction of *R. alpinus* was achieved by mowing, foil covering and manual excavation. Regular and frequent mowing has already been reported to influence *R. alpinus* (Corradini and Artigianelli 1991, Hujerova et al. 2013, St'astna et al. 2010, Tsarik 1987). The frequency of cutting is the most important and, not surprisingly, the more frequent the cutting the more effective is the dock suppression. In our study, we used cutting every 14 days since this period has already been shown to be effective (Tsarik 1987, Zaller 2004). The reduction of cover and biomass by mowing was very gradual compared to foil and excavation. This is congruent with the findings of Courtney (1985) that even five to seven cuts reduced the abundance of dock by only 60 %. When stands are mown less frequently, seedling emergence and seedling survival until next year increases (Tsarik 1987). Nevertheless, successful suppression is possible through regular mowing and removal of the biomass (Zaller 2004), although not in a short time (Pignatti and Pignatti 2014). Combination of mowing and grass seeding proved to be successful, which is congruent with the findings of Corradini and Artigianelli (1991). The competition of grasses and herbs is not enough to restrict docks in the long term (Zaller 2004) and should be combined with some other management. It is important to cut dock at a height of 10 cm to enable other plants to regenerate faster than *Rumex*.

Excavation successfully removed dock plants but it is a very time consuming method. Tillage is usually applied as the ultimate non-chemical control measure on heavy *Rumex* infestations on arable land, and also on grassland, although contrasting results are reported (see review by (Zaller 2004). We removed the upper soil layer with roots and since the rhizomes usually grow at a depth of up to 5 cm (Klimes 1992) or, less frequently, between 10-12 cm (Kliment and Jarolimek 1995), the removal of alpine docks was successful. It is necessary to remove and destroy the roots so they cannot regenerate. New plants can germinate from the remains of root fragments and from the seed bank (Tsarik 1987), so sowing grasses is important in order to suppress young plants that emerge. After three years, only a few small plants were present in the plots, identical to the results of Bucharová (2003), although she used herbicide.

The use of foil (or any other covering material) to reduce the light to weeds or any other invasive species is common practice (Bond and Grundy 2001) and it has been successfully applied to *R. alpinus* (Bechtold and Machatschek 2011). Light availability is a crucial resource in *R. alpinus* stands and control through competition for light should be successful (Zaller 2004). After one year, the docks are destroyed because of light reduction and high temperatures under the foil in the summer period. Foil is less suitable for large areas, especially in mountains with unfavourable climatic conditions.

Treatment with flame reduced the biomass but not the cover and the docks regenerated after the first year. Flame has been successfully used in dock infested grasslands but only on locally infested spots and on single plants (Pötsch 2003), while this method was less suitable in large patches of *R. alpinus,* similar to results in *R. obtusifolius*

(Zaller 2004). In the case of large patches, all plants were damaged by the heat and the herbs were unable to compete with the docks.

Rumex species are rarely grazed by animals and alpine dock is avoided by cattle and horses but readily eaten by goats (Bohner 2005, Ellenberg 1996, Hejcman et al. 2014) and has been used as pig fodder in the past (Wendelberger 1971). In our study, we used cattle and pigs for only one season and this resulted in some suppression of the alpine dock cover but probably more as a result of cattle trampling and tillage by pigs than grazing. Trampling can also reduce the *R. alpinus* above ground biomass (Tsarik 1987). Goats and sheep or combined with cattle graze on docks and effectively remove plants from grasslands (Hejcman et al. 2014) but in the case of mountain pastures in SE Central Europe, grazing of sheep and goats is not common or is traditionally limited to certain localities (part of NW Julian Alps) and the introduction of new animals would require a change in grazing policy related to NATURA 2000.

We must also stress that all grazing experiments from the literature were made in grasslands in which docks are scatter distributed, while alpine dock forms large monodominant stands and such patches are even more avoided by grazing animals.

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Gold nanoparticles do not induce adverse effects on terrestrial isopods *Porcellio scaber* after 14-day exposure

Nanodelci zlata nimajo negativnih učinkov na kopenske rake vrste *Porcellio scaber* po 14-dnevni izpostavitvi

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Abstract: Despite the anticipated environmental release of anthropogenic gold nanoparticles (AuNPs), there is currently not enough data on their potential impact on terrestrial environment. In the current study, we investigated the effects of considerably low concentrations of AuNPs on terrestrial isopods (*Porcellio scaber*) after 14 days of exposure. The effects on mortality, weight change, feeding rate, avoidance/preference feeding behavior, and cell membrane destabilization of digestive gland cells were followed. In parallel, the accumulation of Au in the digestive glands was measured. Our results show that none of the tested parameters was affected in isopods under given exposure doses (10 and 60 µg Au/g dry leaf) and exposure duration. No Au was assimilated in the digestive glands. Also, the same doses of the reference chemical, $AuCl_3$, showed no effect. We conclude that these concentrations of AuNPs are safe for terrestrial isopods. We encourage reporting the results showing no adverse effects of nanoparticles to balance the prevailing publication of their adverse effects. This will help to build a realistic public perception of the environmental risk of nanomaterials.

Keywords: nanoparticles, Au^{3+}, avoidance behavior, bioaccumulation, safety

Izvleček: Kljub naraščujoči uporabi nanodelcev zlata (ND Au) trenutno še vedno ni dovolj podatkov o njihovih potencialnih negativnih učinkih na kopenske organizme. V tej študiji smo proučevali vpliv relativno nizkih koncentracij ND Au na kopenske rake vrste *Porcellio scaber* po 14-dnevni izpostavitvi. Proučevali smo vpliv na smrtnost, maso živali, stopnjo prehranjevanja, izogibalno vedenje in destabilizacijo membrane celic prebavnih žlez. Izmerili smo tudi asimilacijo Au v prebavnih žlezah. Rezultati so pokazali, da testirane koncentracije ND Au (10 in 60 µg Au/g lista) pri kopenskih rakih

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niso vplivale na nobenega izmed testiranih parametrov po danem času izpostavitve. V prebavnih žlezah nismo izmerili Au. Enake rezultate smo dobili tudi z referenčno kemikalijo, soljo $AuCl_3$. Zaključujemo, da so testirane koncentracije ND Au varne za kopenske rake. Naše mnenje je, da se na področju nanotoksikologije trenutno objavlja predvsem rezultate, ki kakorkoli nakazujejo na negativni vpliv nanodelcev na organizme. Zato želimo stimulirati tudi objavo rezultatov, ki kažejo nasprotno. Na ta način bomo pripomogli k bolj uravnoteženemu in realističnemu mnenju javnosti o okoljski varnosti nanomaterialov.

Ključne besede: nanodelci, zlato, izogibalno vedenje, bioakumulacija, varnost

Introduction

Gold is a trace element in lithosphere, hydrosphere, and biosphere. It occurs naturally at concentrations around 4 µg/kg in the Earth's crust, <200 µg/kg in rocks, <2 mg/kg in soils, <1 µg/L in freshwaters and <5 µg/L in marine waters, while waters from auriferous deposits may contain up to 1 mg/L of gold. In living organisms, such as microorganisms from ore fields and marine invertebrates, gold was found at concentrations 30–750 µg/kg (Korobushkina et al. 1983). Gold has no known physiological functions and it is usually considered as inert in the elemental form, Au^0 (Sadler 1976, Eisler 2004). However, exposure to gold jewelry, gold dental restorations, gold implants, and beverages containing flake gold has been linked to contact allergy in susceptible individuals, mostly in the form of dermatitis. It has been proposed that dark discolorations of skin in contact with gold jewelry and dermatitis are associated with formation of the more reactive Au^+ and Au^{3+} species due to extra- or intracellular dissolution of Au^0, e.g. by sweat or in lysosomes (Rapson 1984, Eisler 2004).

Gold nanoparticles (AuNPs) – or the so-called "colloidal gold" – may be of either natural or anthropogenic origin. In the nature, AuNPs are formed by weathering of origin rocks or by microbial precipitaton in auriferous soils (Southam et al. 2009). Production of anthropogenic AuNPs has been increasing due to their use in consumer, industrial and medical products. AuNPs are used for numerous applications, ranging from biosensors to catalysts, electronics, cosmetics, and cancer treatment (Unrine et al. 2010). According to the Consumer Products Inventory, there are currently 25 products (out of 1331 in total) listed containing AuNPs, mostly dietary supplements and cosmetics (Project on Emerging Nanotechnologies, 2015, retrieved on June 21st, 2016). As a result of a variety of uses, environmental release of anthropogenic AuNPs is anticipated and there is a need of further research regarding the impacts of AuNPs on the environment. At the moment, no measured environmental concentration data for AuNPs are available. However, according to Mahapatra et al. (2015), the mean annual predicted environmental concentration of AuNPs in sludge is estimated at 0.120 and 0.150 µg/g for UK and US, respectively, and in sludge-treated soil at 300 and 150 ng/kg yearly for UK and US, respectively. Although yearly concentration is considerably low, it may increase due to continuous application over years.

The bioavailability of AuNPs and their effects on terrestrial invertebrates have been studied in earthworms (*Eisenia fetida*; Unrine et al. 2010, 2012), fruit flies (*Drosophila melanogaster*; Pompa et al. 2011; Sabella et al. 2011, Vecchio et al. 2012a, 2012b) and tobacco hornworms (*Manduca sexta*; Judy et al. 2010, 2012). However, for terrestrial isopod crustaceans, no data on bioavailability or effects of gold ions or AuNPs exists to date. Terrestrial isopods (*Porcellio scaber*) are suitable model terrestrial organisms for testing effects of chemicals, since they enable precise monitoring of the exposure dose together with its consequences on different levels of biological organization (Drobne 1997). Being hyperaccumulators of various metals (Hames and Hopkin 1989), they are especially convenient in studying the biological effects of metal salts and nanoparticles (Pipan-Tkalec et al. 2010, Golobič et al. 2012, Novak et al. 2012).

In isopods, the most sensitive biological endpoints for studying the effects of chemicals are biochemical, histological, and physiological (Drobne 1997). These endpoints can be simultaneously investigated in the digestive glands (hepatopancreas), which are in direct contact with the substances in food. Isopod digestive glands consist of four blind-ending tubes with intestinal, hepatic and pancreatic functions. The digestive gland epithelium is built of two cell types: larger B cells with secretive and absorptive functions, which contain lipid droplets and glycogen, and smaller S cells, which predominantly accumulate metals (Hames and Hopkin 1989). After consumption of metal salt- or NP-spiked food, the digestive gland epithelium can be often found containing elevated concentrations of metal ions accumulated in storage granules (Pipan Tkalec et al. 2010, Golobič et al. 2012). Accumulation of metals in digestive glands in insoluble form is considered as a means of detoxification under exposure to elevated concentrations of metals in food (Hopkin 1990). Destabilization of the digestive gland cell membrane by NPs is a measure of cytotoxicity; however, damaged cell membrane also permits cellular internalization of NPs, which may lead to further cytotoxic effects (Novak et al. 2012). The biological endpoints at the cellular and tissue level can be combined with organism-level endpoints, such as body mass change, mortality, and avoidance behavior (Škarková et al. 2016) to elucidate a broad picture of the effects of the tested NPs on isopods.

The aim of our present study was to investigate the effects of ingested AuNPs and $AuCl_3$ (10 and 60 µg Au/g dry leaf) on terrestrial isopods as well as potential bioaccumulation of gold into digestive gland cells. $AuCl_3$ was used as a positive control to account for potential dissolution of AuNPs inside the isopods' digestive tract (Eisler 2004; Golobič et al. 2012) and to differ between the effects of AuNPs and Au^{3+} ions. We related the data on Au bioaccumulation in digestive gland tissue to the data on the effects of Au exposure, such as mortality, weight change, feeding rate, and cell membrane destabilization of digestive gland cells. A 14-day food selection behavior test was also done to investigate their selection of the Au-spiked food.

Materials and methods

Test chemicals

AuNPs were synthetized by the INMETRO (National Institute of Metrology, Quality and Technology; Rio De Janeiro, Brazil) as a part of the EU FP7 NanoValid project under project label NNV-004. The data on particle size, shape, ζ-potential, and metal content was provided by the supplier. Gold (III) chloride ($AuCl_3$, ≥99.99% trace metals basis, CAS Number 13453-07-1) and gold standard for AAS (1 mg Au/mL, *Trace*CERT®) were purchased from Sigma-Aldrich (Steinheim, Germany). Water used throughout the work (dH_2O) was first deionized and then further purified using Elix 10/Milli-Q Gradient unit (Millipore, Bedford, Massachusetts, USA [pH = 5.7, ρ = 18.5 MΩ·cm]). Physiological solution for *P. scaber* was prepared according to the protocol published in Hagedorn et Ziegler (2002). Tris(hydroxymethyl)aminomethane, NaCl, KCl, $MgCl_2$, and glucose were purchased from Merck (Darmstadt, Germany). All chemicals were of the reagent grade (EMSURE®). For the hepatopancreatic cell membrane stability assay, acridine orange solution (2% in dH_2O) and ethidium bromide solution for fluorescence (~1% in dH_2O) were used, both from Sigma Aldrich. Microwave acid digestion was performed with the reagent grade 65% HNO_3 (Fischer Scientific, Loughborough, Leicester, UK).

Test organisms

Isopods *P. scaber* originated from the synchronized laboratory culture at the Department of Biology, University of Ljubljana, Slovenia. Cultures of *P. scaber* were derived from individuals collected from an unpolluted site in Polhov Gradec, Slovenia (46° 3′ 0″ N, 14° 18′ 0″ E). Animals were kept in a climate chamber at 22 ± 1 °C with a 16/8 h light/dark period (120 and 16 lx, respectively; measured using LI-1000 Data Logger, LI – COR, Nebraska, USA), caged in glass containers with moist loamy sand and peat at the bottom. They were fed with fallen leaves from various trees, with periodical additions of potatoes, fresh vegetables, and apples.

Feeding exposure

Partially decomposed common hazel leaves (*Corylus avellana*) were collected in the Karavanke region, Slovenia (46° 21′ 32.29″ N, 14° 16′ 36.12″ E), for the purpose of the experiment. Leaves were air-dried at room temperature (24 ± 1 °C) and stored in a cardboard box until use. Bigger leaves with minimally damaged leaf lamina were straightened and the serrated leaf edge was cut off. Leaf laminas were cut into pieces of 100 ± 10 mg.

The AuNPs or $AuCl_3$ were suspended in dH_2O using a vortex (20 s, 2000 rpm) to obtain concentrations 10 and 60 µg Au/mL. The higher concentration corresponded to the Au concentration in the original AuNP suspension provided by the supplier (0.006 % w/w), and the lower one was chosen for comparison to the results of our previous studies, e.g. Pipan-Tkalec et al. (2011). No stabilizers were used, and the chemicals were prepared freshly for each experiment. 100 µL of Au NP dispersion or $AuCl_3$ solution per 100 mg of leaf was applied onto the abaxial surfaces of dry leaves. This resulted in two final concentrations of 10 and 60 µg Au/g dry leaf for both sources of Au. Control leaves were spiked with dH_2O only. Spiked leaves were allowed to dry for 24 hours at room temperature. After 24 hours, dry leaves were re-weighed and this data was then used for further calculations.

Only adult isopods of both sexes, and with 30–60 mg body mass were chosen for the experiments. Moulting animals (Zidar et al. 1998) and gravid females were excluded in order to keep the investigated population as homogenous as possible in terms of its physiological state. Each animal was placed individually in plastic Petri dishes (Æ 9 cm), to which individual pieces of Au-treated dry leaves were added. Two experiments were carried out. The first experiment (**Experiment 1**) consisted of the control group and the groups exposed to AuNPs. The second experiment (**Experiment 2**) consisted of the control group and the groups exposed to $AuCl_3$. In both experiments, each experimental group comprised 12 animals. The exposure conditions were the same for both experiments. Petri dishes were placed in a large, plastic-covered glass container and their humidity was maintained by periodical spraying of the internal side of the lids with dH_2O. The experiments were maintained for 14 days in controlled and stable conditions at 22 ± 1 °C, 80% relative humidity (TFA, Dostmann GmbH et Co.KG, Wertheim, Germany), with a 16/8 h light/dark period (120 and 16 lx, respectively) and monitored on a daily basis. The food was not replaced during the exposure period, and fecal pellets were collected weekly.

Post-experimental sample preparation and analysis

After the 14-day exposure period, the animals were transferred to new Petri dishes and fed with uncontaminated hazel leaves for 24 h to depurate Au from their digestive system. The leaves and fecal pellets from the experiments were collected and weighed after drying at room temperature for 24 h. On the 15th day, animal mortality was recorded, and the survived animals were weighed. The experiments were considered valid if the mortality of controls did not exceed 20 % (Hornung et al. 1998). The animals were decapitated, and the hepatopancreas and gut were isolated with tweezers. One gland tube was used for the cell membrane stability assay, and other three gland tubes, the gut and the 'rest' of the body were further processed for the measurments with flame AAS.

Hepatopancreatic cell membrane stability assay

Cell membrane stability was tested with a modified method for the assessment of cell membrane stability, previously described by Valant et al. (2009). A single isolated hepatopancreatic tube was incubated for 5 minutes in a mixture of the fluorescent dyes acridine orange and ethidium bromide and then put on a microscope slide. Fresh samples were photographed and examined by the Axioimager.Z1 fluorescent microscope (Carl Zeiss, Jena, Germany) with two different sets of filters. The excitation filter of 450 to 490 nm and the emission filter of 515 nm were used to visualize acridine orange- and ethidium bromide-stained nuclei, and the excitation filter of 365 nm and the emission filter of 397 nm were used to visualize nuclei stained with ethidium bromide only. Cell membrane integrity was assessed by visual examination of micrographs and classified

from 1 to 10 according to a predefined scale. On the basis of preliminary experiments, the control animals showing less than 10 % of nuclei stained by ethidium bromide were classified as 1 or 2. The animals exposed to AuNPs, $AuCl_3$, but showing less than 10 % of nuclei stained by ethidium bromide, were also classified as 1 or 2 (Valant et al., 2009).

Gold content determination

Each isopod body part was placed on a separate small piece of a filter paper (approximately 4 mm×7 mm size) and stored in a plastic tube. Prior to analysis, samples were acid digested in concentrated HNO_3 in the Milestone Ethos E (Bergamo, Italy) microwave lab station equipped with SK-10 high-pressure segmented rotor and 3 mL quartz microsampling inserts. Digestion was conducted at 180°C and 600 W power, with step 1 (heating) lasting 15 min, step 2 (constant temperature) lasting 10 min, and 45 min cooling to 60°C. Total Au concentrations in the three parts of each animal (one digestive gland, the gut and the 'rest' of the body) were measured by flame AAS (Perkin-Elmer AAnalyst 100, Waltham, Massachusetts, USA). Metal spiking recovery was determined by measuring the Au concentrations on the remnants of leaves after the experiment.

Food selection behavior test

The food selection behavior test (**Experiment 3**) was carried out according to the protocol by Zidar et al. (2004). The preparation of spiked food and the selection of animals were conducted in the same way as in the toxicity endpoint tests. Particular attention was paid to include only the animals with intact antennae. Each of the five experimental groups included 15 animals, which were caged individually in plastic Petri dishes (Æ 9 cm) and offered two hazelnut leaf pieces (approximately 100 mg each) of different shape (square and triangular) for 14 days. The two leaf pieces had been treated differently. The first piece was control (spiked with dH_2O only) and the second one was spiked with a test compound. The tested Au sources and concentrations were the same as in the toxicity endpoint tests, i.e. $AuCl_3$ and AuNPs at concentrations 10 and 60 µg Au/g dry leaf. As a positive control, $CoCl_2 \times 6H_2O$ with the nominal exposure concentration 2000 µg Co^{2+}/g dry leaf was used. In our previous work (data not published), isopods *P. scaber* significantly avoided Co^{2+} contaminated food at the same nominal exposure concentration, therefore we used only 10 animals in this group for ethical reasons. This collectively resulted in 6 combinations of test compounds: "0 (control) vs. 0", "+control (Co^{2+}) vs. 0", "AuNPs 10 vs. 0", "AuNPs 60 vs. 0", "$AuCl_3$ 10 vs 0" and "$AuCl_3$ 60 vs. 0". The experimental conditions and post-experimental procedure were the same as in the toxicity endpoint tests, except that animal dissection and tissue processing were not performed. The total food consumption rate was calculated as the amount of food consumed (both leaves offered) during 14 days, per fresh animal weight. The consumption rate of a single leaf was calculated as the percentage of the total food consumption rate. This data was then used for the calculation of food selection response. Dead animals were excluded from the calculations.

Data analysis

In all experiments, 12 or 15 animals per each tested group were exposed, but the number of analyzed animals after the experiments was lower due to mortality caused by moulting and due to development of marsupia in females; all such animals were excluded from further data processing. The numbers of analyzed animals are presented in the Figures as part of the x-axis labels. Data is presented as mean values, and uncertainties are expressed as standard deviations (SD).

Average animal fresh body mass (per individual) during the experiment was calculated as an arithmetic average of fresh body masses recorded before and after the experiment. Feeding rate (per individual) was calculated by divinding the total mass of consumed leaves during the experiment with the average animal fresh body mass. Since the animals collected for **Experiments 1** and **2** belonged to the same population, the corresponding control groups were tested for homogeneity of variances with the Fliegner-Killeen test and their average body masses and feeding rates were compared by the Mann-Whitney *U*-test using R statistical package (R Development Core Team 2015). Because no significant differences were found ($p>0.05$), the controls were pooled before

further data analysis. Statistical significance of differences between the pooled control and the animals exposed to Au compounds was assessed by the Mann-Whitney *U*-test using OriginPro 8.0 software (OriginLab, Northampton, MA, USA).

Results

Nanoparticle characteristics

Gold NPs were received from the INMETRO as a reddish dispersion in water. As stated by the manufacturer's specifications, the Au NP dispersion contained 0.006 % (w/w) of Au, the nominal average particle size was 15.7 nm, and ζ-potential was -30.4 mV. The shape of nanoparticles was spheroidal (Fig. 1).

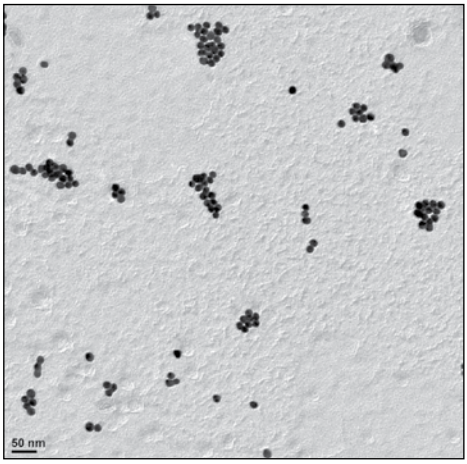

Figure 1. Transmission electron micrograph of Au nanoparticles; the photograph was provided by the INMETRO (National Institute of Metrology, Quality and Technology; Rio de Janeiro, Brazil).

Slika 1. Presevna elektronska mikrografija nanodelcev Au; sliko je posnel INMETRO (Nacionalni inštitut za metrologijo, kakovost in tehnologijo; Rio de Janeiro, Brazilija).

Mortality, body mass change and food consumption of isopods

Fourteen days of exposure to Au compounds had no statistically significant effect on mortality, average body mass (Fig. 2a) or feeding rate (Fig. 2b) of the test animals in comparison to the control in any of the exposure groups (Mann-Whitney *U*-test, $p>0.05$, not marked on Fig. 2).

Digestive gland cell membrane stability

Valant et al. (2009) demonstrated that the digestive gland cell membrane stability value was rarely higher than 2 in the animals from the stock culture, which are in good physiological condition, and this was taken as a benchmark. The higher the value, the more the membrane is destabilized, and the cell membranes are considered completely destabilized when the value is 10. In the present study, in the animals from the stock culture, in control animals and in those exposed to Au-spiked food, the values were never higher than 2, which indicates that neither $AuCl_3$ nor AuNPs affected membrane stability of the digestive gland cells (data not shown).

Gold content in food and animal tissues

The measured metal concentrations on spiked leaves were within 10 % of the nominal values for both tested Au sources at both tested concentrations. No Au was found in the hepatopancreas, the gut or the rest of the body of the animals from any of the exposure regimens, regardless of the Au source or concentration (data not shown).

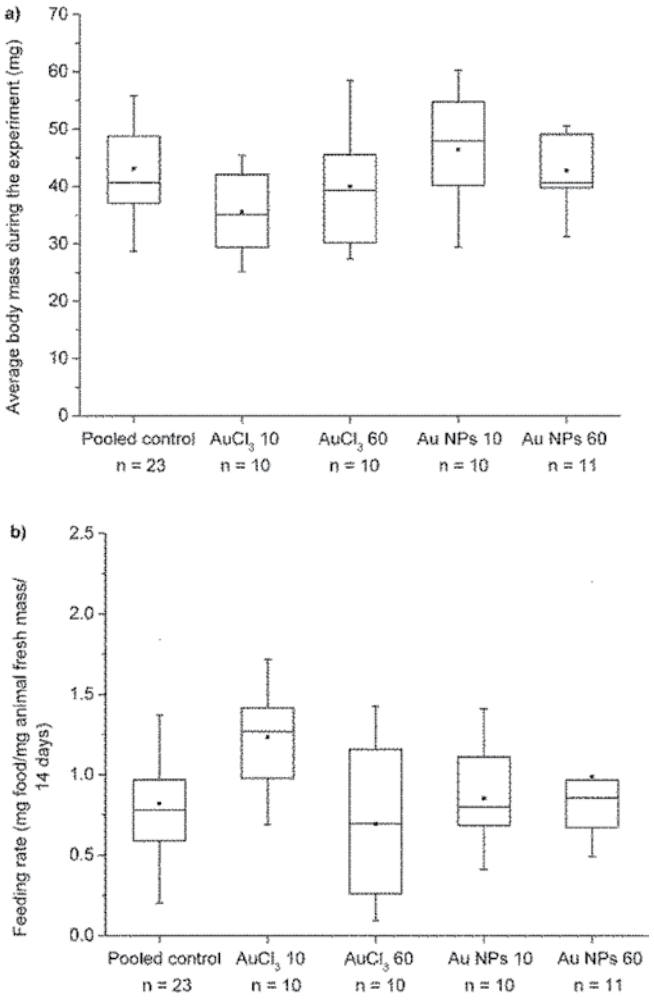

Figure 2. Average body mass **(A)** and feeding rates **(B)** of *P. scaber* isopods during 14-day exposure to Au-spiked food. The animals were fed non-spiked food (control) or food that was spiked with $AuCl_3$ salts ($AuCl_3$ 10 and $AuCl_3$ 60, per nominal Au concentrations) or Au nanoparticles (AuNPs 10 and AuNPs 60, per nominal Au concentrations). The controls from Experiment 1 (with AuNPs) and Experiment 2 (with $AuCl_3$) were pooled because no significant differences between them were found in any of the tested parameters. The nominal exposure concentrations of Au are provided on the x-axis. The symbols on the box plot represent maximum and minimum values (whiskers: ⊥), mean values (■), outliers (–); n = number of specimens in each test group.

Slika 2. Povprečna telesna masa **(A)** in stopnja prehranjevanja **(B)** enakonožcev *P. scaber* med 14-dnevno izpostavitvijo hrani, tretirani z Au. Živali smo hranili z netretirano hrano (kontrola) ali hrano, na katero smo nanesli $AuCl_3$ ($AuCl_3$ 10 in $AuCl_3$ 60, za nominalne koncentracije Au) oziroma nanodelce Au (AuNPs 10 in AuNPs 60, za nominalne koncentracije Au). Kontrolni skupini iz poskusa 1 (z nanodelci Au) in poskusa 2 (z $AuCl_3$) smo združili, saj med njima ni bilo statistično značilnih razlik v nobenem izmed testiranih parametrov. Nominalne izpostavitvene koncentracije Au so navedene na x-osi. Simboli na okvirjih z ročaji predstavljajo minimalne in maksimalne vrednosti distribucije (ročaji: ⊥), povprečja (■) in osamelce (–); n = število osebkov v vsaki testni skupini.

Food selection behavior test

In the **Experiment 3**, avoidance or preference behavior was not demonstrated (Fig. 3). The masses of consumed leaves did not significantly differ (Mann-Whitney *U*-test, $p>0.05$) between the two leaf pieces (control and Au-spiked) for both tested Au compounds (AuNPs and $AuCl_3$) and at both exposure concentrations (10 and 60 µg Au/g dry food). Significant preference for non-spiked over $CoCl_2$-spiked leaves was found in the positive control group (Fig. 3; Mann-Whitney *U*-test, $p<0.001$).

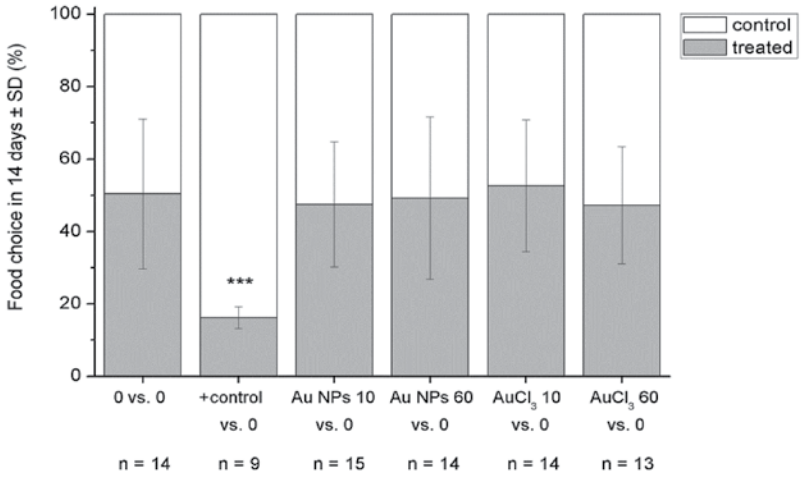

Figure 3. Food selection of *P. scaber* isopods. Animals were exposed to untreated food (0) and food treated with AuNP or $AuCl_3$ simultaneously for 14 days. The nominal exposure concentrations of Au are provided on the x-axis (in µg Au/g dry leaf). $CoCl_2 \times 6H_2O$ with the nominal exposure concentration 2000 µg Co^{2+}/g dry leaf was used as a positive control (+control). The food choice (%) is presented as the ratio between consumption rates of the two offered leaves, expressed as mean percentages of the total food consumption rate for each exposure group. Uncertainties are expressed as standard deviation (SD). Significant differences: $p<0.001$ (***); n = number of specimens in each test group.

Slika 3. Izbira hrane pri *P. scaber*. Živali so bile 14 dni hkrati izpostavljene netretirani hrani (0) and in hrani, na katero smo nanesli $AuCl_3$ oziroma nanodelce Au. Nominalne izpostavitvene koncentracije Au so navedene na x-osi (v µg Au/g suhe mase lista). Kot pozitivno kontrolo (+control) smo uporabili $CoCl_2 \times 6H_2O$ v nominalni izpostavitveni koncentraciji 2000 µg Co^{2+}/g suhe mase lista. Izbira hrane (%) je predstavljena kot razmerje med stopnjama prehranjevanja z dvema ponujenima listoma ter izražena kot povprečna vrednost stopnje prehranjevanja za vsako testno skupino posebej. Negotovost je izražena s standardnimi deviacijami (SD). Statistično značilne razlike: $p<0.001$ (***); n = število osebkov v vsaki testni skupini.

Discussion

In the present study we assesed the influence of AuNPs and $AuCl_3$ on different biological endpoints in the model organisms, terrestrial isopods *P. scaber*. We tested the adverse biological effects of AuNPs and $AuCl_3$ as detectable by common toxicoloxical parameters (body mass change, food consumption rate, metal feeding preference/avoidance behavior, and mortality) and cell membrane integrity assay, which provides information onf the cell membrane destabilization. In parallel, we assessed the Au^{3+} assimilation in digestive glands.

Our results show that AuNPs did not affect *P. scaber* mortality, body mass (Fig. 2a) or food consumption (Fig 2b) when exposed through food at concentrations 10 and 60 µg Au/g for 14 days. In other words, we showed that AuNPs and $AuCl_3$ do not affect the organism-level endpoints in *P. scaber* at relatively low exposure concentrations. Our results are in line with previous reports on earthworms (Unrine et al. 2010). In *E. fetida*, exposure to AuNPs (20 and 55 nm, 5 µg Au/g dry mass spheres) in soil for 28 days did not affect their mortality or growth (Unrine et al. 2010). In contrast, feeding with 15 nm citrate-capped AuNPs at concentrations 3 and 27 µg AuNPs/g food per day reduced the lifespan of *D. melanogaster* for 24 and 41 %, respectively, in comparison to control (average control population half-life was 80 days; Sabella et al. 2011). In the same experimental setup and with the same AuNPs, the lifespan was reduced for 62 % when *D. melanogaster* were exposed to 12 µg AuNPs/g food per day (average control population half-life was 37 days; Pompa et al. 2011). However, the exposure doses for *D. melanogaster* (Pompa et al. 2011, Sabella et al. 2011) were higher than in our study (0.7 and 4.3 µg Au/g food per day for nominal exposure concentrations 10 and 60 µg Au/g dry leaf, respectively), which may partially explain why mortality in *P. scaber* in our study was not elevated in comparison to *D. melanogaster*.

In line with this, we also showed that isopods show no preference/avoidance towards Au-spiked hazelnut leaves for both tested Au compounds ($AuCl_3$ and AuNPs; Fig. 3). This is in agreement with the observation that feeding rate of isopods was unaffected upon Au exposure (Fig. 2b). Avoidance behavior of isopods towards metal-contaminated food has previously been shown (Zidar et al. 2004; Škarková et al. 2016).

Nanoparticles may pass from the gut into the lumen of the *P. scaber* hepatopancreas during digestion, as it has been demonstrated for TiO_2 NPs (Novak et al. 2012) and WO_X nanotubes (Novak et al. 2013), and cause cell membrane destabilization (Novak et al. 2012, 2013). However, neither AuNPs nor $AuCl_3$ caused the reduced cell membrane integrity of hepatopancreatic cells in our current study. The results of cell membrane integrity assay match the data on the Au assimilation. Namely, neither of the *P. scaber* body parts contained any gold, which indicates that AuNPs were not internalized into tissues and Au^{3+} ions were not assimilated into the digestive gland cells. In general, we cannot exclude the possibility that *P. scaber* possesses the assimilation capacity for Au^{3+}, because Au has the affinity for sulphur-bearing ligands (Korobushkina et al. 1983), which are present in the type B granules of the hepatopancreatic S cells (Hopkin 1990) and enable the assimilation of other metals with affinity for sulphur, such as cadmium, copper, lead and mercury (Hopkin 1990), silver (Pipan-Tkalec et al. 2011) and cobalt (Novak et al. 2013). Nor we can exclude the possibility for the dissolution of AuNPs inside the *P. scaber* digestive tract (Golobič et al. 2012), since the dissolution of gold can be induced by biological macromolecules, most notably amino acids and proteins, as well as bacteria (Sadler 1976, Rapson 1982, Korobushkina et al. 1983). However, under employed exposure conditions, the internalization/assimilation of Au into *P. scaber* digestive glands clearly did not occur.

Our results contrast those for *E. fetida* (Unrine et al. 2010), *M. sexta* caterpillars (Judy et al. 2010, 2012), and *D. melanogaster* (Pompa et al. 2011) where AuNPs, administered *via* food, were found in tissues surrounding the digestive tract (including the digestive tract epithelium in *E. fetida*) as well as in the reproductive organs in *D. melanogaster*. The presence and localization of gold was corroborated by transmission electron microscopy (TEM) coupled to energy dispersive spectroscopy ([EDS] Unrine et al., 2010), X-ray absorption near edge spectroscopy (µXANES) and synchrotron X-ray fluorescence microprobe ([µXRF]; Judy et al. 2010, 2012) or high angle annular dark field scanning transmission electron

microscopy ([HAADF-STEM]; Pompa et al. 2011). No evidence of dissolution of AuNPs was observed in the quoted studies, so NPs were likely assimilated intact (Unrine et al. 2010, Judy et al. 2010, Pompa et al. 2011).

In conclusion, our results denote that concentrations of Au salt and AuNPs tested in this work (10 and 60 μg Au/g dry leaf) do not induce adverse effects on terrestrial isopods after 14 days of exposure. These concentrations are higher than currently predicted environmental levels (Mahapatra et al. 2015), which further confirms the finding that AuNPs are safe for isopods. In general, AuNPs are among NPs with the least toxic potential for test organisms commonly employed in environmental studies. Namely, Bondarenko et al. (2016) have used a set of assays to screen seven NMs using 14 different test species and cell lines. The toxicity decreased in the following order: Ag > ZnO > CuO > TiO_2 > MWCNTs > SiO_2 > Au (Bondarenko et al. 2016). According to our perception, the majority of authors in nanotoxicity research encourage the publication of those results that show some kind of effects of nanoparticles on organisms. We therefore suggest that also those data with no documented effects should be published in equal proportion to help built a realistic public perception of the nanomaterial environmental risk.

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Povzetek

Nanodelci zlata se čedalje pogosteje uporabljajo za različne aplikacije, kot je npr. v kozmetiki, barvah in elektrokemijskih senzorjih ter v detekciji, diagnostiki in zdravljenju tumorjev. Kljub temu še vedno ni dovolj podatkov o njihovih potencialnih negativnih učinkih na kopenske organizme. V tej študiji smo proučevali vpliv okoljsko relevantnih koncentracij ND Au na kopenske rake vrste *Porcellio scaber* po 14-dnevni izpostavitvi. Živali smo izpostavili listom, na katere so bili nanešeni nanodelci zlata. Proučevali smo vpliv na smrtnost, maso živali, hitrost prehranjevanja, izogibalno vedenje in destabilizacijo membrane celic prebavnih žlez. Izmerili smo tudi asimilacijo Au v prebavnih žlezah. Rezultati so pokazali, da nobena izmed testiranih koncentracij ND Au (10 in 60 μg Au/g lista) po 14-dnevnem hranjenju ni imela vpliva na kopenske rake. Prav tako nismo izmerili prisotnosti Au v prebavnih žlezah, kar kaže, da se ND Au niso niti raztapljali v prebavnem soku niti vstopali v celice prebavnih žlez. Enake rezultate smo dobili tudi z referenčno kovino, soljo $AuCl_3$. Zaključujemo, da so testirane koncentracije ND Au varne za kopenske rake. Naše mnenje je, da se na področju nanotoksikologije trenutno objavlja predvsem rezultate, ki kakorkoli nakazujejo na negativni vpliv nanodelcev na organizme. Zato želimo stimulirati tudi objavo rezultatov, ki kažejo nasprotno. Na ta način bomo pripomogli k bolj uravnoteženemu ter realističnemu mnenju javnosti o okoljski varnosti nanomaterialov.

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No chronic effects on biochemical biomarkers, feeding and survival of carnolian honeybees (*Apis mellifera carnica)* after exposure to nanosized carbon black and titanium dioxide

Kronična izpostavitev nanomaterialom titanovega dioksida in črnemu ogljiku nima vpliva na biokemijske biomarkerje, prehranjevanje in preživetje kranjske čebele (*Apis mellifera carnica)*

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Abstract: Honeybees (*Apis mellifera*) are important pollinators threatened by environmental pollution, plan protection products and other potential contaminants. Due to an extensive predicted use of engineered nanomaterials (NMs) in agriculture the impact on honeybees should be investigated. We studied the 10-days chronic dietary effect of carbon black (CB) and titanium dioxide (TiO_2) NMs on the antioxidant activities, cholinergic function, feeding behaviour and survival of honeybees. Exposure of honeybees *Apis mellifera carnica* to TiO_2 and CB NMs (1 mg ml^{-1}) did not affect the feeding and survival. No alteration of catalase, acetylcholinesterase and glutathione S-transferase enzymatic activity was noticed in the brain of honeybees, indicating that TiO_2 and CB NMs at the tested exposure dose had no adverse effects on honeybees. Currently predicted environmental concentrations for TiO_2 and CB NMs are significantly lower than the concentration tested in the current study. Based on our findings we conclude that the potential use of TiO_2 and CB NMs in agriculture is currently safe for honeybees at the tested concentration level and presents potential advantages compared to other NMs with known toxic potential.

Keywords: nanopesticide, carbon black nanomaterial, titanium dioxide nanomaterial, acetylcholinesterase, glutathione S-transferase, catalase, feeding behaviour.

Izvleček: Medonosne čebele (*Apis mellifera*) so pomembni opraševalci, ogroženi zaradi onesnaževanja okolja, fitofarmacevtskih sredstev in drugi možnih onesnaževalcev. Zaradi široke predvidene uporabe inženirsko proizvedenih nanomaterialov v poljedelstvu je potrebno raziskati njihov vpliv na medonosne čebele. V tej študiji smo preučili 10-dnevni kronični prehranski učinek nano-črnega ogljika (nČO) in nano-titanovega dioksida ($nTiO_2$) na prehranjevalno vedenje, antioksidativno aktivnost, na delovanje holinergičnega živčnega sistema in preživetje čebel. Pokazali smo, da $nTiO_2$ (1 mg ml^{-1}) in nČO (1 mg ml^{-1}) nista vplivala na hranjenje in preživetje kranjskih čebel *Apis mellifera carnica*. Hranjenje z obema vrstama nanomaterialov

ni vplivalo na aktivnost treh biomarkerskih encimov katalaze, acetilholinesteraze in glutation S-transferaze v možganih čebel, kar pomeni, da nanomateriali verjetno niso imeli škodljivega učinka na čebele. Trenutno ocenjene napovedane vrednosti okoljskih koncentracij za $nTiO_2$ in nČO so znatno nižje od teh, ki smo jih uporabili v sedanji študiji. Na podlagi naših ugotovitev sklepamo, da je morebitna uporaba $nTiO_2$ in nČO NM v kmetijstvu varna za čebele v okviru testiranih koncentracij in predstavlja potencialno prednost v primerjavi z nanomateriali z znanim toksičnim potencialom.

Ključne besede: nanopesticidi, nano-črni ogljik, nano-titanov dioksid, acetilholinesteraza, glutation S-transferaza, katalaza, prehranjevalno vedenje.

Introduction

Honeybees (*Apis mellifera*) are important pollinators and many agricultural crops depend on pollination. Colony collapse disorder (CCD) causes massive deaths in honeybees and significant economic losses (van Engelsdorp et al. 2009). In the recent years it is evident that CCD is caused by the combined action of parasites, pathogens and pesticide stressors (Sánchez-Bayo et al. 2016). Synergistic interactions among the parasitic mite *Varroa*, viral pathogens and pesticides could severely reduce host immune competence potentiating the sensitivity of honeybees to other possible stress agents. Among the stress agents, pesticides are now widely studied while other emerging environmental contaminants, such as nanomaterials (NM) are still highly neglected.

Engineered nanomaterials are extensively used in agriculture to reduce amount of applied plant protection products, minimize nutrient losses in fertilization, and increase yields through an optimized nutrient management (Gogos et al. 2012; Kah and Hofmann 2014). New formulations containing nanomaterials are called "nanopesticides" and "nanofertilisers" (Kah and Hofmann 2014). Up to 3000 of patents and over 100 peer-reviewed publications directly related to nanopesticides have been published until 2011 (Kah and Hofmann 2014), indicating intense research activity in this field.

Nano-sized titanium dioxide (TiO_2 NM) was one of the first nanomaterials commercially available and is used in a wide variety of materials and applications, including self-cleaning surface coatings, light-emitting diodes, solar cells, disinfectant sprays, sporting goods, water-treatment agents, cosmetics and agriculture (IARC/WHO, 2010). These NMs are widely used due to their high stability, anticorrosive and photocatalytic properties. In agriculture, TiO_2 NM is added to pesticide formulation to catalyse the photodegradation of the pesticide organic active ingredient. Examples of such nanoformulations with TiO_2 are: chlorfenapyr (Cao et al. 2005), imidacloprid and avermectin (Guan et al. 2008, 2011). TiO_2 NMs are also interesting in terms of their antimicrobial activity and several studies suggest their suppressing action on bacterial and fungal pathogens growth on crops (Paret et al. 2013 a,b). Until 2013 70 patents including TiO_2 have been registered as nanopesticides (Kah et al. 2013).

Nano-sized carbon black (CB NM) is produced at rates of several million tons per year (Navarro et al. 2008). It is a product of incomplete combustion of fossil fuels and vegetation, and is used in rubber production, as black pigment in printing inks, as electrodes in batteries, and in leather production. In comparison to TiO_2 NM, CB NM is much less explored in terms of potential hazard for the environment. Significantly more information is available for other carbon nanomaterials, such as carbon nanotubes and fullerenes (Jackson et al. 2013).

Insects, including honeybees, are among the least investigated non-target organisms in terms of potential nanomaterial hazard (Garner et al. 2015). Currently only one study on the effect of NM (zinc oxide; ZnO) on honeybees exists (Milivojević et al. 2015). Honeybees (*Apis mellifera*) are potentially exposed to TiO_2 and CB NM due to their foraging behaviour via contact with contaminated plants as well as water droplets. Flying bees, beehives or flowers attracting the bees may come into contact with NM also *via* traffic dust contaminated with TiO_2 and CB NM (Perez et al. 2010).

In the present work, we aimed to investigate the chronic effects of CB and TiO_2 NM on feeding behaviour, survival and stress enzyme activities in the honeybee brain. Three commonly applied biomarkers of exposure and effect were measured: antioxidant enzymes catalase (CAT) and glutathione S-transferase (GST), and neurotoxic biomarker acetylcholinesterase (AChE) (Jemec et al. 2010). Catalase decomposes hydrogen peroxide (Halliwell and Gutteridge 2007). GSTs are a family of detoxification enzymes, which catalyse the conjugation of glutathione with xenobiotics and cytotoxic aldehydes produced during lipid peroxidation. GSTs are considered as both antioxidant and detoxification enzymes (Barata et al. 2005). Acetylcholinesterase plays an established role in cholinergic transmission by hydrolysing the neurotransmitter acetylcholine thereby terminating the synaptic transmission (Kim and Lee 2013). In addition, a number of non-neuronal functions of AChE have also been proposed (Karczmar 2010). A disruption of the honeybees' neuronal cholinergic signalling affects their orientation, olfactory learning and navigation abilities, which results in their failure to return to hives, even potentially leading to CCD (Farooqui 2013). Knowledge on the effect of pollutants on the cholinergic system of honeybee brain is therefore important for understanding the potential environmental hazard of NMs.

Materials and methods

Chemicals

The following chemicals were purchased from Sigma Aldrich (Germany): monobasic and dibasic potassium phosphate, 1-chloro-2,4-dinitrobenzene, L-glutathione (reduced form), 5,5′-dithiobis-2-nitrobenzoic acid, sodium hydrogen carbonate, acetylthiocholine chloride, sodium sulphate and ethylenediaminetetraacetic acid. BCA Protein Assay Reagent A and Reagent B were purchased from Pierce (US). All chemicals were of the highest commercially available grade, typically >99%. CB nanopowder was provided by PlasmaChem GmbH (Berlin, Germany) and TiO_2 NM was provided by Nanologica (Sweden) in the framework of the EU FP7 NanoValid project.

Preparation and characterisation of NM suspensions

A suspension of TiO_2 NM or CB NM was prepared by adding nano powder to 1.5 M sucrose solution in milli-Q water with sonication (PIO Iskra, Sonnys 2GT; 40 kHz, 2x100 W) of the suspension for 24 h. For better comparison with the effects of ZnO NM on honeybees, published by Milivojević et al. 2015, where the same experimental set-up and tested concentrations were applied, we used final nominal concentration 1 mg mL-1 of NMs in sucrose.

The properties of CB NM were as reported by Mesarič et al. (2013). CB is composed of amorphous, globular primary nanoparticles with diameter of about 20 nm (**Supplementary information Fig.S1A**). The primary sizes of TiO_2 NM were in range between 110 and 170 nm, showing a large size distribution (**Supplementary information Fig.S1B**). The secondary size of NMs in the 1.5 M sucrose solution was not measured since the size of NM aggregates/agglomerates alters in different fluids present in the honeybee digestive system. Therefore, it is important to bear in mind that the data on the size of aggregates in the sucrose solution have no actual correlation with the actual size of NM in the digestive system of honeybees.

Test animals

Adult summer honeybee workers (*Apis mellifera carnica*, Pollman 1879) were randomly collected inside the hive from colonies that were maintained according to the good beekeeping practice at the Biotechnical Faculty, University of Ljubljana, Slovenia. Honeybees were then transferred to wooden cages (9.5 x 4 x 7.5 cm) and supplied *ad libitum* with water and 1.5 M sucrose solution in gravity feeders until all bees were collected. All bees were maintained in cages for 1 h at 27 °C before treatments.

Honeybee exposure to TiO_2 and CB NM via food

A group of 120 bees was divided into 6 groups of 20 animals. Each group of bees (20 specimens) was placed into separate wooden cage. Two cages with a control group were fed with 1.5 M sucrose

solution only, two groups of bees received a suspension of TiO_2 NM in 1.5 M sucrose (1 mg mL^{-1} TiO_2) and two groups were fed with a suspension of CB NM in 1.5 M sucrose (1 mg mL^{-1} CB). Each cage received a syringe with tap water. The sucrose solution and water were renewed every 2 days. The cages were placed for 10 days in an incubator at 27 °C and 95% relative humidity during the overall exposure period. The feeding was estimated as the volume of total consumed solutions/suspensions per exposure group after 10 days. Mortality of bees was monitored during 10 days of the experiment.

Brain dissection and homogenization

After 10-days of dietary exposure to NM, the brains were isolated from honeybees according to Carreck et al. (2013). Isolated brains were submersed into a droplet of honeybee Ringer solution, both hypopharyngeal and postcerebral glands were removed. The brains were then stored at -20 °C until analysis. Individual brains were homogenized in 200 mL of 100 mM potassium phosphate buffer (pH 7.4) per sample. Individual homogenates were centrifuged for 15 min at 12,092 *g* and 4 °C, and the supernatants were stored at -20 °C for enzyme analyse.

Enzyme and protein assays

AChE, GST and CAT activity measurements

AChE activity was analysed according to Ellman et al. (1961), GST activity was analysed according to the method of Habig et al. (1974) and CAT activity was determined according to Jemec et al. (2008). All procedures were adapted as described in Milivojević et al. (2015) and Jemec et al. (2008). AChE activity was expressed in nmoles of hydrolyzed acetylthiocholine chloride/min/mg protein (extinction coefficient ε_{405}=13,600 M^{-1} cm^{-1}). GST activity was expressed in nmoles of conjugated GSH/min/mg protein (extinction coefficient ε_{340}=9600 M^{-1} cm^{-1}). The CAT activity was expressed as µmoles of degraded hydrogen peroxide/min/mg protein (extinction coefficient, ε_{240} = 43.6 M^{-1} cm^{-1}).

Protein quantification

Protein concentration in the supernatant of honeybee brain homogenates was analyzed using a BCA™ Protein Assay Kit, a modification of the bicinchoninic acid protein assay (Pierce, Rockford, IL, USA).

Statistical analysis

The significant differences between the control and exposed groups of animals were determined by Kruskal-Wallis analysis and Mann-Whitney U test ($p<0.05$) using OriginPro software.

Results

Feeding and survival

After 10-days of exposure to CB NM the total volume of consumed suspensions of CB NM (1 mg mL^{-1}) in 2 groups of 40 individuals was similar to the total volume of consumed sucrose solution in 2 groups of 40 individuals indicating that CB NM did not affect the feeding when comparing treated and control groups of honeybees (Fig.1). In honeybees (2 groups, n=40) exposed to the TiO_2 NM (1 mg mL^{-1}), we observed a slightly (13.9 %) higher food uptake than in honeybees fed with control sucrose solution (Fig.1), but this increase was not statistically significant. The total volume of consumed suspensions of CB NM, TiO_2 NM and control sucrose solution in 10-days were 15.8 ml (8 mL and 7.8 mL), 18 mL (9 mL and 9 mL) and 16 mL (8 mL and 8 mL) per treatment (and per groups), respectively (Fig.1).

The chronic 10-days oral exposure of honeybees to TiO_2 (1 mg mL^{-1}) and CB NM (1 mg mL^{-1}) did not affect the survival in both treated groups (data not shown). There was no morality in all groups during the 10-days exposure period.

Enzyme activities after exposure to tested substances

Chronic 10-days exposure to TiO_2 NM (1 mg mL^{-1}) or CB NM (1 mg mL^{-1}) did not alter significantly the activities of brain AChE, CAT and GST (Fig.2) (Kruskal-Wallis analysis and

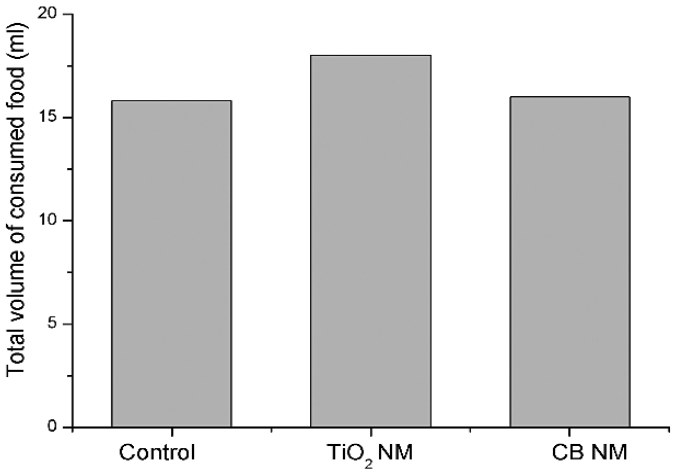

Figure1: The effect of titanium dioxide (TiO_2 NM) and nano carbon black (CB NM) on honeybee feeding. Total volume of consumed food (mL) in bees after 10-days exposure to TiO_2 NMs suspension (1 mg mL^{-1}) and to CB NM suspension (1 mg mL^{-1}) is shown (N=40).

Slika 1: Vpliv nano-črnega ogljika (nČO) in nano-titanovega dioksida ($nTiO_2$) na prehranjevanje medonosne čebele. Prikazana je skupna količina porabljene hrane (mL) pri čebelah po 10-dnevni izpostavljenosti suspenziji $nTiO_2$ (1 mg mL^{-1}) ali nČO (1 mg mL^{-1}) (N = 40).

Mann-Whitney U test ($p<0.05$)). The mean (±SE) activity of AChE in bee brains (Fig. 2A) after chronic oral exposure to TiO_2 NM and CB NM were 2.08±0.16 and 2.08± 0.16 nmol/min/mg protein, respectively, and were similar to control values (1.91±0.15 nmol/min/mg protein). The mean activity of GST in bee brains (Fig. 2B) after chronic oral exposure to TiO_2 NM and CB NM were 4.15±0.13 and 4.81± 0.12 nmol/min/mg protein, respectively, and were similar to the control values (4.59±0.15 nmol/min/mg protein). The mean activities of CAT in bee brains (Fig. 2C) after chronic oral exposure to TiO_2 NMs and CB NMs were 0.64 ± 0.046 and 0.77 ± 0.044 µmol/min/mg protein, respectively, and were similar to control values (0.72 ± 0.048 µmol min/mg protein).

Discussion

The results of the present study show that the TiO_2 (1 mg mL^{-1}) and CB NMs (1 mg mL^{-1}) do not cause any adverse effects, neither sub-lethal nor lethal, on honeybees *Apis mellifera carnica* after a 10-days chronic dietary exposure. No effects on survival, feeding behaviour, antioxidant activities (CAT and GST), and cholinergic enzyme activity (AChE) in the brain of honeybees was observed.

We have previously anticipated that TiO_2 NM and CB NM might not be highly toxic to honeybees, since these two materials have been previously recognised as presumably inert, e.g. having little biological interaction with the test organisms (Bondarenko et al. 2016). Namely, Bondarenko et al. (2016) have used a set of assays to screen seven NMs using 14 different test species and cell lines. The toxicity decreased in the following order: Ag>ZnO>CuO>carbon nanotubes>Au>SiO_2=TiO_2. It is now well established that the toxicity of the NMs is mainly driven by two basic intrinsic properties of NMs: high solubility (e.g. Ag, CuO and ZnO) and high aspect ratio (describes the proportional relationship between its diameter and its length) (e.g. carbon have high aspect ratio). TiO_2 and CB NMs are neither soluble in aqueous media nor have high aspect ratio, which could explain their low toxic potential.

In contrast to soluble NMs, both TiO_2 and CB NMs have high adsorption potential for the body

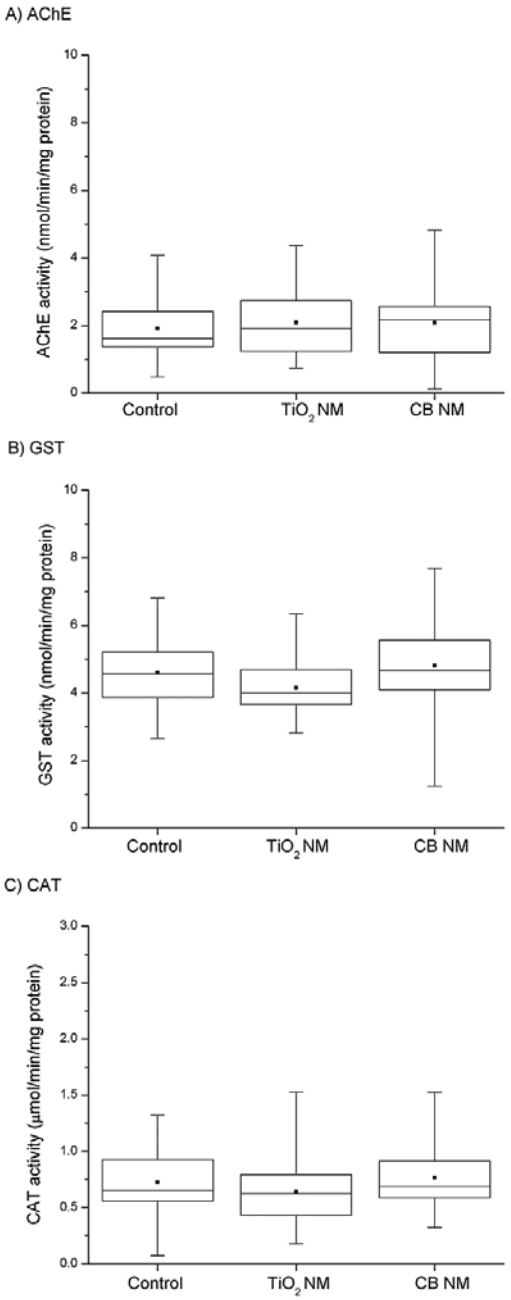

Figure 2: Acetylcholinesterase(A), glutathione-S-transferase (B), and catalase (C) activity in honeybee brains after chronic exposure to TiO_2 NM (1 mg mL^{-1}) and CB NMs (1 mg mL^{-1}) (N=40).

Slika 2: Aktivnost acetilholinesteraze (A), glutation S-transferaze (B) in katalaze v možganih čebel po kronični izpostavitvi $nTiO_2$ (1 mg mL^{-1}) ali nČO (1 mg mL^{-1}) (N=40).

surface which was shown to induce adverse effects on organisms (Xia et al. 2011; Mesarič et al. 2015a, b; Nielsen et al. 2008). CB NM adsorbed on the sperm and embryo of brown algae *Fucus serratus*, to body surface of *A. salina* larvae (Mesarič et al. 2015a) and to sperm of sea urchin (*Paracentrotus lividus*) (Mesarič et al. 2015b), resulted in abnormal development of algae, inhibition of larvae swimming, and reduced fertilisation of sea urchin eggs, respectively. The adsorption of TiO_2 NM on crustaceans *Daphnia magna* (Dabrunz et al. 2011) and algae (Aruoja et al. 2009) had a negative effect on the immobility and growth, respectively. Based on available literature data on TiO_2 and CB NM and their high adsorption potential these NM could adsorb onto the digestive tract surface of honeybees, which may result in feeding disruption. However, this potential effect was not confirmed during 10-days exposure of honeybees to 1 mg mL^{-1} of CB and TiO_2 NMs.

Chronic exposure of honeybees to CB and TiO_2 NMs did not significantly change the feeding, but we observed a slightly higher food uptake in honeybees fed with TiO_2 NM in comparison to honeybees that received only control sucrose solution. Behavioural response such as feeding alteration due to possible stress agents could occur before the alterations in biochemical biomarkers (Hellou 2011). Currently, no data in the literature are available confirming that honeybees are able to sense metals. The effect of different metals on honeybees behaviour was explored only by few studies (Hladun et al. 2012; Burden et al. 2016; Søvik et al. 2015), and only in our last study the effects of metallic NMs was addressed (Milivojević et al. 2015). In our study with tested ZnO NMs we found that chronic exposure did not alter the feeding in honeybees whereas Zn^{2+} salt increased the feeding (Milivojević et al. 2015). Due to potential environmental burden of CB and TiO_2 NMs it would be important to investigate the possible preference/avoidance towards solutions contaminated with these NMs.

Catalase, GST and AChE are among the most commonly applied biochemical biomarkers of toxicant-induced physiological changes in organisms (Jemec et al. 2010). It has been previously shown that 1 mg mL^{-1} of CB NM caused an increase of GST and AChE activities in *Artemia salina* after 48 h exposure (Mesarič et al. 2015a), while no alteration of CAT was reported. CB or TiO_2 NMs (0.005 mg mL^{-1}) increased the activities of CAT and GST in the digestive gland and gills of mussels *Mytilus galloprovincialis* after 24 h (Canesi et al. 2010), while 0.001 mg mL^{-1} of CB NM decreased the AChE activity in gastrulae of sea urchin (*Paracentrotus lividus*) after 24 h (Mesarič et al. 2015b). In the present work, CAT and GST were not altered in the head of honeybees, which indicates that most probably no oxidative stress and detoxification process occurred. Also, the activity of AChE was not changed which is in contrast to the effect of ZnO NM previously reported for honeybees (Milivojević et al. 2015). We can conclude that both TiO_2 and CB NMs have low neurotoxic potential in honeybees. In the study of Milivojević et al. (2015) the alteration of AChE was predominately explained as an effect of released $Zn^{2+}$from ZnO NM. Nevertheless, in the study of Romih et al. (2015), the authors suggested that both dissolved ions and NMs in the subtoxic range could be responsible for the activation of different metabolic pathways in the hepatopancreases of crustacean *Porcellio scaber*. They have found that the ZnO NM induced different metabolic responses from those induced by Zn^{2+} salt. Based on this study it could be also the case with TiO_2 and CB NMs but at the moment no data are available to support this theory. We can conclude that both TiO_2 and CB NMs have low neurotoxic potential in honeybees.

At the moment no data on the realistic environmental concentrations of nano TiO_2 and nano CB are available. Predicted environmental concentration (PEC) for TiO_2 NM was estimated at 107 mg kg^{-1} for wastewater treatment plant sludge, and 21 ng L^{-1} for surface waters (data from 2009) (Gottschalk 2009). TiO_2 NM production market is constantly increasing and these NMs are expected to have higher release into soil, water and air in the future (Keller et al. 2013). The upper quantity of TiO_2 NM estimated to pass through the waste water system in 2010 was nearly 48,000 t/year, with a potential for over 38,000 t/year to be added to the soil, and 32 000 t/year to landfill mainly through application of biosolids, but also small quantities through atmospheric deposition (1600 t/year) (Keller et al. 2013). Carbon nanomaterials are also among those with high expected production, but their emission rates are lower compared to TiO_2

NM (e.g. 10 times lower for carbon nanotubes) (Keller et al. 2013). We were unable to find the PEC values for nano CB, but PEC values have been calculated for non-nano CB based on the data obtained in the period 1999-2010 (Screening Assessment for the Challenge 2013). The estimated PEC from industrial emission (inks and paints industry) was 6.6 mgL^{-1} for river, which is based on a total of 4 336 447 kg of CB used/year.

In conclusion, currently estimated PEC values for TiO_2 and CB NMs are significantly lower than those tested in this study (1 mg mL^{-1}). Future production and release rates of these two NMs are expected to be very high (Keller et al. 2013). Therefore it is reasonable to analyze the potential hazard of high TiO_2 and CB NMs exposure concentrations (up to 1 mg mL^{-1}). The current study reveals that both TiO_2 and CB NM have no adverse effects, neither sub-lethal nor lethal, on honeybees after a 10-days exposure. In this regard, the use of carbon and titanium NMs in agriculture, instead of those with more hazardous potential, could have an advantage.

Acknowledgements

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Povzetek

Medonosne čebele (*Apis mellifera*) ogrožajo različni dejavniki, med katere lahko štejemo tudi povečano proizvodnjo in uporabo različnih nanomaterialov v kmetijstvu v obliki nano-pesticidov in nano-gnojil. Vpliv kovin na čebele, sploh v obliki nanodelcev, je zelo slabo raziskan. Zato smo se odločili, da preučimo učinke dveh vrst nanomaterialov, ki veljata za dokaj inertna v smislu raztapljanja in interakcije z biološkimi sistemi: nano-črnega ogljika (nČO) in nano-titanovega dioksida ($nTiO_2$). Odrasle čebele delavke kranjske čebele (*Apis mellifera carnica)* smo kronično (10 dni) hranili s suspenzijama $nTiO_2$ (1 mg mL^{-1}, n=40) ali nČO (1 mg mL^{-1}, n=40), v 1.5 M raztopini saharoze. Kontrolna skupina čebel (n=40) je bila hranjena samo z 1.5 M raztopino saharoze. Ugotovili smo, da 10-dnevno hranjenje z $nTiO_2$ ali nČO ni vplivalo na preživetje ali stopnjo prehranjevanja čebel. Prav tako $nTiO_2$ ali nČO nista vplivala na aktivnosti dveh antioksidativnih encimov, katalazo in glutation S-transferazo ter na aktivnost pokazatelja delovanja holinergičnega živčnega sistema encima acetilholinesteraza v možganih čebel. Ti rezultati kažejo na to, da uporabljeni nanomateriali verjetno niso imeli škodljivega učinka na čebele. Trenutno ocenjene predvidene vrednosti okoljskih koncentracij za $nTiO_2$ in nČO so znatno nižje od teh, ki smo jih uporabili v sedanji raziskavi. Sklepamo, da je morebitna uporaba $nTiO_2$ in nČO v kmetijstvu primernejša od tistih pripravkov, ki imajo več škodljivih učinkov na organizme.

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Nekatere anatomske značilnosti skeleta sive čaplje, *Ardea cinerea*

Some anatomical characteristics of the skeleton of grey heron, *Ardea cinerea*

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Izvleček: Na štirih skeletih sive čaplje (*Ardea cinerea*) smo proučili morfološke lastnosti kosti in skeletnih sklopov, predvsem nekatere segmente lobanje, hrbtenice, plečnega obroča, prsnice in medeničnega obroča. Za lobanjo je značilna izrazita kraniofacialna upogibna cona (*zona flexoria craniofacialis*), dolga čelnica (*os frontale*) z izrazito vzdolžno depresijo (*depressio frontalis*), dolg postorbitalni lok (*arcus postorbitalis*), obsežna senčnična jama (*fossa temporalis*) in dodatna plitka podsenčnična jama (*fossa subtemporalis*). Parasfenoidni ročaj (*rostrum parasphenoidale*) je izrazit, krilatki (*ossa pterygoidea*) pa močni in premi. Pri vratnih vretencih je značilna močnejša kalcifikacija šestega vretenca in njegova toga povezanost s petim. V področju notarija (*notarium*) vretenca niso zraščena: med zadnjim vratnim in prvim prsnim vretencem je jasen sklep. Tudi prva tri prsna vretenca niso medsebojno zraščena, pač pa tesneje povezana v ventralnem delu. Četrto prsno vretence je izrazito pregibno (prosto), kot pri drugih ptičih. Peto prsno vretence je pridruženo sinsakrumu. Ključnici sta koščeno zraščeni v vilice (*furcula*), njuna apofiza pa tvori sinostozo z vrhom prsničnega gredlja (*apex carinae*). Krokarnici se končujeta na prsnici vsaka v svojem sklepnem žlebu (*sulcus articularis coracoideus*), in sicer tako, da je sternalni konec desne krokarnice položen nekoliko preko leve. Na ročaju prsnice (*manubrium sterni*) manjka notranji trn (*spina interna*), na njegovem mestu je kratek žleb. Osnovni morfološki opis dopolnjujejo slike kosti oz. skeleta in dva rentgenska posnetka na živi živali, predstavljeni so tudi rezultati meritev posameznih kosti.

Ključne besede: anatomija, skelet, ptiči, siva čaplja

Abstract: In the skeletons of four grey herons (*Ardea cinerea*), we studied the morphological characteristics of bones and skeletal groups, particularly certain segments of the skull, spine, pectoral girdle, sternum and pelvic girdle. The skull is characterized by a pronounced craniofacial bending zone (*zona flexoria craniofacialis*), long frontal bone (*os frontale*) with a distinct longitudinal depression (*depressio frontalis*), long postorbital arch (*arcus postorbitalis*), extensive temporal fossa (*fossa temporalis*) and additional shallow subtemporal fossa (*fossa subtemporalis*). The parasphenoid rostrum (*rostrum parasphenoidale*) is distinctive, pterygoids (*ossa*

pterygoidea) are strong and straight. Cervical vertebrae are characterized by stronger calcification of the sixth vertebra and its rigid link to the fifth. At the notarium region vertebrae are not fused: a joint is clearly seen between the last cervical and first thoracic vertebrae. The first three thoracic vertebrae are not fused, however, they are tightly connected at the ventral section. The fifth dorsal vertebra is associated with the synsacrum. The clavicles are fused into the fork (*furcula*) and their apophysis forms a synostosis with the top of the sternal keel (*apex carinae*). Coracoids end at the sternum, each in its own groove (*sulcus articularis coracoideus*), in a way that the sternal end of the right coracoid is placed slightly over the left. On the handle of the sternum (*manubrium s. rostrum sterni*) an internal thorn (*spina interna*) is missing, with a short groove in its place. The basic morphological description is complemented by images of bones or the skeleton, and two X-ray images of a live animal. Presented are also the measurements of individual bones.

Keywords: anatomy, skeleton, birds, *Ardea cinerea*

Uvod

Siva čaplja (*Ardea cinerea*) je močvirski ptič iz družine čapelj (Ardeidae). Naseljuje Evropo, vzhodno Azijo, zahodno Kitajsko in Afriko. Odrasla žival tehta od 1000 do 2000 g (povprečno 1700 g), visoka je od 90 do 100 cm, razpon peruti meri od 175 do 190 cm. Dnevno potrebuje okoli 400 g hrane. Prehranjuje se z manjšimi vretenčarji, predvsem ribami, dvoživkami, mišmi in voluharicami (Cramp, 1994).

Družino Ardeidae smo še nedavno uvrščali v red močvirnikov (Ciconiiformes), ki je združeval v glavnem močvirske tropske ptiče z dolgimi nogami in dolgim kljunom, ki brodijo po vodi in plenijo stoje. Na podlagi analize DNK pa je mednarodni ornitološki kongres (IOC) prerazporedil družino čapelj v red veslonožcev (Pelecaniformes) (Gill in Donsker, 2010).

Pri opisovanju in poimenovanju kosti in skeleta smo se naslonili predvsem na naslednjo literaturo: Fürbringer (1888, 1902), Boas (1929, 1933), Payne in Risley (1976), King in McLelland (1984), Rigler (1985, 1990), Kellner (1986), Baumel in sod. (1993), NAV (2012), Livezey in Zusi (2006) in Golob (2011).

Pri izhodiščni raziskavi skeleta sive čaplje (Zajc, 2010) nismo našli ustrezne domače literature, v svetovni literaturi pa je več člankov, ki obravnavajo čaplje (Ardeidae). Posebej je treba omeniti primerjalno analizo čapelj, ki sta jo opravila Payne in Risley (1976) in morfometrični opis postkranialnega skeleta čapelj v doktorski disertaciji Kellner-jeve (1986). Posamezne lastnosti skeleta čapelj so opisane v Nomina Anatomica Avium (Baumel in sod., 1993) in v delu Livezey-a in Zusi-ja (2006). V okviru priprave članka o anatomskem poimenovanju kosti ptičev (Janžekovič in sod., 2015) smo opravili poglobljeno analizo skeleta sive čaplje. Pričakujemo, da bo vsebina članka koristila tudi v veterinarski praksi, saj so pri sivi čaplji razmeroma pogoste poškodbe z zlomi kosti.

Namen raziskave je opisati anatomske značilnosti izbranih kosti sive čaplje, predvsem lobanje, hrbtenice in privesnega okostja, nekatere tudi izmeriti.

Material in metode

V raziskavo smo vključili štiri skelete sive čaplje, dva hrani Oddelek za biologijo, Fakulteta za naravoslovje in matematiko, Univerza v Mariboru, dva pa Prirodoslovni muzej Slovenije, Ljubljana.

Posamezne kosti smo opisali, izmerili in fotografirali.

Opis

Opredelili in opisali smo posamezne kosti in dele skeleta. Pri tem smo se naslonili na literaturo, ki jo navajamo v uvodu.

Merjenje

Kosti smo merili s kljunastim merilom z natančnostjo 0,1 mm, daljše kosti (> 16 cm) pa z natančnostjo 1 mm. Morfometrične spremenljivke smo povzeli po Cohen-u in Serjeantson-u (1996), predstavljene so tudi v Zajc-evi (2010) diplomski nalogi. Izračunali smo povprečne vrednosti za posamezne kosti (x) in standardni odklon (*SD*).

Fotografiranje

Fotografije smo posneli s fotoaparatom Canon PowerShot SX120 IS v največji resoluciji 3648x2736 slikovnih pik in Canon EOS 350D digital. Pri fotografiranju smo uporabili temnejšo podlago, zaradi boljšega kontrasta. Fotografiranje je izvedel Z. Golob.

Rentgen

Rentgenske slike smo posneli z aparatom FUJIFILM FCR CAPSULA x Model CR-IR 357.

Rezultati in razprava

Rezultati so razvrščeni po sklopih: skelet glave, hrbtenica z rebri in prsnico, kosti plečnega obroča in peruti ter kosti medeničnega obroča in medeničnih okončin. Nekateri rezultati so opremljeni z izsledki v literaturi in kratko razpravo. Pri predstavitvi privesnega skeleta podajamo predvsem biometrične lastnosti.

Skelet glave

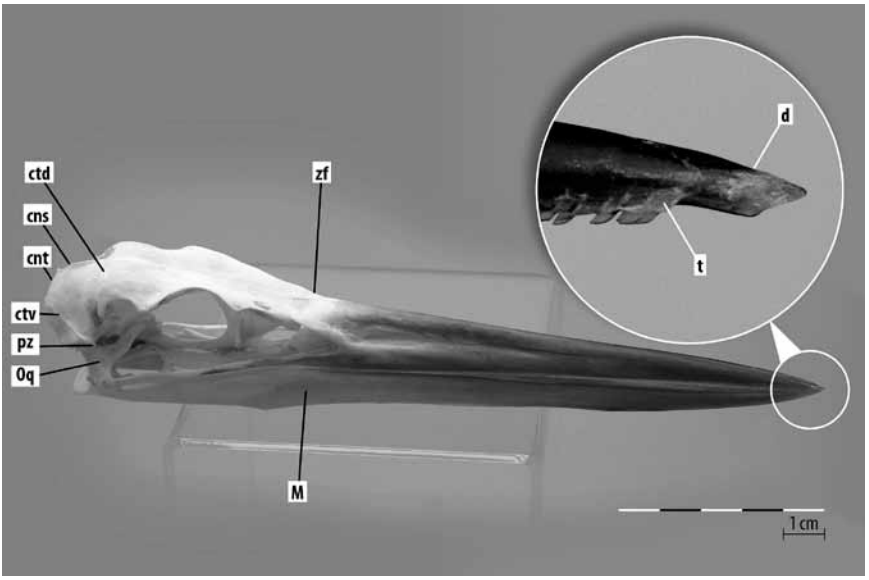
Dorzalna površina lobanje

Lobanja (*cranium*) skupaj s kljunom (*rostrum maxillae et mandibulae*) meri v dolžino povprečno 19 cm, in sicer okrog 12,2 cm od konice kljuna do kraniofacialne upogibne cone (*zona flexoria craniofacialis*) in okrog 6,6 cm od upogibne cone do kavdalnega konca lobanje; povprečna višina lobanje je 3,6 cm (Tab. 1).

Kljun je pri čaplji dolg in suličast (Sl. 1), kraniofacialna upogibna cona pa se nahaja na začetku vzdolžne depresije čelnice (*depressio frontalis*). Zadnji del te depresije je v obliki medianega jarka, ki se končuje pri prečnem senčničnem grebenu (*crista temporalis transversa*; Baumel in sod., 1993), ki ga imenujemo tudi dorzalni senčnični greben (*crista temporalis dorsalis*; Livezey in Zusi, 2006). Od tod naprej teče sagitalni tilnični greben (*crista nuchalis sagitalis*), ki se konča pri prečnem tilničnem grebenu (*crista nuchalis transversa*). Od tega grebena proti veliki odprtini (*foramen magnum*) pa se nahaja sagitalna malomožganska štrlina (*prominentia cerebellaris*). Le-ta je pri sivi čaplji slabo izražena, opazen je le nizek greben te štrline. Dorzalni senčnični greben (*crista temporalis dorsalis*) je pri sivi čaplji zglajen (Sl. 1 in 3). Lateralno se končuje s postorbitalnim podaljškom (*processus postorbitalis*), ki je kratek. Od sagitalnega tilničnega grebena in izza postorbitalnega podaljška se s temenice (*os parietale*) spušča proti senčnici (*os squamosum s. temporale*) žlebasta ugreznina, senčnična jama (*fossa temporalis*).

Tabela 1: Meritve lobanje (cm) s srednjimi vrednostmi (x) in standardnim odklonom (*SD*).
Table 1: Measurements of the skull (cm), with mean (x) and standard deviation (*SD*).

Oznaka živali Specimen	Dolžina kljuna Beak length	Dolžina lobanje Skull length	Celotna dolžina Total length	Višina lobanje Skull height
1	14,1	6,8	20,9	3,2
2	11,2	6,2	17,4	3,3
3	10,5	6,0	17,0	3,9
4	13,1	7,2	20,5	3,8
$x \pm SD$	12,2 ± 1,7	6,6 ± 0,6	19,0 ± 2,0	3,6 ± 0,4



Slika 1: Lobanja sive čaplje z desne strani.
Figure 1: The grey heron skull from right side.
Legenda / Legend: cns = *crista nuchalis sagitalis*, cnt = *crista nuchalis transversa*, ctd = *crista temporalis dorsalis*, ctv = *crista temporalis ventralis*, d = *dens,* M = mandibula, Oq = *os quadratum*, pz = *processus zygomaticus* t = *tomium*, zf = *zona flexoria craniofacialis*.

Kavdalno omejuje senčnično jamo spodnji senčnični greben (*crista temporalis ventralis*; Livezey in Zusi, 2006), ki se oddeli od prečnega tilničnega grebena (*crista nuchalis transversa*) in se končuje s kratkim ličničnim podaljškom (*processus zygomaticus*) (Sl. 3, pz). Ventrolateralno omejuje senčnično jamo rostralni greben senčnice (*crista temporalis rostralis*; Livezey in Zusi, 2006). Izrazi *crista temporalis dorsalis*, *crista temporalis ventralis* in *crista temporalis rostralis* so skladni s sedanjo anatomsko terminologijo in primernejši od izrazov *crista temporalis superior*, *crista temporalis inferior* in *crista temporalis anterior*, ki sta jih uporabila Pascotto-va in Donatelli (2003) pri opisu skeleta lobanje družine Momotidae. Izrazi *anterior*, *posterior*, *superior* in *inferior* se le izjemoma uporabljajo pri štirinožnih živalih (NAV, 2012). Pod senčnično jamo, na senčnici nad otičnim podaljškom kvadratne kosti, se nahaja plitka podsenčnična jama, *fossa subtemporalis* (Sl. 3, fst).

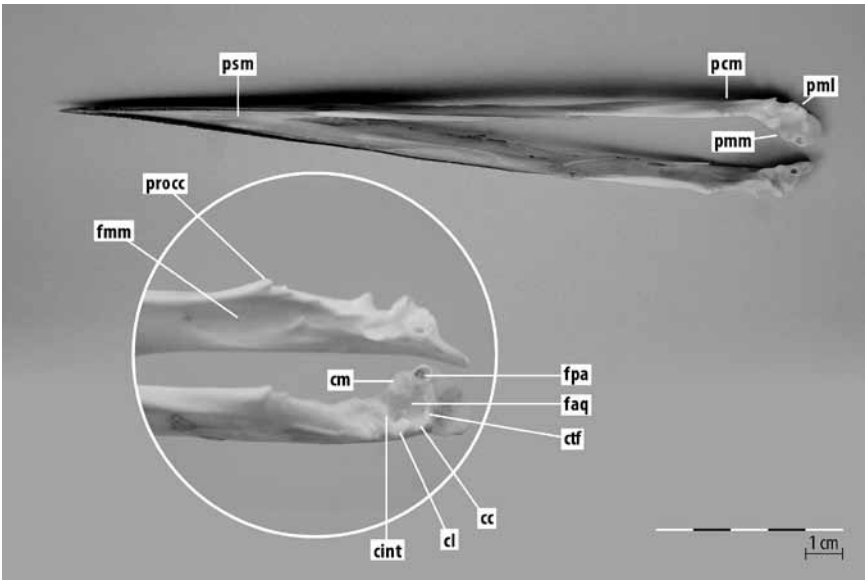
Spodnja čeljustnica (*mandibula*)

Mandibula sive čaplje ima dolg zobni (*os dentale*) oz. simfizni del (*pars symphysialis*), ki predstavlja osnovo obeh mandibularnih vej (*rami mandibulae*); sledita vmesni (*pars intermedia*) in kavdalni del (*pars caudalis*). Obe čeljustnici sta ravni, v sprednjem, tj. zobnem področju pa je mandibula nekoliko usločena, manj izrazito tudi maksila (Sl. 1). Na tem mestu je opaziti med čeljustnicama manjšo zev. Rožena prevleka (*ramphoteca*) tvori na mandibuli in maksili zobčast 1 do 2 mm visok roženi tomij (*tomium*) (Sl. 1, t). Zobci tomija so v področju usločenih delov čeljustnic izraziti in kavdalno usmerjeni. Na konici mandibule tvori ramfoteka oster koničast zaključek, medtem ko je pri maksili le-ta zadebeljen v »zob« z ostrim koncem (Sl. 1, d). Močno keratinizirana ramfoteka se razteza od vrha rostrumov v kavdalni smeri do ravni mandibularnega kota (*angulus mandibularis*).

Na svojem kavdalnem koncu, nasproti kvadratne kosti, se veji mandibule razširita in tvorita dva medsebojno povezana podaljška, medialnega (*processus medialis*) in lateralnega

(*processus lateralis*) (Sl. 2, pmm, pml). Med njima je kotanja (*fossa articularis quadratica*), ki služi artikulaciji s čvrši mandibularnega podaljška (*processus mandibularis*) kvadratne kosti (*os quadratum*) (Sl. 1 in 3). Sklepne ploskve oz. ponvice (*cotylae fossae articularis*) so pri sivi čaplji tri: medialna, lateralna in kavdalna (Sl. 2, cm, cl, cc). Pod medialno ponvico (*cotyla medialis*) se od medialne strani v notranjost sklepne kotanje razteza žleb (*sulcus intercotylaris*), pred njim pa je poševen greben (*crista intercotylaris*) z grbico (*tuberculum intercotylare*). Za medialno ponvico se nahaja sklepna zračna odprtina (*foramen pneumaticum articulare*, Sl. 2, fpa). Končni del mandibule, za sklepnim področjem s kvadratno kostjo, je značilno podkvasto podaljšan (Sl. 2 in 3), dorzokavdalna površina tega podaljška pa je nekoliko ugreznjena (*fossa caudalis s. posterior*, Sl. 3, fc). Ta jama služi pripetju mišice spuščevalke mandibule (*musculus depressor mandibulae*), ki ima svoje izhodišče v podsenčični jami (*fossa subtemporalis*) med prečnim tilničnim grebenom in senčnično jamo (Baumel in sod., 1993). Na meji s sklepnim delom mandibule se nahaja prečen greben (*crista transversa fossae*), ki se razteza med medialnim in lateralnim podaljškom mandibule (Sl. 2). Na grebenu se nahaja grbica (*tuberculum*).

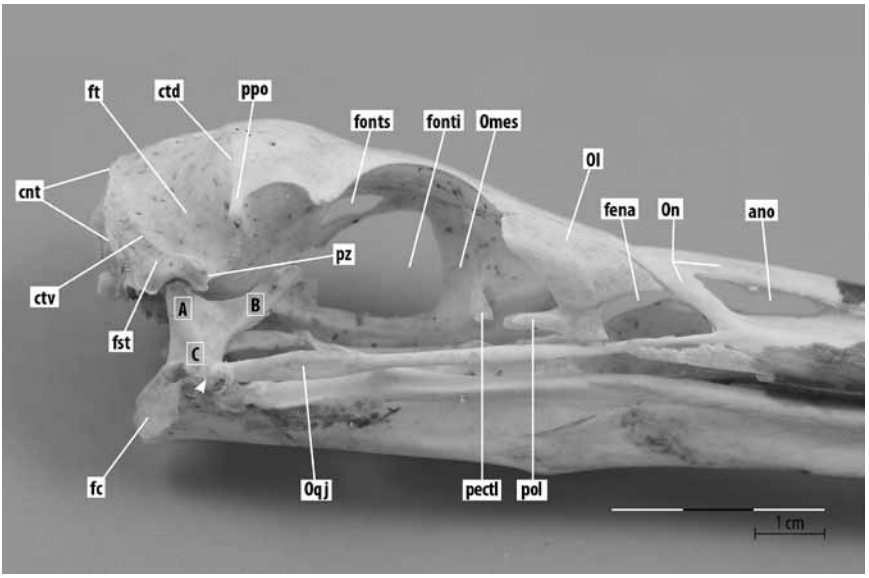
Rostralno od sklepnega področja je na medialni strani mandibularne veje ovalna jama (*fossa medialis mandibulare*) (Sl. 2, fmn), ki ima na sprednjem koncu odprtino v mandibularni kanal; imenujemo jo tudi vhodna kotanja nevrovaskularnega kanala (*fossa aditus canalis neurovascularis*) (Baumel in sod., 1993). Na kavdalnem koncu ventralnega roba te kotanje se nahaja psevdotemporalna grbica (*tuberculum pseudotemporale*), nasadiščno mesto za tetivo psevdotemporalne površinske mišice (*m. pseudotemporalis superficialis*) (Baumel in sod., 1993).



Slika 2: Mandibula sive čaplje z dorzalne strani, v okviru kavdalni del z laterodorzalne strani.
Figure 2: The grey heron mandible, dorsal view. In the circle is enlarged caudal part of the mandible, laterodorsal view.
Legenda / Legend: cc = *cotyla caudalis*, cint = *crista intercotylaris*, cl = *cotyla lateralis*, cm = *cotyla medialis*, ctf = *crista transversa fossae*, faq = *fossa articularis quadratica*, fmm = *fossa medialis mandibulae*, fpa = *foramen pneumaticum articulare*, pcm = *pars caudalis mandibulae*, pml = *processus mandibulae lateralis*, pmm = *processus mandibulae medialis*, procc = *processus coronoideus*, psm = *pars symphysialis mandibulae*.

Kvadratna kost (*os quadratum*)

Kvadratna kost je osrednja sestavina skeleta lobanje v lateroventralnem področju (Sl. 1 in 3). Sestoji iz telesa in treh glavnih podaljškov, ti so mandibularni (*processus mandibularis*), orbitalni (*processus orbitalis*) in otični podaljšek (*processus oticus*). Na mandibularnem podaljšku so štirje sklepni čvrši, in to trije na ventralni površini za stik z mandibulo (*condylus caudalis*, *condylus lateralis* in *condylus rostralis*) in eden kraniomedialno za stik s krilatko (*condylus pterygoideus*). Kraniolateralno se na tem podaljšku nahaja sklepna ponvica (*cotyla quadratojugalis*), za sklep s kvadratojarmno kostjo (Sl. 3, glava puščice). Otični podaljšek ima dva sklepna čvrša, za stik s senčnico in z otičnim stebričkom (*pila otica*; op.: kompleks zlitih otičnih koščic, ki vsebuje med drugim koščeni labirint notranjega ušesa; Baumel in sod., 1993). Končni del orbitalnega podaljška se lopatasto razširi in se prislanja na orbito, na katero se rahlo pripenja. Na posušenem skeletu je orbitalni podaljšek dejansko nekoliko odmaknjen od podlage.



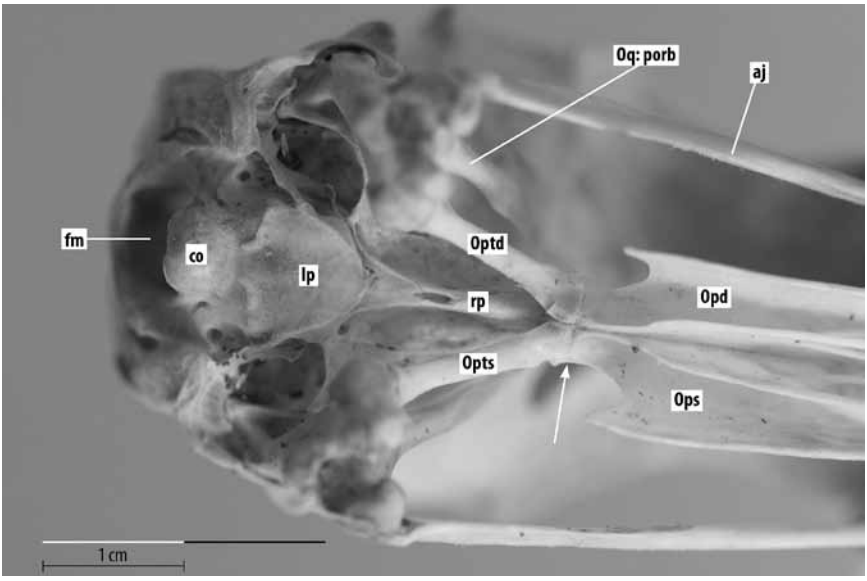
Slika 3: Zadnji del lobanje in spodnje čeljustnice z desne strani. Glava puščice označuje sklep med kvadratno kostjo in kvadratnojarmno kostjo.

Figure 3: Posterior part of the skull and mandible from the right side. The arrow-head indicates the joint between quadrate and quadratojugal bones.

Legenda / Legend: ano = *apertura nasi ossea*, cnt = *crista nuchalis transversa*, ctd = *crista temporalis dorsalis*, ctv = *crista temporalis ventralis*, fc = *fossa caudalis s. posterior*, fena = *fenestra antorbitalis*, fonti = *fonticulus interorbitalis*, fonts = *fonticulus supraorbitalis*, ft = *fossa temporalis*, fst = *fossa subtemporalis*, Ol = *os lacrimale*, On = *os nasale*, Omes = os mesethmoidale, Oqj = *os quadratojugale*, pectl = *processus ectethmoidalis lateralis*, pol = *processus orbitalis lacrimalis*, ppo = *processus postorbitalis*, pz = *processus zygomaticus*. A, B, C = *os quadratum*: A = *processus oticus*, B = *processus orbitalis*, C = *processus mandibularis*.

Krilatka (*os pterygoideum*) **in parasfenoidni ročaj** (*rostrum parasphenoidale*)

Krilatka je v paru, z značilnim položajem na spodnji površini lobanje (Sl. 4, Optd, Opts). Na koncu proti mandibularnemu podaljšku kvadratne kosti ima sklepno ponvico, pri nebnici (*os palatinum*) pa tvorita obe veji obsežnejše sklepno področje skupaj s parasfenoidnim ročajem (*rostrum parasphenoidale*) (Sl. 4, puščica). Pri čaplji je krilatka iztegnjena (ravna), medtem ko je pri nekaterih ptičih lokasta (npr. *Mergus*) ali upognjena (npr. *Vanellus*) (Baumel in sod., 1993). Za parasfenoidnim ročajem se nahaja parasfenoidna plošča (*lamina parasphenoidalis*), ki sega do podnožja zatilničnega čvrša (*condylus occipitalis*) (Sl. 4, co). To podnožje je pravzaprav ozek pas bazilarne zatilnice (*os basioccipitale*). Končni del parasfenoidne plošče pokriva mamilarna hribčka (*tuberculi basilare*), ki sta na rostralnih kotih bazilarne zatilnice.



Slika 4: Lobanja z ventralne strani. Puščica označuje sklepno področje med krilatkama in nebnicama.
Figure 4: The gray heron skull from ventral side. Arrow indicates the joint between palatines and pterygoids.
Legenda / Legend: aj = *arcus jugale*, co = *condylus occipitalis*, fm = *foramen magnum*, lp = *lamina parasphenoidalis*, Opd = *os palatinum dextrum*, Ops = *os palatinum sinistrum*, Optd = *os pterygoideum dextrum*, Opts = *os pterygoideum sinistrum*, Oq:porb = *os quadratum: processus orbitalis*, rp = *rostrum parasphenoidale*.

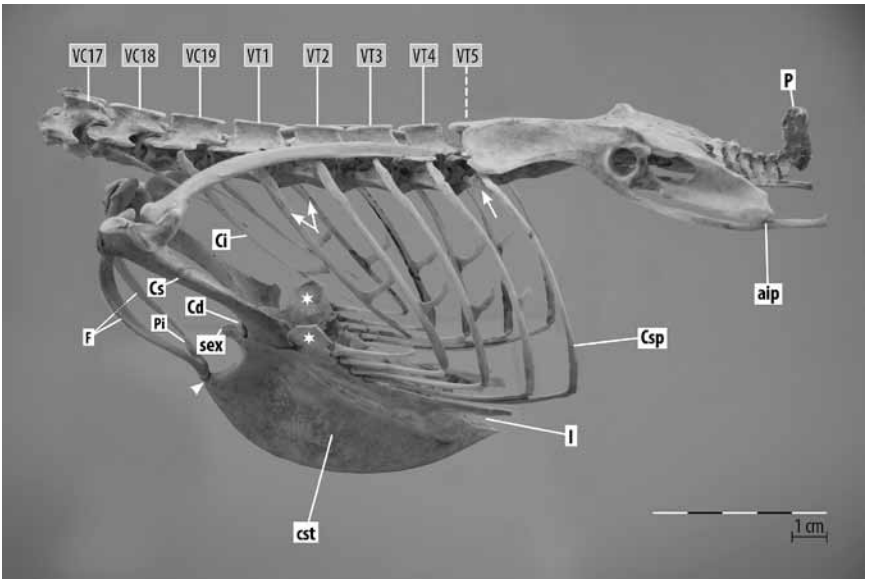
Hrbtenica (columna vertebralis)

Hrbtenica čaplje sestoji – kot pri drugih ptičih – iz vratnih in prsnih vretenc, sinsakruma, prostih repnih vretenc in pigostila. Najznačilnejše je področje vratu. Vrat je dolg in pri odraslih živalih esasto ukrivljen tudi med letom, pri lovljenju plena pa ga žival lahko sunkovito iztegne.

Vratna vretenca *(vertebrae cervicales)*

Vse proučene živali so imele po 19 vratnih vretenc. Predzadnje in zadnje nosita par nepopolnih reber (*costae incompletae*), s to razliko, da imata rebri zadnjega vratnega vretenca kavljasti podaljšek (*processus uncinatus*), rebri predzadnjega pa ne (Sl. 5). Skladno z opredelitvijo v NAA (Baumel in sod., 1993) smo za prvo prsno vretence smatrali tisto, ki nosi par popolnih pravih reber (*costae completae verae*) (Sl. 5, dvoglava puščica).

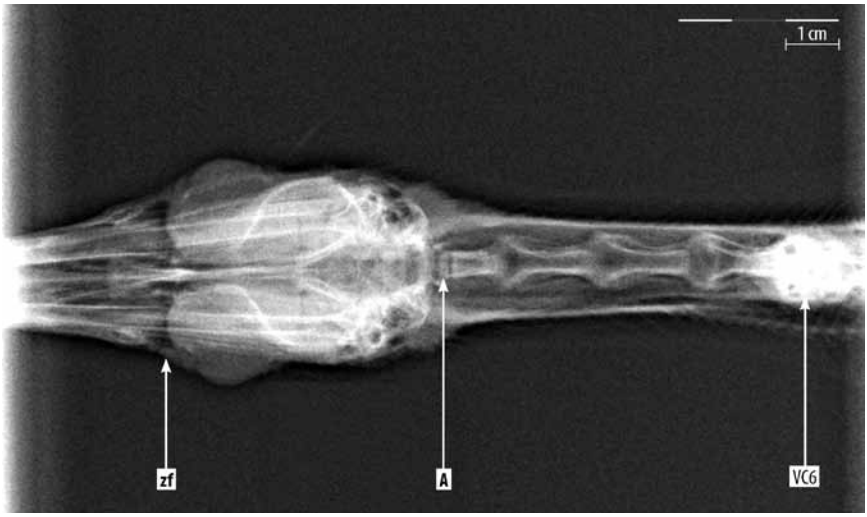
Boas (1929) je pri sivi čaplji ugotovil 16 oz. 17 vratnih vretenc. Razliko med njegovo in našo navedbo je mogoče pripisati drugačnemu metodološkemu kriteriju; domnevamo, da je Boas zadnji dve vratni vretenci štel med prsna. Boas je vratni del hrbtenice razdelil v tri segmente in v drugem segmentu ugotovil skupino sublateralno zraščenih vretenc. Pri preverjanju njegove trditve smo na rentgenskih posnetkih vratu ugotovili močnejšo kalcifikacijo pri šestem vratnem vretencu (Sl. 6) in pri skupini vretenc od 12-tega do 16-tega vretenca (Sl. 7). Potrebne so nadaljnje raziskave vratnega področja hrbtenice pri sivih čapljah različne starosti.



Slika 5: Zadnja tri vratna vretenca, prsna vretenca, medenični obroč, prosta repna vretenca, pigostil, plečnica, krokarnici, furkula, rebra in prsnica, slikano z leve strani. Enoglava puščica označuje glavico nepravega rebra, dvoglava puščica prvi par pravih reber, glava puščice pa sklep med furkulo in vrhom gredlja.

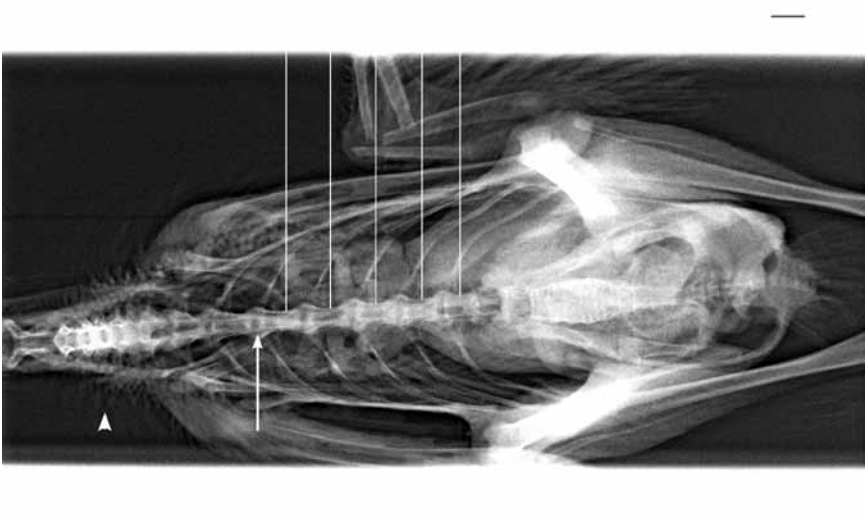
Figure 5: The last three cervical vertebrae, thoracic vertebrae, pelvic girdle, free caudal vertebrae, pygostyle, scapula, coracoid, furcula, ribs and keel from the left view. One-headed arrow marks the head of the false rib, two-headed arrow the first pair of true ribs, and the arrow-head the joint between furcula and the tip of the keel.

Legenda / Legend: aip = *articulatio ischio-pubica*, Cd = *coracoideum dextrum* (sternalni konec desne krokarnice, ki deloma sega preko leve), Ci = *costa incompleta*, Cs= *coracoideum sinistrum*, Csp = *costa spuria*, cst = *carina sterni*, F = *furcula*, l = *incisura sterni*, P = *pygostylus*, pi = *processus interclavicularis*, sex = *spina externa*, VC17, VC18, VC19 = *vertebra cervicalis* 17, 18, 19, VT1, VT2, VT3, VT4, VT5 = *vertebra thoracalis* 1, 2, 3, 4, 5, * = *processus craniolateralis sterni*.



Slika 6: Rentgenski posnetek lobanje in prvih šest vratnih vretenc z ventralne strani. Odrasla žival. Opazna je tesna povezanost med petim in šestim vretencem.

Figure 6: Radiograph of the skull and first six cervical vertebrae from the ventral side. An adult specimen. A close relationship between the fifth and sixth vertebrae is visible.
Legenda / Legend: A = *atlas*, VC6 = *vertebra cervicalis* 6, zf = *zona flexoria craniofacialis*.



Slika 7: Rentgenski posnetek trupa z dorzalne strani. Izrazitejša kalcifikacija se nahaja na področju od 12. do 16. vratnega vretenca (glava puščice) in prsnih vretenc. Notarija ni mogoče jasno opredeliti: medvretenčni sklepi se lepo vidijo (puščica: sklep med zadnjim vratnim in prvim prsnim vretencem); 1, 2, 3, 4, 5 = prvo, drugo, tretje, četrto in peto prsno vretence.

Figure 7: Radiograph of the torso from the dorsal side. Pronounced calcification is located in the area from 12th to 16th cervical vertebrae (the arrow-head) and thoracic vertebrae. The notarium cannot be clearly defined: intervertebral joints are visible (arrow: joint between the last cervical and first thoracic vertebra); 1, 2, 3, 4, 5 = the first, second, third, fourth and fifth thoracic vertebrae.

Prsna vretenca (*vertebrae thoracicae*)

Prsni del hrbtenice sestoji iz petih vretenc. Zadnje vratno in prva tri prsna vretenca so pri mnogih ptičih vrstah zlita in tvorijo enoto notarij (*notarium*). Pri sivi čaplji je stanje nekoliko drugačno, kajti med zadnjim vratnim in prvim prsnim vretencem se nahaja izrazit sklep (Sl. 7; puščica). Lateroventralni deli prvih treh prsnih vretenčnih teles so tesneje medsebojno povezani, trnaste podaljške (*processus spinosi*) pa pokriva močna vezivna opna, ki sega od zadnjega vratnega vretenca do preacetabularnega krila črevnic.

Notarijevo področje čaplje se torej razlikuje od tipičnega notarija, saj manjkajo koščene medvretenčne povezave med vrhovi trnastih podaljškov, med stranskimi podaljški in na ventralni strani. Na rentgenskih posnetkih je sicer opaziti pri vretencih notarijevega področja nekoliko izrazitejšo kalcifikacijo, a tudi medvretenčne sklepe (Sl. 7). Menimo, da je med vretenci notarija sive čaplje pričakovati določeno pregibnost. Četrto prsno vretence je prosto. Prsna vretenca tvorijo sklepe s popolnimi rebri (*costae complete*) in sicer prva štiri s pravimi rebri (*costae completae verae*), peto, ki spada v sinsakrum, pa ima par popolnih nepravih reber (*costae completae spuriae*). Slednji nimata kavljastega podaljška, njun vertebralni del pa je prek rebrne grčice (*tuberculum*) zraščen s prečnim podaljškom (*processus transversus*) petega prsnega vretenca in ta s preacetabularnim delom črevnice (Sl. 9).

Sinsakrum (*synsacrum*)

Preacetabularni del sinsakruma sestoji iz enega prsnega in štirih ledvenih vretenc, dve ali tri križna vretenca oblikujejo acetabularni del sinsakruma in šest zlitih repnih vretenc postacetabularni del sinsakruma. Boas (1933) navaja pri sinsakrumu sive čaplje skupno 14 ali 15 zraščenih vretenc. Med zadnjim prsnim in prvim ledvenim vretencem je pri mlajših živalih opaziti sklepno progo, medtem ko so naslednja vretenca sinsakruma že ankilozno zraščena. Sinsakrum je sinostozno zraščen s kolčnicama (*ossa coxae*).

Prosta repna vretenca (*vertebrae caudales liberae*)

Prostih repnih vretenc je pet, ki imajo dobro razvite prečne podaljške (Sl. 9 in 10).

Pigostil (*pygostylus*)

Pigostil ali repni opornik je enojna in skoraj pravokotno navzgor obrnjena kost (Sl. 5). Oblikuje bazo (*basis pygostyli*), ki ima na kranialnem koncu sklepno ploskev (*facies articularis cranialis*) za sklep z zadnjim prostim repnim vretencem. Plošča pigostila (*lamina pygostyli*) meri pri sivi čaplji 15 x 7 mm.

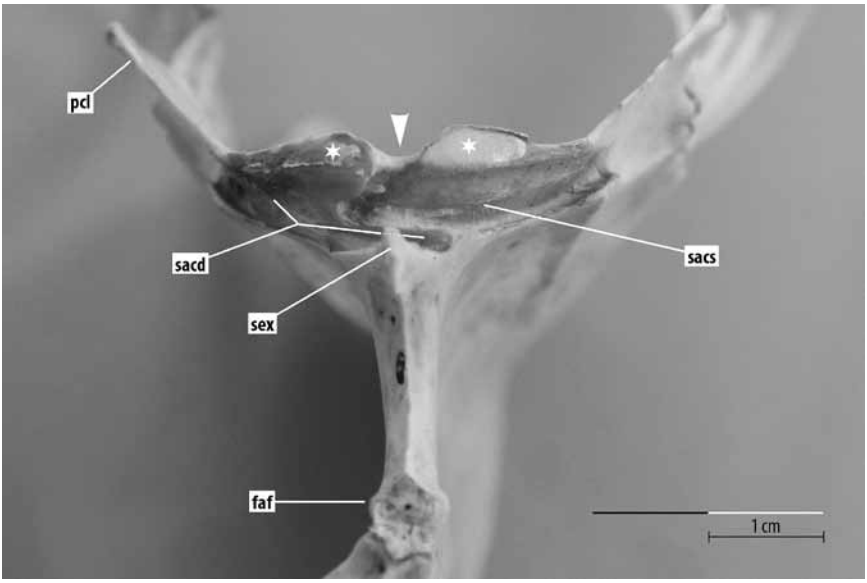
Rebra (*costae*)

Zadnji dve vratni vretenci nosita para nepopolnih reber (*costae incompletae*) (slika 5); pri rebrih zadnjega vratnega vretenca je prisoten kavljasti podaljšek (*processus uncinatus*). Prsna vretenca nosijo popolna prava rebra (*costae completae verae*) in prvo ledveno popolni nepravi rebri (*costae completae spuriae*), ki pa nimata kavljastega podaljška (Sl. 5, Csp).

Prsnica (*sternum*)

Prsnica je dobro razvita in z izrazitim gredljem (*carina sterni*) (Sl. 5, cst). Pri odrasli čaplji meri v dolžino 9 cm. Njen kranialni konec predstavlja rebrni del (*costosternum*) in kavdalni konec ksifoidni del (*xiphosternum* ali *metasternum*) (Fürbringer, 1888).

Na kranialnem koncu prsnice se nahajata sklepna žlebova za krokarnici (*sulci articularis coracoidei*) (Sl. 8, sacd/sacs). Pri čaplji se krokarnici v svojem distalnem delu ne končujeta v skupni ravnini, ampak se nekoliko prekrižata: desna krokarnica je pomaknjena deloma preko leve in njen konec je zato nekoliko ventralnejši od konca leve krokarnice. Takšno oz. podobno umestitev krokarnic na prsnici so opisali tudi pri turakih (Musophagidae) (Fürbringer, 1888), pri nekaterih cevonoscih (Procellariiformes) ter pri *Buteo*, Falkoniformes, *Bubo* in *Ichtyornis* (Baumel in sod., 1993).



Slika 8: Prsnica s sprednje strani. Posebej so označeni sklepni ploskvi na notranji ustni (*), žleb v mediani ravnini (glava puščice), krokarnična sklepna žleba (sacd, sacs), zunanji trn (sex) kljuna ali ročaja prsnice (*rostrum s. manubrium sterni*) in (hrapava) sklepna ploskev za vilice (faf).

Figure 8: Sternum – frontal view.
Legenda / Legend: faf = *facies articularis furculae*, pcl = *processus craniolateralis sterni*, sacd / sacs = *sulcus articularis coracoideus dexter / sinister*, sex = *spina externa rostri*.

Pod sklepnim področjem s krokarnicama se v kranialni smeri razteza okrog 8 mm dolg bilateralno stisnjen podaljšek, zunanji trn (*spina externa*) (Sl. 5 in 8, sex), ki predstavlja najizrazitejši del prsničnega kljuna ali ročaja (*rostrum s. manubrium sterni*). Notranji trn (*spina interna*) pri čaplji manjka, na njegovem mestu pa se nahaja zareza oz. kratek žleb (Sl. 8). Grebenasta zunanja ustna (*labrum externum*) se nahaja pred umestitvijo končnega dela krokarnic v prečna sklepna žlebova (*sulci articularis coracoidei*) in se razteza lateralno od zunanjega trna. Nad sklepnim žlebom za krokarnico je notranja ustna (*labrum internum)*. Levi in desni del notranje ustne oblikujeta posebno ovalno in na medialnem koncu nekoliko razpotegnjeno sklepno ploskev za krokarnico (Sl. 8, zvezdici).

Dorzalna ali visceralna površina prsnice je vzdolžno kotanjasta, obsega pa srčni in jetrni del (*pars cardiaca et pars hepatica*). Pred srčnim delom se nahaja obsežnejša zračna odprtina (*foramen pneumaticum*). Za sklepnim področjem s krokarnicama se strani prsnice dvigata v izrazit kraniolateralni podaljšek (*processus craniolateralis*) in se nato spustita v sklepno področje z rebri. V tem sklepnem področju so pri čaplji po štiri sklepne ponvice za sklep s sternalnim koncem popolnih pravih reber. Pri opazovanju s strani vidimo, da se omenjene sklepne ponvice nahajajo na kratkih prsnorebrnih sklepnih podaljških (*proccessus articularis sternocostalis*), pred vsakim sklepnim podaljškom pa je majhna a izrazita zareza z globeljo. Rebrni rob prsnice (*margo costalis sterni*) se od sklepnega področja z rebri nadaljuje v ksifoidni del prsnice, ki je na kavdalnem koncu zarezan v obliki črke V (Sl. 5). Gledano od zadaj (dorzokavdalno oz. ventralno) vidimo obe zarezi, levo in desno, ki spominjata na črko W.

Apendikularni skelet (skeleton appendiculare)

Plečni obroč (*ossa cinguli*)

Plečnica (*scapula*) je izrazito sabljaste oblike (Sl. 5) in sega kavdalno do sprednjega roba črevnice. Povprečna dolžina obeh plečnic je 8,4 cm

(Tab. 2 in 15). Skupaj s krokarnico in dorzalnim okrajkom ključnice (*extremitas omalis claviculae*) sooblikuje trikostni kanal.

Tabela 2: Meritve na plečnici (*scapula*; v cm); izračun povprečnih vrednosti (*x*) in standardnega odklona (SD) za levo in desno plečnico ter za obe skupaj.
Table 2: Measurements of the scapula (cm), with mean (*x*) and standard deviation (*SD*). L-left, D-right scapula.

Živali Specimen	Celotna dolžina Total length		Največja kranialna diagonala Maximal cranial diagonal	
	L	D	L	D
1	8,8	8,8	1,6	1,6
2	8,5	8,6	1,4	1,4
3	7,7	7,9	1,4	1,5
4	8,6	8,5	1,6	1,5
x	8,4	8,5	1,5	1,5
SD	0,5	0,4	0,1	0,1
L + D	8,4 ± 0,4		1,5 ± 0,1	

Ključnici (*claviculae*) sta ventralno v apofiznem področju koščeno zraščeni v vilice ali furkulo (*furcula*), končni del vilic pa oblikuje sklep s konico gredlja (*apex carinae*) (Sl. 5). Fürbringer (1902) je opisal ta sklep kot sinostozo. Pri sivi čaplji opazimo v apofiznem delu vilic (*apophysis furculae*) izrazit medključnični podaljšek (*processus interclavicularis*), ki meri okrog 7 x 2 mm in se razteza proksimalno v kot med obema vejama furkule (Sl. 5, pi). Dorzalni konec ključnice ima podaljšek (*processus acrocoracoideus*) in sklepno ploskev za stik s krokarnico (*facies articularis acrocoracoidea*). Ostrčev podaljšek ključnice (*processus acromialis claviculae*) oblikuje sklepni stik z ostrcem plečnice (*acromion*).

Krokarnica (*os coracoideum*) je krepka kost, dolga okrog 6,9 cm (Tab. 3 in 15). Dorzalni konec (*extremitas omalis coracoidei*) je s sklepi in vezmi čvrsto povezan s plečnico, vilicami in nadlahtnico, ventralni konec (*extremitas sternalis coracoidei*) pa s prsnico. Krokarnici sta v sklepnem področju s prsnico poravnani tako, da sega desna nekoliko navzpred od leve (Sl. 5, Cd), vsaka pa je umeščena v svoj sklepni žleb na prsnici (*sulcus articularis coracoideus*) (Sl. 8, sacd/sacs).

Tabela 3: Meritve leve in desne krokarnice (*os coracoideum*): njene celotne dolžine, bazalne širine, širine sklepne površine in dolžine njene medialne strani (v cm), posebej za levo in desno ter za obe skupaj (L+D).
Table 3: Measurements of the coracoid (cm), with mean (*x*) and standard deviation (*SD*). L-left, D-right coracoid.

Živali Specimen	Celotna dolžina krokarnice Total lenght		Bazalna širina Basal breadth		Širina sklepne površine Breadth of the joint surface		Dolžina med. strani Length of the medial side	
	L	D	L	D	L	D	L	D
1	7,1	6,8	3,0	3,0	2,3	2,2	6,5	6,4
2	6,7	6,7	2,6	2,8	2,0	2,1	6,1	6,0
3	6,8	6,8	2,3	2,4	2,0	2,1	5,8	5,9
4	7,1	7,2	2,1	2,2	2,7	2,7	6,3	6,2
x	6,9	6,9	2,5	2,5	2,3	2,3	6,2	6,1
SD	0,2	0,2	0,4	0,4	0,3	0,3	0,3	0,2
L + D	6,9 ± 0,2		2,6 ± 0,4		2,3 ± 0,3		6,2 ± 0,2	

Kosti prsnih okončine (*ossa alae*)

Pri čaplji je **nadlahtnica** (*humerus*) dolga, saj meri pri odrasli živali okrog 17,6 cm (Tab. 4). Na proksimalnem koncu nadlahtnice se nahaja glava (*caput humeri*) in dobro razvit prsni greben (*crista deltopectoralis*) za nasadišče prsnih mišic (kranialna površina). Na kavdalni površini glave nadlahtnice leži odprtina (*foramen pneumaticum*), skozi katero vstopa stranski betič ključnične zračne vrečke. **Koželjnica** (*radius*) je vzdolžno usločena in meri okrog 20 cm (Tab. 5). **Komolčnica** (*ulna*) je prav tako usločena in meri okrog 21 cm (Tab. 6). Njen ventralni rob ima papile za nasadišče vezi peresnih čebulic sekundarnih letalnih peres (*papillae remigales ventrales*), ki jih je pri sivi čaplji 12. Kavdalni konec koželjnice in komolčnice se v sklepu povezuje z nadlahtnico, kranialni pa oblikuje sklepno povezavo z zapestjem. **Zapestnici** (*ossa carpi*), koželjnična zapestnica (*os carpi radiale*) in komolčična zapestnica (*os carpi ulnare*) sta enako dolgi, in to 1,1 cm. **Zapestnodlančna kost** (*carpometacarpus*) je dolga 9,3 cm (Tab. 7). Dolžina **prstnic** (*ossa digitorum manus*) je predstavljena v preglednici (Tab. 8). Kostna osnova iztegnjene peruti meri pri sivi čaplji okrog 54 cm.

Tabela 4: Meritve nadlahtnice (*humerus*): njene dolžine, največje širine na proksimalnem koncu, najmanjše širine telesa in največje širine na distalnem koncu (v cm), posebej za levo in desno ter za obe skupaj (L+D), z aritmetičnimi sredinami (*x*) in standardnimi odkloni (*SD*).

Table 4: Measurements of the humerus (cm), with mean (*x*) and standard deviation (*SD*). L-left, D-right humerus.

Živali Specimen	Celotna dolžina nadlahtnice Total lenght		Največja širina proksimalnega konca Maximal breadth of the proximal end		Najmanjša širina telesa Minimal breadth of the corpus		Največja širina dist. konca Maximal breadth of the distal end	
	L	D	L	D	L	D	L	D
1	17,7	18,0	2,7	2,7	1,0	1,0	2,4	2,5
2	17,5	17,5	2,6	2,6	1,0	1,0	2,2	2,2
3	16,7	16,8	2,4	2,5	0,9	0,9	2,1	2,1
4	18,1	18,4	2,6	2,5	0,9	1,0	2,3	2,1
x	17,5	17,7	2,6	2,6	1,0	1,0	2,3	2,2
SD	0,6	0,7	0,1	0,1	0,1	0,1	0,1	0,2
L + D	17,6 ± 0,6		2,6 ± 0,1		1,0 ± 0,1		2,2 ± 0,2	

Tabela 5: Meritve koželjnice (*radius*): njene dolžine, najmanjše širine debla in največje širine na distalnem koncu (v cm), posebej za levo in desno ter za obe skupaj (L + D), z aritmetičnimi sredinami (*x*) in standardnimi odkloni (*SD*).

Table 5: Measurements of the radius (cm), with mean (*x*) and standard deviation (*SD*). L-left, D-right radius.

Žival Specimen	Celotna dolžina Total lenght		Najmanjša širina debla Minimal breadth of corpus		Največja distalna širina Maximal distal breadth	
	L	D	L	D	L	D
1	20,1	20,1	0,4	0,4	1,3	1,2
2	19,7	19,6	0,4	0,4	1,1	1,1
3	19,5	19.4	0,4	0,4	1,0	1,0
4	20,3	20,2	0,4	0,5	1,0	1,1
x	19,9	19,8	0,4	0,4	1,1	1,1
SD	0,4	0,4	0,0	0,1	0,1	0,1
L+D	19,9 ± 0,4		0,41 ± 0,04		1,1 ± 0,1	

Tabela 6: Meritve komolčnice (*ulna*): njene dolžine, največje širine na proksimalnem koncu, diagonalne širine proksimalnega konca, najmanjše širine telesa ter diagonalne širine distalnega konca (v cm), posebej za levo in desno ter za obe skupaj (L + D), z aritmetičnimi sredinami (*x*) in standardnimi odkloni (*SD*).

Table 6: Measurements of the ulna (cm), with mean (*x*) and standard deviation (*SD*). L-left, D-right ulna.

Žival Specimen	Dolžina Total lenght		Širina proksimalnega konca Breadth of the proximal end		Diagonala proks. konca Diagonal of the proximal end		Najmanjša širina telesa Minimal breadth of the corpus		Diagonala distal. konca Diagonal of the distal end	
	L	D	L	D	L	D	L	D	L	D
1	20,8	21,0	1,7	1,7	1,9	1,8	0,7	0,7	1,3	1,3
2	20,6	20,5	1,5	1,5	1,8	1,7	0,6	0,6	1,3	1,3
3	20,3	20,2	1,5	1,6	1,2	1,3	0,5	0,7	1,3	1,3
4	21,4	21,5	1,6	1,6	1,5	1,5	0,6	0,7	1,3	1,4
x	20,8	20,8	1,6	1,6	1,6	1,6	0,6	0,7	1,3	1,3
SD	0,5	0,6	0,1	0,1	0,3	0,2	0,1	0,1	0,0	0,1
L+D	20,8 ± 0,5		1,6 ± 0,1		1,6 ± 0,3		0,6 ± 0,1		1,3 ± 0,04	

Tabela 7: Meritve zapestnodlančnice (*carpometacarpus*): njene dolžine, dolžine manjše dlančnice, največje širine proksimalnega konca in diagonalne širine na distalnem koncu (v cm); posebej za levo in desno ter za obe skupaj (L + D), z aritmetičnimi sredinami (*x*) in standardnimi odkloni (*SD*).

Table 7: Measurements of the carpometacarpus (cm), with mean (*x*) and standard deviation (*SD*). L-left, D-right carpometacarpus.

Žival Specimen	Skupna dolžina Total length		Dolžina manjše dlančnice Length of the minor metacarpal		Največja proks. širina Maximal proximal breadth		Diagonala distalnega konca Diagonal of the distal end	
	L	D	L	D	L	D	L	D
1	9,6	9,6	9,5	9,5	1,7	1,8	1,4	1,3
2	9,2	9,2	9,1	9,1	1,6	1,6	1,2	1,2
3	8,9	8,9	8,7	8,7	1,3	1,4	1,1	1,0
4	9,7	9,6	9,3	9,2	1,6	1,7	1,2	1,1
x	9,4	9,3	9,2	9,1	1,6	1,6	1,2	1,2
SD	0,4	0,3	0,3	0,3	0,2	0,2	0,1	0,1
L+D	9,3 ± 0,3		9,1 ± 0,3		1,6 ± 0,2		1,2 ± 0,1	

Tabela 8: Dolžina bazalne in terminalne prstnice večjega in dolžina prstnice manjšega prsta peruti (v cm); posebej za levo in desno stran ter za obe skupaj (L + D), z aritmetičnimi sredinami (*x*) in standardnimi odkloni (*SD*).

Table 8: Measurements of the basal and terminal phalanx of the major digit and length of the minor digit (cm), with mean (*x*) and standard deviation (*SD*). L-left, D-right digit.

Žival Specimen	Večji prst: bazalna prstnica Basal phalanx of the major digit		Večji prst: terminalna prstnica Terminal phalanx of the major digit		Dolžina manjšega prsta Length of the minor digit	
	L	D	L	D	L	D
1	3,5	3,4	3,1	3,1	1,6	1,6
2	3,1	3,1	2,7	2,7	1,4	1,4
3	3,0	3,1	2,6	2,7	1,1	1,0
4	3,4	3,3	2,9	3,0	1,3	1,3
x	3,3	3,2	2,8	2,9	1,4	1,3
SD	0,2	0,2	0,2	0,2	0,2	0,3
L+D	3,2 ± 0,2		2,9 ± 0,2		1,3 ± 0,2	

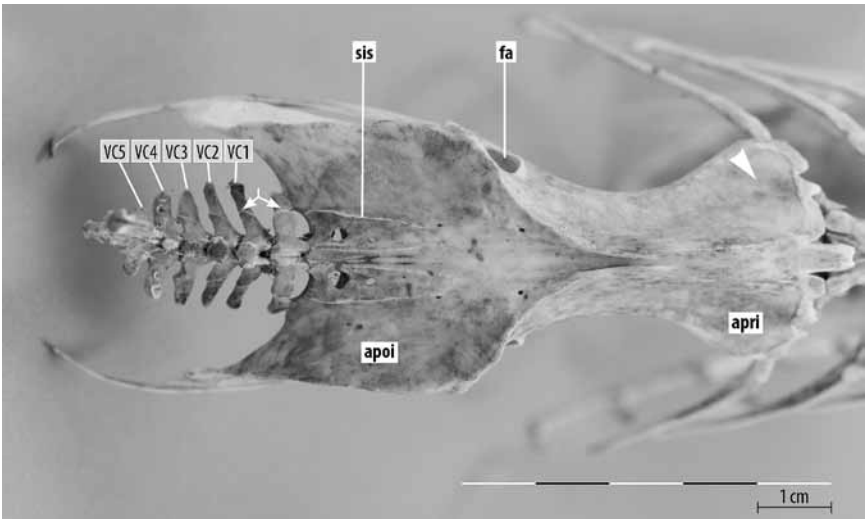
*Kosti medeničnega obroča in okončin (*ossa cinguli membri pelvici*)*

Medenica (*pelvis*)

Pri odrasli sivi čaplji so medenične kosti **črevnica** (*ilium*), **sednica** (*ischium*) in **dimeljnica** (*pubis*) zlite v **kolčnico** (*os coxae*). Kolčnici sta zrasli s sinsakrumom pa tudi druga z drugo v področju preacetabularnih kril, nad dorzalnim grebenom sinsakruma (Sl. 9). Zraslost s sinsakrumom je temeljita, tako da črevničnokrižničnega šiva (*sutura iliosynsacralis*) v področju postacetabularnih kril črevnic ni opaziti. Ta šiv je npr. pri kokoši in puranu izrazit. Se pa na mestu zrasti s kavdalnim koncem sinsakruma, med prečnimi podaljški zlitih vretenc sinsakruma in krilom črevnice (tj. na mestu omenjenega šiva), nahaja sinostozen sklep (*synostosis iliosynsacralis*), ki oblikuje greben (Sl. 9, sis). V tem področju so pri sinsakrumu tri odprtine, *foramina intertransversaria*. Sicer pa se omenjeni greben nadaljuje s presledki prek medvretenčnih prostorov na prečnih podaljških prvih štirih prostih repnih vretenc (Sl. 9, dvoglava puščica).

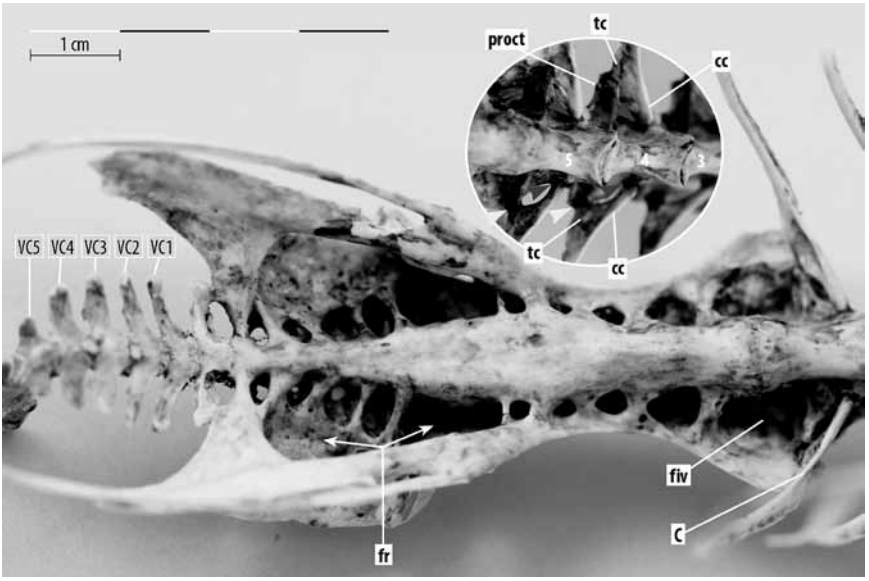
Pri čaplji je črevnična dorzalna kotanja preacetabularnih kril (*fossa iliaca dorsalis*) plitka, plitka je tudi kavdalna črevnična kotanja (*fossa iliocaudalis*) na koncu postacetabularnih kril črevnice. Črevničnosednična odprtina (*foramen ilioischiadicum*) je pri sivi čaplji ovalna, zadelana odprtina (*foramen obturatum*) pa se v kavdalni smeri nadaljuje v podaljšano sedničnodimeljnično okno (*fenestra ischiopubica*) (Sl. 5).

Medenica sive čaplje je razmeroma ozka, v področju sinsakruma meri v dolžino okrog 8,0 cm in v širino okrog 4,0 cm, zato sta tudi kotanji za umestitev ledvic ozki in razpotegnjeni. Kotanja na ventralni strani preacetabularnih kril (*fossa iliaca ventralis*) (Sl. 10, fiv) omogoča umestitev kranialnega dela ledvic. V področju postacetabularnih kril črevnice je ledvična kotanja (*fossa renalis*) (Sl. 10, fr), ki je iz dveh z žlebom povezanih delov, manjšega kranialnega in obsežnejšega kavdalnega. Kranialni del (*pars ischiadica fossae*) se nahaja v področju acetabuluma in kavdalni del (*pars pudenda fossae*) v področju črevničnosednične odprtine (*foramen ilioischiadicum*).



Slika 9: Področje sinsakruma, kolčnic, prostih repnih vretenc in pigostila z dorzalne strani. Sklepna odprtina kolčnice (fa) predeli medenični obroč na preacetabularni, acetabularni in postacetabularni del. Preacetabularni krili črevnice sta medsebojno zrasli, postacetabularni krili pa tvorita s sinsakrumom črevničnosinsakralno sinostozo (sis). Puščici: prečni podaljšek vretenc (*processus transversus*), glava puščice: *fossa iliaca dorsalis*.

Figure 9: The synsacrum area, hip, free caudal vertebrae and pygostyl; dorsal view.
Legenda / Legend: apoi = *ala postacetabularis ilii*, apri = *ala preacetabularis ilii*, fa = *foramen acetabuli,* sis = *synostosis iliosynsacralis,* VC1, VC2, VC3, VC4, VC5 = *vertebra caudalis libera* 1, 2, 3, 4, 5. Arrows = *processus transversus*, Head of arrow = *fossa iliaca dorsalis.*



Slika 10: Obroč medenične okončine z ventralne strani, v okviru področje tretjega, četrtega in petega prsnega vretenca. Izrazitejši globeli sta ventralna črevnična jama (fiv) in ledvična jama (fr), slednja je predeljena na kranialni sednični del (*pars ischiadica fossae*) in kavdalni dimeljni del (*pars pudenda fossae*). Prosta repna vretenca (VC1, 2, 3, 4, 5). V okviru: Nepravo popolno rebro z glavico (*cc*) in grčico (*tc*). 3, 4, 5 = tretje, četrto (prosto) in peto prsno vretence, temna glava puščice = sklep glavice rebra (*cc*) s telesom vretenca (*corpus vertebrae*) na lateralni površini vretenca, svetla glava puščice = sklep kvrgice rebra (*tc*) s prečnim podaljškom (*proct*) vretenca.

Figure 10: The pelvic girdle from ventral side with the third, fourth and fifth thoracic vertebrae in the circle. **Legenda / Legend**: 3, 4, 5 = *vertebrae thoracicae* 3, 4, 5, C = *costa completae spuria,* cc = *capitulum costae*, fiv = *fossa iliaca ventralis,* fr = *fossa renalis,* proct = *processus transversus*, tc = *tuberculum costae*, VC1, VC2, VC3, VC4, VC5 = *vertebra caudalis libera* 1, 2, 3, 4, 5.

Tabela 9: Meritve sokrižnice (*synsacrum*) in medenice (*pelvis*) (v cm) z aritmetičnimi sredinami (*x*) in standardnimi odkloni (*SD*).

Table 9: Measurements of the synsacrum (cm), with mean (*x*) and standard deviation (*SD*).

Živali Specimen	Celotna dolžina Total length	Dolžina medenice Pelvis length	Dolžina vzdolž sinsakralnih vretenc Synsacrum vetebrae length	Najmanjša širina Minimal breadth	Širina v sredini Middle breadth
1	9,4	9	9,6	1,8	4,0
2	9,2	8,9	7,4	1,7	3,6
3	10,5	9,1	7,8	1,6	3,4
4	11,5	9,8	8,5	1,8	4,0
x	10,2	9,2	8,3	1,7	3,8
SD	1,1	0,4	1,0	0,1	0,3

Kosti medeničnih okončin (ossa membri pelvici)

Stegnenica (*femur*) je razmeroma kratka, saj pri odrasli živali meri okrog 9,0 cm (Tab. 10 in 15), **goleničnonartna kost** (*os tibiotarsus*) pa okrog 20,8 cm (Tab. 11 in 15). **Mečnica** (*fibula*) je v poprečju dolga 9,7 cm (razpon od 9,0 do 10,2 cm) in sega približno do sredine goleničnonartne kosti.

Nartnostopalna kost (*tarsometatarsus*) meri v dolžino povprečno 15,3 cm, največja širina proksimalnega konca meri povprečno 1,4 cm, najmanjša širina telesa 0,6 cm in največja širina distalnega konca 1,4 cm (Tab. 12). Distalni konec nartnostopalne kosti ima tri ločene in izrazite sklepne površine v obliki valja (*trochleae metatarsi II, III et IV*). Meritve prstnic zadnje okončine so prikazane v tabelah 13 in 14. Prvi prst (*hallux*) je obrnjen nazaj; meri 5,6 cm v dolžino in ima dve prstnici. Drugi prst (*digitus secundus*) meri okrog 8 cm in ima tri prstnice, tretji prst (*digitus tertius)* meri 10,5 cm in je najdaljši, četrti prst (*digitus quartus*) ima pet prstnic in je dolg 8,6 cm. Drugi, tretji in četrti prst so obrnjeni naprej (anizodaktilni tip noge).

Payne in Risley (1976) sta predstavila osnovne značilnosti skeleta čapelj (Ardeidae) in jih primerjala med vrstami te družine, kakor tudi z nekaterimi drugimi skupinami (Balaenicipitidae, Scopidae, Ciconiidae, Threskiornithidae, Phoenicopteridae). Med proučenimi omenjata tudi dva skeleta sive čaplje (*Ardea cinerea*), ki pa v rezultatih nista prikazana. Našim rezultatom so primerljivi podatki, ki sta jih navedla za ameriško sivo čapljo (*Ardea herodias*; 12 skeletov). To je skladno s splošno sprejetim stališčem, da sta siva čaplja (*Ardea cinerea*) in ameriška siva čaplja (*Ardea herodias*) v tesnem sorodstvu in sta si podobni.

Kellner-jeva (1986) je proučila postkranialni skelet evropskih čapelj (*Ardea cinerea*, *Ardea purpurea*, *Casmerodius albus*, *Egretta garzetta*, *Ardeola ralloides*, *Bubulcus ibis*, *Nycticorax nycticorax*, *Ixobrychus minutus*, *Botaurus stellaris*). Opisala in izmerila je prsnico (*sternum*), krokarnico (*os coracoideum*), plečnico (*scapula*), vilice (*furcula*), nadlahtnico (*humerus*), koželjnico (*radius*), komolčnico (*ulna*), zapestnodlančnico (*carpmetacarpus*), medenico (*pelvis*), stegnenico (*femur*), goleničnonartno kost (*tibiotarsus*) in nartostopalnico (*tarsometatarsus*). Ugotovila je trend sekundarnega spolnega dimorfizma, samci imajo nekoliko krepkejše kosti, vendar pa te razlike niso statistično značilne. Iz njenih podatkov za sivo čapljo smo izračunali poprečne vrednosti za vse živali, ki jih je izmerila (tj. za samce, samice in živali z neugotovljenim spolom) in dobili vrednosti, ki se ne razlikujejo od naših rezultatov oz. so razlike neznatne (Tab. 15). Poleg meritev omenjenih kosti smo v naši raziskavi proučili še morfometrične parametre lobanje ter prstnic in prstov.

Tabela 10: Meritve stegnenice (*femur*; v cm): posebej za levo in desno stegnenico ter za obe skupaj (L+D); z aritmetičnimi sredinami (*x*) in standardnimi odkloni (*SD*).
Table 10: Measurements of the femur (cm), with mean (*x*) and standard deviation (*SD*). L-left, D-right femur.

Žival Specimen	Dolžina Length		Največja proks. širina Maximal proximal breadth		Najmanjša širina telesa Minimal corpus breadth		Največja distalna širina Maximal distal breadth		Največja distal. globina Maximal distal depth	
	L	D	L	D	L	D	L	D	L	D
1	9,4	9,4	1,6	1,6	0,7	0,7	1,6	1,6	1,4	1,4
2	8,9	8,8	1,5	1,5	0,7	0,6	1,5	1,5	1,4	1,3
3	8,5	8,5	1,3	1,4	0,8	0,8	1,2	1,2	1,2	1,1
4	9,2	9,2	1,6	1,7	0,8	0,8	1,5	1,5	1,3	1,3
x	9,0	9,0	1,5	1,6	0,8	0,7	1,5	1,5	1,3	1,3
SD	0,4	0,4	0,1	0,1	0,1	0,1	0,2	0,2	0,1	0,1
L+D	9,0 ± 0,4		1,5 ± 0,1		0,7 ± 0,1		1,5 ± 0,2		1,3 ± 0,1	

Tabela 11: Meritve goleničnonartne kosti (*tibiotarsus*; v cm), leve in desne ter obeh skupaj (L+D).
Table 11: Measurements of the tibiotarsus (cm), with mean (*x*) and standard deviation (*SD*). L-left, D-right tibiotarsus.

Žival Specimen	Celotna dolžina Total length		Najmanjša širina telesa Minimal corpus breadth		Globina distal. konca Distal end depth	
	L	D	L	D	L	D
1	21,5	21,3	0,6	0,5	1,5	1,5
2	20,2	20,4	0,5	0,5	1,3	1,3
3	19,0	19,1	0,5	0,5	1,3	1,3
4	22,3	22,2	0,5	0,6	1,4	1,4
x	20,8	20,8	0,5	0,5	1,4	1,4
SD	1,5	1,3	0,1	0,1	0,1	0,1
L+D	20,8 ± 1,3		0,5 ± 0,05		1,4 ± 0,1	

Tabela 12: Meritve nartostopalnice (*tarsometatarsus*; v cm): posebej leve in desne ter obeh (L + D).
Table 12: Measurements of the tarsometatarsus (cm), with mean (*x*) and standard deviation (*SD*). L-left, D-right tarsometatsarsus.

Žival Specimen	Celotna dolžina Total length		Največja proks. širina Maximal proximal breadth		Najmanjša širina telesa Minimal corpus breadth		Največja distalna širina Maximal distal breadth	
	L	D	L	D	L	D	L	D
1	15,8	15,8	1,5	1,5	0,6	0,6	1,5	1,5
2	14,8	14,8	1,3	1,4	0,5	0,6	1,3	1,3
3	14,1	14,0	1,2	1,3	0,6	0,5	1,3	1,3
4	16,7	16,7	1,4	1,5	0,6	0,5	1,4	1,4
x	15,4	15,3	1,4	1,4	0,6	0,6	1,4	1,4
SD	1,1	1,2	0,1	0,1	0,1	0,1	0,1	0,1
L+D	15,3 ± 1,1		1,4 ± 0,1		0,6 ± 0,1		1,4 ± 0,1	

Tabela 13: Dolžina posameznih prstnic (v cm) pri vseh štirih prstih (x±SD), leve in desne okončine; n = 4.
Table 13: Measurements of the pedal phalanges (cm), with mean (*x*) and standard deviation (*SD*); n = 4. L-left, D-right phalanges.

Prstnica Phalanges	Prst I Digit I		Prst II Digit II		Prst III Digit III		Prst IV Digit IV	
	L	D	L	D	L	D	L	D
1	3,6 ± 0,2	3,6 ± 0,2	3,7 ± 0,2	3,7 ± 0,1	3,3 ± 0,1	3,3 ± 0,2	2,3 ± 0,2	2,3 ± 0,2
2	2,0 ± 0,3	2,0 ± 0,3	2,8 ± 0,2	2,8 ± 0,2	3,4 ± 0,2	3,4 ± 0,3	2,0 ± 0,2	1,9 ± 0,2
3			1,6 ± 0,3	1,6 ± 0,3	2,4 ± 0,4	2,4 ± 0,3	1,7 ± 0,1	1,7 ± 0,2
4					1,5 ± 0,3	1,5 ± 0,3	1,5 ± 0,2	1,6 ± 0,3
5							1,2 ± 0,2	1,1 ± 03

Tabela 14: Skupna dolžina prstnic (v cm) pri posameznem prstu in izračunana povprečna vrednost za vse proučene živali (n = 4) (*x*, *SD*).
Table 14: Total length of the digits (cm), with mean (*x*) and standard deviation (*SD*). L-left, D-right digits.

Žival Specimen	Prst I Digit I		Prst II Digit II		Prst III Digit III		Prst IV Digit IV	
	L	D	L	D	L	D	L	D
1	6,2	6,2	8,7	8,6	11,5	11,7	9,4	9,6
2	5,3	5,3	7,5	7,5	9,9	10,0	8,4	8,3
3	5,3	5,4	7,5	7,4	9,6	9,6	7,5	7,3
4	5,7	5,7	8,4	8,3	11,2	10,9	9,3	9,0
x	5,6	5,7	8,0	8,0	10,6	10,5	8,7	8,6
SD	0,4	0,4	0,6	0,6	0,9	0,9	0,9	1,0
L + D	5,6 ± 0,4		8,0 ± 0,6		10,6 ± 0,9		8,6 ± 0,9	

Tabela 15: Primerjava rezultatov naših meritev (v cm) z rezultati Kellner-jeve (1986) za dolžino plečnice (*scapula*), krokarnice (*coracoid*), nadlahtnice (*humerus*), komolčnice (*ulna*), stegnenice (*femur*) in golenonart nice (*tibiotarsus*).
Table 15: Comparasion of measurements of this work and Kellner's (1986).

	Dolžina plečnice Scapula length	Dolžina krokarnice Coracoid length	Dolžina nadlahtnice Humerus length	Dolžina komolčnice Ulna length	Dolžina stegnenice Femur length	Dolžina golenonartnice Tibiotarsus length
Naše delo This work	8,4 ± 0,4	6,9 ± 0,2	17,6 ± 0,6	20,8 ± 0,5	9,0 ± 0,4	20, 8 ± 1,3
Kellner 1986 *	8,6 ± 0,4	6,8 ± 0,3	17,7 ± 0,6	20,9 ± 0,8	9,1 ± 0,3	21,1 ± 1,1

* Preračunano iz srednjih vrednosti skupin Kellner-jeve (samci, samice, neznan spol) in števila živali v posamezni skupini.
* Calculated from the mean values of the Kellner's groups (males, females, not determined sex) and number of animals in each group.

Povzetek

Predstavljen je skelet sive čaplje (*Ardea cinerea*), in to nekatere kosti lobanje, vretenca v področju vratu, prsi in sinsakruma, plečni in medenični obroč ter prsnica. Za sivo čaplje je značilen dolg kljun, opremljen z nazobčenim tomijem, izrazita kraniofacialna upogibna cona (*zona flexoria craniofacialis*), dolga čelnica z značilno depresijo (*depressio frontalis*) v nadočesnem področju in izrazit postorbitalni lok, ki kavdalno prehaja v kratek postorbitalni podaljšek (*processus postorbitalis*). Podrobneje je opisan kompleks zadnjega dela mandibule, kvadratne kosti in krilne kosti ter njihovih sklepnih področij. Za sivo čaplje so značilni dolg parasfenoidni ročaj (*rostrum parasphenoidale*), obsežna senčnična kotanja (*fossa temporalis*) in dodatna plitka podsenčnična kotanja (*fossa subtemporalis*).

V vratnem delu hrbtenice imajo poseben pomen nosač, okretač, šesto vretence, skupina vretenc od 12-tega do 16-tega in zadnje vratno vretence. Šesto vretence in omenjena skupina vretenc (tj. od 12-tega do 16-tega) so močneje kalcificirana od ostalih. Zadnje vratno in prva tri prsna vretenca so v svojem distalnem delu tesneje medsebojno speta, medtem ko so v dorzalnem področju bolj prosta. Področje notarija torej ni iz zraščenih vretenc, manjkata tudi dorzalni (*crista dorsalis*) in lateralni (*lamina transversa notarii*) koščeni greben. Pač pa je izrazita koščena zraščenost sinsakruma in kolčnic. Na mestu šiva med črevnico in sinsakrumom v postacetabularnem področju se nahaja čvrsta sinostozna

zrast (*synostosis iliosynsacralis*), ki se grebenasto dviga nad površino.

Ključnici sta v apofiznem področju koščeno zraščeni v vilice (*furcula*), ki tvorijo koščen sklep z vrhom gredlja (*apex carinae*). Krokarnici se v področju prsnice deloma prekrižata, desna je umeščena nekoliko navzpred in ventralno od leve, in oblikujeta vsaka svoj sklep s prsnico (*sulcus articularis coracoideus*). Na kranialnem koncu držaja prsnice (*manubrium s. rostrum sterni*) se nahaja izrazit stransko sploščen izrastek (*spina externa*), medtem ko notranji izrastek manjka in je na njegovem mestu kratek žleb. Na notranji ustni (*labrum internum*) sta sklepni površini za sinovialni sklep s krokarnicama, obsežnejše sklepno področje pa predstavljata krokarnična žleba.

V delu so prikazane še nekatere druge značilnosti skeleta sive čaplje in v preglednicah rezultati meritev posameznih kosti.

Summary

Skeleton of the grey heron (*Ardea cinerea*) has been presented, particularly with regard to certain skull bones, vertebrae of the neck, thorax and synsacrum, pectoral girdle and pelvic girdle bones, and sternum. The grey heron's skull is characterized by a long rostrum equipped with the serrated tomium, pronounced craniofacial flexion zone (*zona flexoria craniofacialis*), long frontal bone with depression (*depressio frontalis*) and distinct postorbital arch (*arcus postorbitalis*), which caudally proceeds into a short postorbital process. A complex of the caudal part of mandibula, quadrate bone and pterygoid bone, and of their joint domains is described in detail. The long parasphenoid rostrum (*rostrum parasphenoidale*), large temporal fossa and shallow subtemporal fossa are characteristic for the grey heron.

In the cervical part of the spine, the first, second, sixth and last vertebrae, as well as the region from the twelfth to sixteenth vertebrae, are especially significant. The sixth vertebra and the mentioned group of vertebrae (from 12th to 16th cervical vertebrae) are more intensively calcified than others. The last cervical vertebra and the first three thoracic vertebrae are tightly connected in the ventral part and loosen in the dorsal part. Therefore, the notarium region is not composed of the fused vertebrae and the dorsal and lateral osseous crests (*crista dorsalis et lamina transversa notarii*) are also missing. However, there is a clear osseous fusion between the synsacrum and hip bone. On the suture place between ilium and synsacrum in the postacetabular region is a firm fusion (*synostosis iliosynsacralis*) elevating over surface and forming a crest.

The clavicles are fused into the fork (*furcula*) and their apophysis forms a synostosis with the sternal keel apex (*apex carinae*). The coracoids end at the sternum, each in its own groove (*sulcus articularis coracoideus*); the right coracoid is placed partly ahead and ventrally to the left coracoid. At the cranial end of the rostrum sterni expressive flattened process (*spina externa*) is found, however, the internal process (*spina interna*) is missing, and a short groove is in its place. At labrium internum the joint facets for synovial joints with coracoids are placed, however, widely extended joint area belongs to the coracoid joint grooves (*sulci articularis coracoidei*).

Also some other characteristics of the grey heron skeleton and measurements of individual bones are presented.

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Effect of short exposure to electro-oxidation treatment on *Planktothrix rubescens*

Učinek kratke izpostavitve elektro-oksidaciji na *Planktothrix rubescens*

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Abstract: Cyanobacterial blooms in fresh water bodies have a serious negative impact on human, animal and environmental health. The impact of electro-oxidation of water containing *Planktothrix rubescens* in electrolytic cell equipped with diamond electrodes has been tested. The treatment had an immediate effect on cyanobacterial phycocyanin and chlorophyll fluorescence response and total cell biovolume. Cyanobacterial number reduced for 80 % 130 h after a single treatment of 500 mL of a water sample through electrolytic cell with a flow rate 1 L/min. Obtained results indicate a great potential of the method for in-lake cyanobacterial control.

Keywords: Electrolytic cell, boron doped diamond electrode, cyanobacterial bloom control, hydroxyl radicals

Izvleček: Pojav cianobakterijskega cveta v sladkih vodah negativno vpliva na zdravje ljudi, na živali in okolje. Z elektrolitsko celico, opremljeno z borom dopiranima diamantima elektrodama, smo povzročili elektrooksidacijo in testirali njen vpliv na *Planktotrix rubescens*. Tretiranje je imelo takojšen vpliv na fluorescenco klorofila, na fluorescenco cianobakterijskega fikocianina in na celotni biovolumen cianobakterijskih celic. Volumen vzorca je bil 500 ml, pretok skozi elektrolitsko celico pa je znašal 1l/min. 130 ur po tretiranju se je število cianobakterijskih celic zmanjšalo za 80 %. Rezultati kažejo na velik potencial te metode pri nadzoru cianobakterijske populacije.

Ključne besede: elektrolitska celica, z borom dopirana diamantna elektroda, cianobakterijski cvet, hidroksilni radikali

Introduction

Increased cyanobacterial concentrations and the resulting toxin formation have negative ecological, biogeochemical, health-related and economic impacts (Paerl et al. 2011). A toxic cyanobacterial species *Planktothrix rubescens* inhabits deep lakes and can bloom throughout the entire year (Sedmak et. al. 2008). A variety of conventional water treatments (chlorination, coagulation, filtration) have been developed to minimize the harmful effects of cyanobacterial bloom, which are based on the removal of the cyanobacterial biomass and do not completely remove microcystins from water (Falconer et al. 1989, Anderson et al., 2009, Pantelić et al. 2013).

Introduction of advanced oxidation technologies (ozonation, photochemical degradation, Fenton processes, sonolysis) has made total removal of cyanotoxins possible (de la Cruz et al. 2011, Barrington et al. 2013). The inactivation of cyanobacteria by electrochemical oxidation (ECO) in electrolytic cell equipped with high-performance electrodes represents a perspective alternative for water treatment with great potential, being economical, environmentally friendly and offering higher treatment efficiency (Zhank et al. 2009). Boron-doped diamond anode (BDDA) is known to have the highest potential of forming hydroxyl radicals (˙OH) and exceptional chemical inertness and durability. Produced ˙OH are powerful non-selective oxidizing agents, capable to react with organic matter. The aim of our work was to test the effect of the method for the reduction of *P. rubescens* biomass from natural water sample.

Materials and methods

The ECO treatment was performed in electrolytic cell, equipped with two 60 cm^2 large BDD electrodes (Condias, Germany), serving as anode and cathode, placed parallel 2 mm apart and forming 12 ml treatment chamber. The effect of the ECO was studied on *P. rubescens* taken from Lake Bled, Slovenia. The complete water sample (500 ml) with *P. rubescens* starting biovolume of 56 mm^3/L was pumped through the electrolytic cell at a constant flow rate of 1 L/min. The achieved ECO time on the electrodes was 0.72 s using 3 A current intensity. Submersible Sensors (Cyclops 7, Turner Designs, USA) were used for detecting the change in fluorescence of chlorophyll a (CHL) and phycocyanin (PC) after exposed stress in electrolytic cell. The effect of ECO was also monitored by determination of cyanobacterial biovolume (CEN EN 15204 2006) and the extraction of CHL (ISO 10260 2001) before and immediately after the treatment, and then every 24 hours for 5 days.

Results

Treating *P. rubescens* with BDD resulted in immediate effect in PC and CHL fluorescence (Fig. 1, results for CHL not shown). After a transient increase of the PC and CHL fluorescence, they both reduced up to 75 % in treated sample 130 h after the ECO compared to the control. Extraction of CHL supported these results, as its concentration dropped for 80 % (not shown). ECO also affected the cyanobacterial biovolume. It reduced for 21 % immediately after the treatment and for 80 % 130 h later.

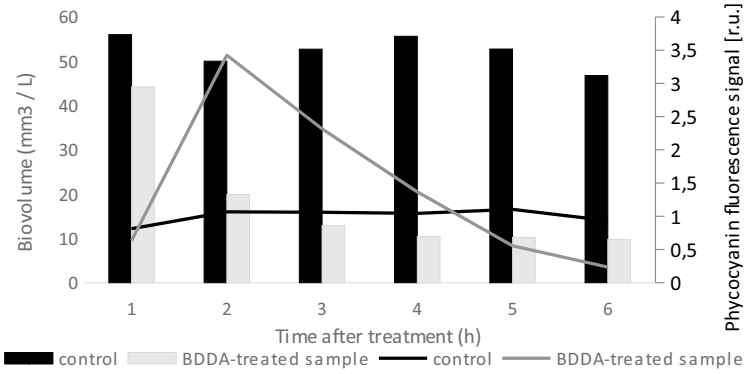

Figure 1: Phycocyanin fluorescence (line) and cyanobacterial biovolume (bar) in treated and control samples after the electro-oxidation treatment.

Slika 1: Fluorescenca fikocianina (črti) in biovolumen cianobakterij (stolpci) v kontrolnih in tretiranih vzorcih po elektro-oksidacijskem tretiranju.

Discussion

The three selected monitoring approaches enabled us to follow immediate and postponed effects of ECO treatment on *P. rubescens*. Besides an immediate biovolume reduction and therefore a die-off of a part of the population, a transient increase of fluorescence of PC and CHL immediately after the treatment was observed. This can be contributed to the stress response of the survived part of the cells, where the photosynthetic apparatus was damaged. After Zilinskas and Glick (1981), the increased fluorescence is the result of decoupling of PS II reaction centers from CHL, to which the energy from PC can no longer be transferred. The damages were detrimental; resulting in greatly reduced PC and CHL signals below the control level after 4 days (Fig. 1) and were in positive correlation with low concentration of extracted CHL and biovolume, showing on 80 % reduction of the *P. rubescens* population. Although the ECO time was very short, the immediate and postponed effects of ECO were observed even though ˙OH radicals are known to be short-lived (Pryor 1986). This indicates on gradual decrease of the cyanobacterial population, which is beneficial also from the point of cyanotoxins release. The use of BBD electrode has already proved to be effective also in extracellular cyanotoxins degradation and inactivation (Meglič et al. 2016). The effect of ECO using BDD electrodes on degradation of cyanotoxins in natural water samples is planned to be closely followed in our future experiments, as well as the effect of ECO on other biota for which larger scale experiments are planned.

Conclusions

Short-term exposure of natural water sample to ECO treatment applying BDD electrodes in laboratory environment caused a visible stress response of *P. rubescens*, resulting in an evident decrease in biovolume and concentration of CHL, indicating on efficiency of the novel method as an in-lake cyanobacterial control method. Measurement of the fluorescence of cyanobacterial pigments enabled the quantification and probably also the determination of their physiological state. Performance of simultaneous on-line fluorescence measurements immediately after the ECO treatment are therefore suggested in the case of further activities in natural environment to promptly react on increased density of cyanobacteria or their regrowth after the treatment and to keep the natural biodiversity balance.

Povzetek

Cvetenje cianobakterij v sladkovodnih telesih predstavlja resno težavo za zdravje ljudi in živali ter za okolje. Testirali smo vpliv okolju prijazne metode, elektro-oksidacije z diamantnimi elektrodami, na številčnost cianobakterij. Uporabili smo vodo iz Blejskega jezera, ki je vsebovala *P. rubescens*. Pri tretiranju smo 500 ml vzorca vode spustili skozi elektrolitsko celico z delovnim volumnom 12 ml in tokom 3 A. S pretokom 1 L/min smo dosegli elektrooksidacijski čas 0,72 s. Učinek tretiranja na fluorescenčni signal fikocianina in klorofila ter na skupni celični biovolumen je bil takojšen. Fluorescenca in koncentracija klorofila sta 130 ur po tretiranju upadli za 75 in 80 %, prav tako biovolumen cianobakterij. Rezultati kažejo, da ima uporabljena metoda velik potencial za izvajanje kontrole nad cianobakterijami v jezerih.

Acknowledgements

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INSTRUCTIONS FOR AUTHORS

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SCIENTIFIC ARTICLES are comprehensive descriptions of original research and include a theoretical survey of the topic, a detailed presentation of results with discussion and conclusion, and a bibliography according to the IMRAD outline (Introduction, Methods, Results, and Discussion). In this category ABS also publishes methodological articles, in so far as they present an original method, which was not previously published elsewhere, or they present a new and original usage of an established method. The originality is judged by the editorial board if necessary after a consultation with the referees. The recommended length of an article including tables, graphs, and illustrations is up to fifteen (15) pages; lines must be double-spaced. Scientific articles shall be subject to peer review by two experts in the field.

REVIEW ARTICLES will be published in the journal after consultation between the editorial board and the author. Review articles may be longer than fifteen (15) pages.

BRIEF NOTES are original articles from various biological fields (systematics, biochemistry, genetics, physiology, microbiology, ecology, etc.) that do not include a detailed theoretical discussion. Their aim is to acquaint readers with preliminary or partial results of research. They should not be longer than five (5) pages. Brief note articles shall be subject to peer review by one expert in the field.

CONGRESS NEWS acquaints readers with the content and conclusions of important congresses and seminars at home and abroad.

ASSOCIATION NEWS reports on the work of Slovene biology associations.

2. Originality of Articles

Manuscripts submitted for publication in *Acta Biologica Slovenica* should not contain previously published material and should not be under consideration for publication elsewhere.

3. Language

Articles and notes should be submitted in English, or as an exception in Slovene if the topic is very local. As a rule, congress and association news will appear in Slovene.

4. Titles of Articles

Title must be short, informative, and understandable. It must be written in English and in Slovene language. The title should be followed by the name and full address of the authors (and if possible, fax number and/or e-mail address). The affiliation and address of each author should be clearly marked as well as who is the corresponding author.

5. Abstract

The abstract must give concise information about the objective, the methods used, the results obtained, and the conclusions. The suitable length for scientific articles is up to 250 words, and for brief note articles, 100 words. Article must have an abstract in both English and Slovene.

6. Keywords

There should be no more than ten (10) keywords; they must reflect the field of research covered in the article. Authors must add keywords in English to articles written in Slovene.

7. Running title

This is a shorter version of the title that should contain no more than 60 characters with spaces.

8. Introduction
The introduction must refer only to topics presented in the article or brief note.

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Articles should not contain more than ten (10) illustrations (graphs, dendrograms, pictures, photos etc.) and tables, and their positions in the article should be clearly indicated. All illustrative material should be provided in electronic form. Tables should be submitted on separate pages (only horizontal lines should be used in tables). Titles of tables and illustrations and their legends should be in both Slovene and English. Tables and illustrations should be cited shortly in the text (Tab. 1 or Tabs. 1-2, Fig. 1 or Figs. 1-2; Tab. 1 and Sl. 1). A full name is used in the legend title (e.g. Figure 1, Table 2 etc.), written bold, followed by a short title of the figure or table, also in bold. Subpanels of a figure have to be unambiguously indicated with capital letters (A, B, …). Explanations associated with subpanels are given alphabetically, each starting with bold capital letter (**A**), a hyphen and followed by the text.

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All the figures have to be submitted in the electronic form. The ABS publishes figures either in pure black and white or in halftones. Authors are kindly asked to prepare their figures in the correct form to avoid unnecessary delays in preparation for print, especially due to problems with insufficient contrast and resolution. Clarity and resolution of the information presented in graphical form is the responsibility of the author. Editors reserve the right to reject unclear and poorly readable pictures and graphical depictions. The resolution should be 300 d.p.i. minimum for halftones and 600 d.p.i. for pure black and white. The smallest numbers and lettering on the figure should not be smaller than 8 points (2 mm height). The thickness of lines should not be smaller than 0.5 points. The permitted font families are Times, Times New Roman, Helvetica and Arial, whereby all figures in the same article should have the same font type. The figures should be prepared in TIFF, EPS or PDF format, whereby TIFF (ending *.tif) is the preferred type. When saving figures in TIFF format we recommend the use of LZW or ZIP compression in order to reduce the file sizes. The photographs can be submitted in JPEG format (ending *.jpg) with low compression ratio. Editors reserve the right to reject the photos of poor quality. Before submitting a figure in EPS format make sure first, that all the characters are rendered correctly (e.g. by opening the file first in the programs Ghostview or GSview – depending on the operation system or in Adobe Photoshop). With PDF format make sure that lossless compression (LZW or ZIP) was used in the creation of the *.pdf file (JPEG, the default setting, is not suitable). Figures created in Microsoft Word, Excel, PowerPoint etc. will not be accepted without the conversion into one of the before mentioned formats. The same goes for graphics from other graphical programs (CorelDraw, Adobe Illustrator, etc.). The figures should be prepared in final size, published in the magazine. The dimensions are 12.5 cm maximum width and 19 cm maximum height (width and height of the text on a page).

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Articles shall end with a summary of the main findings which may be written in point form.

12. Summary
Articles written in Slovene must contain a more extensive English summary. The reverse also applies.

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References shall be cited in the text. If a reference work by one author is cited, we write Allan (1995) or (Allan 1995); if a work by two authors is cited, (Trinajstić and Franjić 1994); if a work by three or more authors is cited, (Pullin et al. 1995); and if the reference appears in several works, (Honsig-Erlenburg et al. 1992, Ward 1994a, Allan 1995, Pullin et al. 1995). If several works by the same author

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Mielke, M.S., Almeida, A.A.F., Gomes, F.P., Aguilar, M.A.G., Mangabeira, P.A.O., 2003. Leaf gas exchange, chlorophyll fluorescence and growth responses of Genipa americana seedlings to soil flooding. Experimental Botany, 50 (1), 221–231.

Books, chapters from books, reports, and congress anthologies use the following forms:

Allan, J.D., 1995. Stream Ecology. Structure and Function of Running Waters, 1st ed. Chapman & Hall, London, 388 pp.

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Toman, M.J., 1992. Mikrobiološke značilnosti bioloških čistilnih naprav. Zbornik referatov s posvetovanja DZVS, Gozd Martuljek, pp. 1-7.

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A manuscript element checklist (For a manuscript in Slovene language the same checklist is appropriately applied with a mirroring sequence of Slovene and English parts):
English title – (Times New Roman 14, bold)
Slovene title – (Times New Roman 14, bold)

Names of authors with clearly indicated addresses, affiliations and the name of the corresponding author – (Times New Roman 12)
Author(s) address(es) / institutional addresses – (Times New Roman 12)
Fax and/or e-mail of the corresponding author – (Times New Roman 12)
Keywords in English – (Times New Roman 12)
Keywords in Slovene – (Times New Roman 12)
Running title – (Times New Roman 12)
Abstract in English (Times New Roman 12, title – Times New Roman 14 bold)

Abstract in Slovene – (Times New Roman 12, title – Times New Roman 14 bold)
Introduction – (Times New Roman 12, title – Times New Roman 14 bold)
Material and methods – (Times New Roman 12, title – Times New Roman 14 bold)
Results – (Times New Roman 12, title – Times New Roman 14 bold)
Discussion – (Times New Roman 12, title – Times New Roman 14 bold)
Summary in Slovene – (Times New Roman 12, title – Times New Roman 14 bold)
Figure legends; each in English and in Slovene – (Times New Roman 12, title – Times New Roman 14 bold, figure designation and figure title – Times New Roman 12 bold)
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Literature – (Times New Roman 12, title – Times New Roman 14 bold)
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