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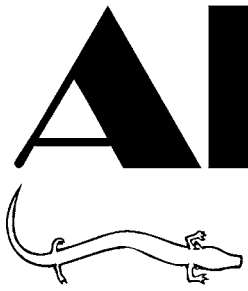

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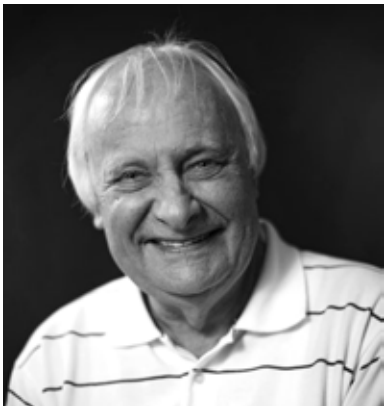
In memoriam: profesor dr. Pavel Ličar (21. april 1935-3. oktober 2015)



Leto dni je minilo, ko smo se poslovili od profesorja, sodelavca in prijatelja Pavla Ličarja. Ob spominu nanj želim izpostaviti tiste lastnosti in posebnosti iz njegovega življenja, ki so najbolj zaznamovale razvoj slovenske biologije in obdarovale študente in sodelavce, ki smo imeli srečo, da smo z njim delali in živeli.

Asistenta Pavla Ličarja sem prvič srečala pri vajah iz Splošne zoologije leta 1972. Biološki bruci, željni novih znanj o življenju, smo uživali v njegovem pedagoškem zanosu in prijetnem vzdušju v zoološkem praktikumu, v stavbi Filozofske fakultete na Aškerčevi ulici v Ljubljani. Skoraj trideset generacij študentov prvih letnikov biologije, veterine, živilstva, zootehnike in Pedagoške fakultete je z njim prepotovalo svet živih bitij, od molekul in celic do organizmov in skupnosti , ki ga je s predanostjo in nazornostjo pričaral med vajami in predavanji s področij splošne zoologije in biologije celice v letih 1968 do 1997.

Kot dolgoletni predsednik študijske komisije na Oddelku za biologijo je prof. Ličar organiziral in vodil informativne dneve za gimnazijce in sprejemal bruce v kraljestvo biologije. Ko je študent stopil v njegov svet se je počutil zaželenega, varnega in cenjenega. Probleme je reševal predano in s humorjem, le redko se je vznejevoljil. Talent vreden občudovanja!

Profesor Pavel Ličar je bil namestnik predstojnika in predstojnik VTO za biologijo v zelo zahtevnih časih načrtovanja gradnje biološkega središča v obdobju od leta 1977 do 1981. V zvezi s prostorsko stisko biologije in gradnjo biološkega središča je bil aktiven na mnogih področjih in je vztrajno predstavljal problematiko v javnih medijih. Bil je dolgoletni predstojnik katedre za zoologijo, vse do upokojitve. Kot član odborov za vprašanja znanstveno- raziskovalnega in vzgojno-izobraževalnega dela univerze je pomembno prispeval k vključevanju biologije v širše nacionalno in mednarodno okolje.

Zaslužil si je priznanji za prizadevno in uspešno delo ter za prispevek k razvoju Biotehniške fakultete, ki ju je prejel junija 1982 ob 35 letnici in ob 50 letnici BF leta 1997.

Ni vsak rojen pedagog, prof. Pavlu Ličarju pa je bilo to dano in študenti so ga imeli radi. Dejstvo, ki ga tudi danes potrjujejo številne generacije diplomiranih biologov, ki jih je navdušil za študij in delo na področju biologije. Na oddelku za biologijo je bil zaposlen skoraj 30 let in zapustil je bogato dediščino. Temeljno znanje

biologije je posredoval več tisočim študentom. Učil je v obdobju , ko še ni bilo računalnikov in digitalnih projekcij, v časih ko sta le krede vešča roka in slikovita beseda lahko pričarali študentom najlepše slike iz sveta živali. Njegova predavanja so bila zanimiva, slikovita in zelo duhovita! Kot mentor je bil prijeten sogovornik, ki je cenil in vzpodbujal samoiniciativnost in ustvarjalnost. Z besedami, vzpodbudami in zgledom je širil veselje do pedagoškega dela in raziskovanja.

Raziskovalno delo prof. Pavla Ličarja na področjih funkcionalne morfologije, histologije, anatomije in fiziologije je bilo usmerjeno predvsem v raziskave biologije rakov. Bil je dolgoletni koordinator programskega sklopa Struktura in funkcija organizmov in vodja tematskega sklopa Primerjalne biološke študije pri izopodih. Sprva je raziskoval pigmente v krilih metuljev, v nadaljevanju pa se je usmeril v raziskave strukture in funkcije prebavnega sistema površinskih in jamskih rakov enakonožcev. Bil je med prvimi na biologiji, ki je raziskoval biološke strukture z novim vrstičnim elektronskim mikroskopom. Filtrirne strukture v želodcu vodnih osličkov so zaživele v vsej svoji kompleksnosti. Ko je raziskoval zmogljivost in delovanje hitinskih filtrov je v navdušenju izjavil, da bodo morda nekoč v čistilnih napravah uporabljali podobne mehanske filtre, kot jih je opisal v svojih delih. Povezoval se je tudi z drugimi zoološkimi skupinami in svoje raziskovalne dosežke predstavil na mnogih srečanjih doma in v tujini. S sodelavci je razvijal histološke tehnike za pripravo raznolikih bioloških vzorcev in uvajal novo metodologijo za pripravo vzorcev za vrstično elektronsko mikroskopijo. Na tem področju je opravil pionirsko delo za slovensko zoologijo. S področja raziskav strukture in funkcije živali je objavil kar nekaj znanstvenih in strokovnih prispevkov. Zelo pomembno je, da je pisal tudi v slovenskem jeziku in objavljal v Biološkem vestniku in v zbornikih SAZU.

Ostaja nam spomin na njegov optimizem, dobrovoljnost in predanost delu! S svojim zgledom je dokazal, da je čustvena inteligenca še kako pomembna za uspeh v delovnem okolju! Njegova toplina, pristna komunikacija, empatija, humor in družabne veščine, ki jih je zasejal v generacije študentov ostajajo in nas vzpodbujajo, da sledimo njegovemu slogu poučevanja in sodelovanja s študenti.

Imeli smo srečo vsi, ki smo ga poznali in šli z njim skozi lepo obdobje! Bilo je samoumevno, da smo se pogovarjali, skupaj razmišljali in se smejali. Vse je bilo samoumevno, samo konec ne! Žal z ljudmi umira tudi čas!

Jasna Štrus

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Four decades of multidisciplinary studies on isopods: a tribute to Pavel Ličar

Štiri desetletja interdisciplinarnih raziskav rakov enakonožcev (Crustacea:Isopoda): v spomin Pavlu Ličarju

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Abstract: In this paper we review the research on aquatic and terrestrial isopods during the last four decades at the Chair of Zoology, Department of Biology, Biotechnical Faculty, University of Ljubljana. Isopods have attracted substantial attention from our research team in the following areas: functional morphology and developmental biology, host-microbiota specific interactions, ecotoxicology, and systematics and evolution. We present the rationale for using two isopod species as our central model organisms: the waterlouse (*Asellus aquaticus*) and the woodlouse (*Porcellio scaber*). We summarize the most important and interesting findings about the structure and function of the integument and digestive systems of several amphibious and terrestrial woodlice species during molting and developmental stages, the importance of *P. scaber* as a model organism in the study of arthropod-microbe interactions, and its central role as a test model in terrestrial ecotoxicity studies. We highlight the role that *A. aquaticus* has played in studying the evolution of subterranean biodiversity and in the evolution of troglomorphies. In addition to the retrospective view on our research with isopods we also present the scope of our future research, and the importance for zoology (biology). We wish to dedicate this work to our late co-worker, Prof. Dr. Pavel Ličar, who devoted much of his research into studying the digestive system of freshwater asellids (Isopoda: Asellota).

Keywords: isopods, terrestrial, aquatic, functional morphology, developmental biology, host-microbiota interactions, terrestrial isopod, aquatic isopod, ecotoxicology, systematics, evolution

Izvleček: V članku predstavljamo raziskave biologije rakov enakonožcev, ki že nekaj desetletij potekajo na Katedri za zoologijo Oddelka za biologijo Biotehniške fakultete Univerze v Ljubljani. Rake enakonožce proučujemo na različnih raziskovalnih področjih, kot so funkcionalna morfologija in razvojna biologija, študij interakcij med gostiteljem in mikroorganizmi, ekotoksikologija ter sistematika in evolucija. V prispev-

ku navajamo razloge za preučevanje dveh vrst rakov enakonožcev: navadnega prašička (*Porcellio scaber*) in vodnega oslička (*Asellus aquaticus*), ki sta odlična modelna organizma za omenjene raziskave. V prispevku povzemamo glavne ugotovitve raziskav zgradbe in delovanja integumenta in prebavnega sistema med levitvijo in razvojem amfibijskih in kopenskih mokric, pomen vrste *P. scaber* kot modelnega organizma za študij specifičnih interakcij z mikrobi ter njegovo osrednjo vlogo v kopenski ekotoksikologiji. Izpostavljamo tudi vlogo vrste *A. aquaticus* kot modelnega organizma za študij evolucije jamske pestrosti in troglomorfij. Poleg zgodovinskega pregleda raziskav na posameznih področjih, predstavljamo tudi najpomembnejše rezultate novejših raziskav, njihov pomen za zoologijo (biologijo) ter pomen za prihodnost. Članek je posvečen lani preminulemu sodelavcu prof. dr. Pavlu Ličarju, ki je raziskoval predvsem prebavila vodnih rakov enakonožcev iz skupine Asellota.

Ključne besede: raki enakonožci, kopenski raki, vodni raki, funkcionalna morfologija, razvojna biologija, interakcija gostitelj-mikroorganizmi, ekotoksikologija, sistematika, evolucija

Introduction

Research on peracarid crustaceans, mostly amphipods and isopods at the Chair of Zoology, has a long history, dating back to 1965 with the first publications on taxonomy of *Asellus aquaticus* (Sket 1965a,b, 1967) and has so far resulted in 319 publications of Prof. Sket and his co-workers. Ličar (1970) and, Ličar and Sket (1971) published their first papers on the stomach morphology of water lice in the journal *Acta Biologica Slovenica* (formerly Biološki vestnik). Since then, aquatic and terrestrial isopods have attracted substantial interest from different researchers at the Chair of Zoology, Department of Biology, Biotechnical Faculty, University of Ljubljana. In this paper we review our research on isopods undertaken in the last four decades to commemorate Professor Ličar's work.

Pavel Ličar devoted his academic career to aquatic isopods, mostly to *Asellus aquaticus* (Isopoda:Asellota). He investigated the structure and function of the isopod digestive system. His main scientific questions were related to morphological characteristics of the isopod stomach and its possible role as an identification key in taxonomy. From a morphological viewpoint, the stomach is by far the most complicated part of the digestive tract of isopod crustaceans. The structure of the stomach varies between species but in many there is a dorsal groove into which indigestible material is channelled and a ventral part which transports filtered food to the digestive caeca, the hepatopancreas. He described the morphological characteristics of the stomach of *A. aquaticus* with emphasis on the fine structure of filters. In general, the isopod stomach is composed of an anterior primary filter, the adjacent masticatory areas, the posterior secondary filter on the lateral sides of the protuberations, called inferomedianum, and the inferolateralia. Ingested food can be filtered twice: first via the primary filter and then the secondary filter, both of which are composed of finely branched cuticular spines and plumose setae which can separate particles of different sizes. In the following years a detailed descriptions of the stomachs of different species of Asellidae were presented (Ličar 1976). He contributed significantly towards the understanding of the mechanical properties of primary filters (Ličar 1977, Ličar et al. 1979). It was stated, that the stomach structure of different genera of Asellidae is rather uniform, even similar to that of Janiridae, while Stenasellidae are remarkably varied and diverse; in specialized species the masticatory parts are vestigial.

A new approach in phylogenetic studies was research into the reproductive cycles of epigean and hypogean species of *A. aquaticus* which was an exciting topic for young researchers in his lab (Štrus and Blejec 1983). Ličar's work on stomach ultrastructure was the basis for international cooperation with researchers at the Institute of Zoology, University of Heidelberg (Germany) (Štrus et al. 1985, Storch and Štrus 1989, Drobne et al. 1991a, Štrus and Storch 2004)

and the University of Reading, United Kingdom (Drobne and Hopkin 1994). Studies on the stomach structure of terrestrial isopods were extended to ecotoxicological studies on digestive glands of the model organism *Porcellio scaber* (Drobne and Hopkin 1995). The structure of the digestive system of strictly terrestrial isopods was compared with the digestive system of amphibious species *Ligia italica* and *Ligidium hypnorum* (Štrus et al. 1995), both important species in the phylogenetic studies of woodlice.

A significant moment in the research of Prof. Pavel Ličar was when a scanning electron microscope (SEM; Stereoscan 600, Cambridge Instruments, UK) was installed at the Department of Biology in 1975, only a decade after the first commercial SEM appeared on the market. In the following years, his ideas and work expanded from the functional morphology of the stomach in aquatic isopods to also encompass amphibious and strictly terrestrial isopod species as a basic approach for phylogenetic studies of isopod crustaceans (Drobne et al. 1991b). It was confirmed that the mastication of food is an adaptation to terrestrial food sources, evidenced by masticatory structures in the stomach which are not so obvious in aquatic species. Amphibious and terrestrial isopods are fully adapted for life on land. Woodlice, suborder Oniscidea, are the only truly terrestrial group of crustaceans and show various adaptations to life on land. Research on the structure and function of the stomach was expanded towards other parts of the digestive system and other organic systems including integument and respiratory structures.

In 1999, the Department of Biology celebrated the 80th anniversary of the foundation. Almost 30-years of academic research of Professor Pavel Ličar was summarised in the book of abstracts. Together with his close collaborators Jasna Štrus and Damjana Drobne, past, present and future research directions and perspectives were presented (Drobne et al. 1999a). Almost 20 years later, aquatic and terrestrial isopods still attract a lot of attention from different directions including structure and function of digestive system, integument and molting, embryology, immunology, ecotoxicology, and nanobiology. Here, the achievements of the last four decades and perspectives in research on isopods at the Department of Biology are presented.

Presentation of two key model isopod species

The terrestrial isopod Porcellio scaber (Isopoda: Oniscidea)

More than one third of described isopod species (approximately 9000) belong to terrestrial Oniscidea or woodlice, also known as "*slaters, sowbugs and pillbugs"* (Schmalfuss H. 2003). Terrestrial isopods are widely distributed, occurring from Iceland to South America and South Africa (Harding and Sutton 1985). They play an important role in plant litter decomposition by breaking down organic materials such as fallen leaves into smaller fragments (Hassall et al. 1987), they affect the soil by physical transportation of litter materials, and alter microbial activity. The terrestrial isopod *Porcellio scaber* (Latreille, 1804) (Slovenian "navadni prašiček") (Fig. 1 A) is ubiquitous and common on tree trunks and walls, on waste ground, and in gardens and grassland (Harding and Sutton et al. 1985).

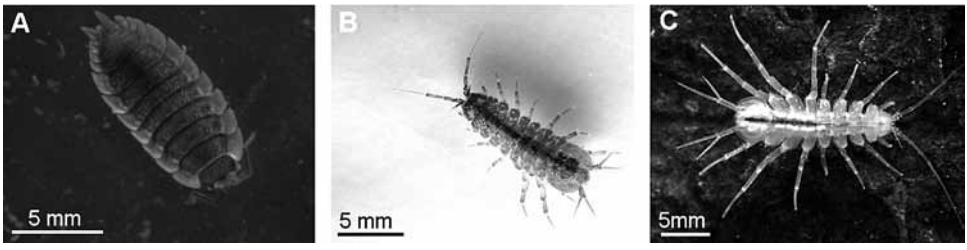

Figure 1: The terrestrial and aquatic isopods: (**A**)-The terrestrial isopod *Porcellio scaber* and (**B**)-aquatic isopods *Asellus aquaticus aquaticus*: the surface ecomorph and (**C**)-*A. a. cavernicolus*: the cave ecomorph.

Slika 1: Kopenski in vodni raki enakokožci. (**A**)- Kopenski rak enakonožec vrste *Porcellio scaber* in (**B**)-vodni osliček *Asellus aquaticus aquaticus*: kopenski ekomorf in (**C**)- *A. a. cavernicolus*: jamski ekomorf.

The aquatic isopod Asellus aquaticus (Isopoda: Asellota)

Asellus aquaticus (Linné, 1758) (Slovenian "vodni osliček") is one of the most studied and well-known aquatic macroinvertebrates in Europe (Fig. 1B). Its common names include "*water slater, waterlouse, aquatic sowbug and water hoglouse*". Its range reaches across the entire continent except for the Pyrenean Peninsula and some smaller Mediterranean areas (Birštejn 1951, Williams 1962, Argano 1979, Sket 1994, Henry and Magniez 1983, 1995, Henry et al. 1996). This generalist species is able to thrive in all types of fresh to slightly brackish surface waters. Its tolerance to organic pollution ranks *A. aquaticus* as an pollution indicator and a successful member of the 'α-mesosaprobic' community (Liebmann 1962). While most of its range is inhabited by the nominotypical *A. a. aquaticus*, extensive racial diversification is present in S and SE Europe (Sket 1965a, Prevorčnik et al. 2004, Verovnik et al. 2003, 2004, 2005). Moreover, in two comparatively small karstic areas (E Romania and NW Dinaric karst) a number of local populations have invaded and adapted to subterranean fresh waters (Sket 1994, Henry et al. 1996, Turk et al. 1996, Turk-Prevorčnik and Blejec 1998, Prevorčnik et al. 2004) (Fig. 1 C).

Isopods as key experimental models

Functional morphology of the integument and the digestive system of woodlice during molting and development

Woodlice are crustaceans that are well adapted to terrestrial life. Coping with the restrictions of living life on land has resulted in many important adaptations and changes to the animals nutrition, digestion, gas/water balance and reproduction strategies. The functional morphology of the digestive system was studied in different woodlice species and described thoroughly in the model species *P. scaber* (Lane 1988, Hames and Hopkin 1989, Štrus et al 2008). Comparative studies of the digestive system in amphibious woodlice *Ligidium hypnorum* (Drobne et al. 1991a), and *Ligia italica* (Štrus et al. 1995) showed important differences in the structure and function of the stomach, alimentary canal and in the presence or absence of endodermal midgut. Woodlice are decomposers but can feed on almost anything. Food is mechanically and enzymatically broken down in the foregut, efficiently filtered in the stomach (Fig. 2A) and transported to the midgut glands where the secretion of digestive juices and absorption of nutrients take place (Štrus et al. 1985). The ultrastructure of masticatory regions and filters in stomachs of different isopod crustaceans was described in both asellids (Ličar 1976) and woodlice (Storch 1987). Undigested material is transported to the hindgut which is entirely covered by cuticle and composed of the anterior chamber, with absorptive and transport functions, and the osmoregulatory papillate region with the rectum, where water is resorbed and fecal pellets form. Microbiota which are associated with the cuticle surface are important as a food source but can also aid in food digestion (Kostanjšek et al. 2006). A description of the role of microbiota in the digestive system of woodlice is presented in the subsequent chapter.

Growth and reproduction in woodlice is governed by frequent molting which demands constant synthesis of new cuticles of the integument and alimentary canal. Molt cycles of amphibious and terrestrial woodlice have been described (Zidar et al. 1998) and cuticle ultrastructure and calcification were studied with various microscopic and analytical techniques implemented at the Chair of Zoology and in cooperation with labs worldwide (Štrus and Blejec 2001, Štrus et al. 2008, Žnidaršič et al. 2010, Mrak et al. 2012, Vittori et al. 2013). The exoskeleton of woodlice is a multilayered calcified chitinous-proteinaceous matrix (Figs. 2B,C) which varies in its elasticity/rigidity depending on the body region, between individual animals and degree of species terrestrialization (Vittori and Štrus 2014). Cuticle is secreted during both embryonic development and molting in adults. In premolt animals, both old and new cuticles can be visualized due to apolysis and subsequent deposition of preecdysal cuticular layers (Fig. 2D). Resorption of organic and mineral components from the detached cuticle and formation of spherules in the ecdysal space are the most prominent features observed in the exoskeleton of premolt animals (Fig. 2E). Due to frequent

biphasic molting (Fig. 2F) and different cuticle types woodlice are excellent models for studying extracellular matrix secretion and calcification.

Embryonic and postembryonic development of crustaceans has been gaining an increasing amount of research interest in functional morphology and evolutionary developmental biology (Browne et al. 2005, Hejnol et al. 2006, Scholtz et al. 2009, Mittmann et al. 2014, Alwes and Scholtz 2014, Stamataki and Pavlopoulos 2016). In our research group the morphogenesis of the digestive system and integument has been studied in the isopod *P. scaber* building on previous expertise and knowledge of the anatomy, ultrastructure and development of this organism (Milatovič et al. 2010). In laboratory rearing conditions the development from the released fertilized eggs till embryo hatching lasts about 25 days and after that marsupial mancas continue to develop within the female brood pouch (marsupium) for approximately 10 days (Figs. 3A and 3B). Based on morphological data, 19 sequential embryonic stages and 3 stages of marsupial mancas can be distinguished (Milatovič et al. 2010, Mrak et al. 2012). The microscopic anatomy of the digestive system in embryos and mancas was investigated by Štrus et al. (2008). Differentiation of the ectodermal stomodeum and proctodeum into the complex foregut and hindgut respectively, was revealed at the histological and ultrastructural levels. Morphogenesis of the endodermal midgut gland tubes was explained. In the early embryos the midgut gland primordia that contain yolk and lipid globules are visible. In late embryogenesis the midgut gland epithelium consists of two cell types, as is characteristic for adults. The ultrastructural aspects of the hindgut cuticle differentiation during development were analysed by Mrak et al. (2015). The hindgut in late embryos of stages 16 and 18 is lined by a precuticular matrix, structurally similar to that of the epidermis. The first hindgut cuticle formation is evident in stages 18 and 19, before hatching (Fig. 3C). The hindgut cuticle in marsupial mancas is thinner in comparison to adults, the electron dense epicuticular layer in particular, and the structural differences between the cuticle in the anterior chamber and papillate region are not evident yet. The stomach in late marsupial mancas is already differentiated in complex cuticular structures (Fig. 3D). Exoskeletal cuticle differentiation was studied by Mrak et al. (2014). The ultrastructural organization and composition of precuticular matrices and exoskeletal cuticle in embryos and marsupial mancas were revealed. Two successively formed precuticular matrices were evident in mid-stage and late-stage embryos. The precuticular matrix is composed of the outermost ruffled lamina and subjacent loose material with no lamellar pattern characteristic for the cuticle and differs from the cuticle also regarding its organic scaffold composition as shown by wheat germ agglutinin labelling. In late embryos of stages 18 and 19 the first cuticle formation was observed (Fig. 3E), including elaboration of the surface scales and differentiation of characteristic cuticular layers. In marsupial mancas a new exoskeletal cuticle is secreted and according to the progression of development gradually becomes more similar to the cuticle of adults in respect to the ultrastructure, organic scaffold and calcification (Fig. 3F). An integrative part of cuticle differentiation is also the establishment of connections between cuticle and muscles via tendon cells. Tendon cells ultrastructure, including the linkages to cuticle at the apical membrane and myotendinous junctions to underlying muscle cells, is established in the prehatching embryos and marsupial mancas (Žnidaršič et al. 2012).

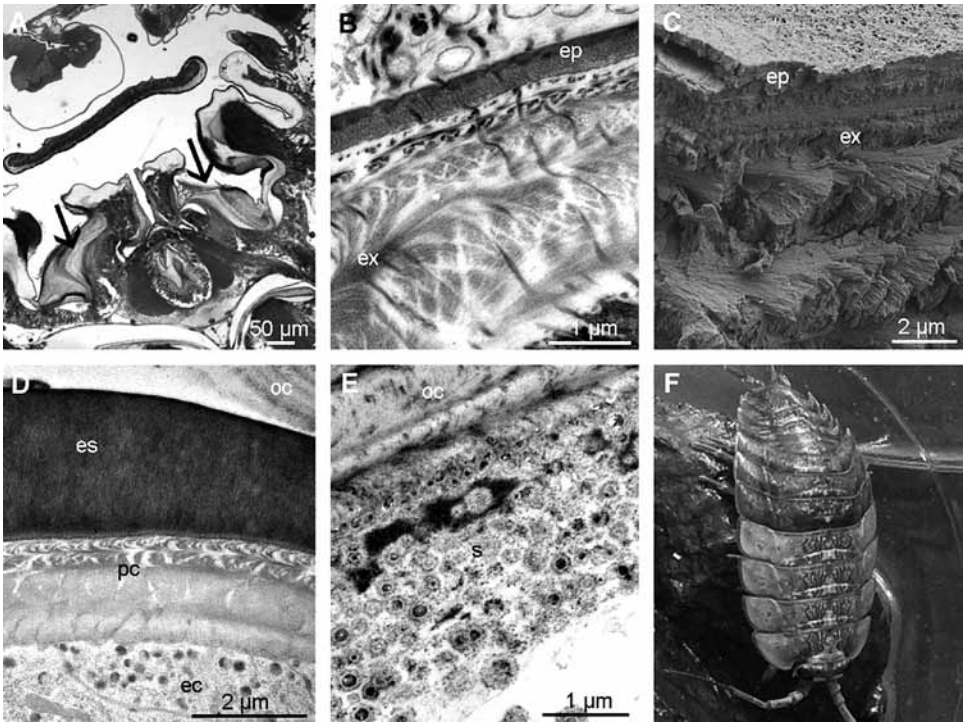

Figure 2: Cuticle structure and function in terrestrial isopods. (**A**)-Histological section of stomach lined by cuticle with prominent filtering areas (arrows) in adult *Porcellio scaber*. (**B**)-TEM micrograph of preecdysal exoskeletal cuticle ultrastructure in *Ligia palassii* showing the epicuticle (ep) and exocuticle (ex). (**C**)-SEM micrograph of preecdysal exoskeletal cuticle in *Ligia palassii* with layered epicuticle (ep) and exocuticle (ex) composed of helicoidally arranged chitin-protein fibers. (**D**)-TEM micrograph of exoskeletal cuticle in premolt *Ligia italica*; during apolysis old cuticle (oc) is detached and the preecdysal cuticle (pc) with epicuticular scale (es) is formed by epidermal cells (ec). (**E**)-TEM micrograph of resorbing lamella of the old cuticle (oc) with spherules (S) containing electron dense material. (**F**)-An intramolt male of *Ligia palassii* with shed posterior cuticle (animal size approximately 3 cm).

Slika 2: Struktura in funkcija kutikule pri kopenskih rakih enakonožcih: (**A**)- Histološki prerez želodca z izrazitimi kutikularnimi filtri (puščici) odrasle živali *Porcellio scaber*. (**B**)-TEM mikrografija nove kutikule eksoskeleta v fazi predlevitve, ki prikazuje epikutikulo (ep) in eksokutikulo (ex) pri vrsti *Ligia palassii*. (**C**)-SEM mikrografija kutikule eksoskeleta pred levitvijo prikazuje slojevito epikutikulo (ep) in eksokutikulo (ex) iz helikoidno urejenih hitinsko-proteinskih vlaken pri vrsti *Ligia palassii*. (**D**)-TEM mikrografija kutikule eksoskeleta v fazi predlevitve pri vrsti *Ligia italica*; med apolizo se stara kutikula (oc) odmakne od epitela, epidermalne celice (ec) pa tvorijo novo kutikulo (pc) z epikutikularnimi luskami (es). (**E**)-TEM mikrografija stare kutikule (oc) med resorpcijo in sferul (S), ki vsebujejo elektronsko gost material. (**F**)-Samec vrste *Ligia palassii* v fazi medlevitve, ko je že odvrgel posteriorno kutikulo (velikost živali je približno 3 cm).

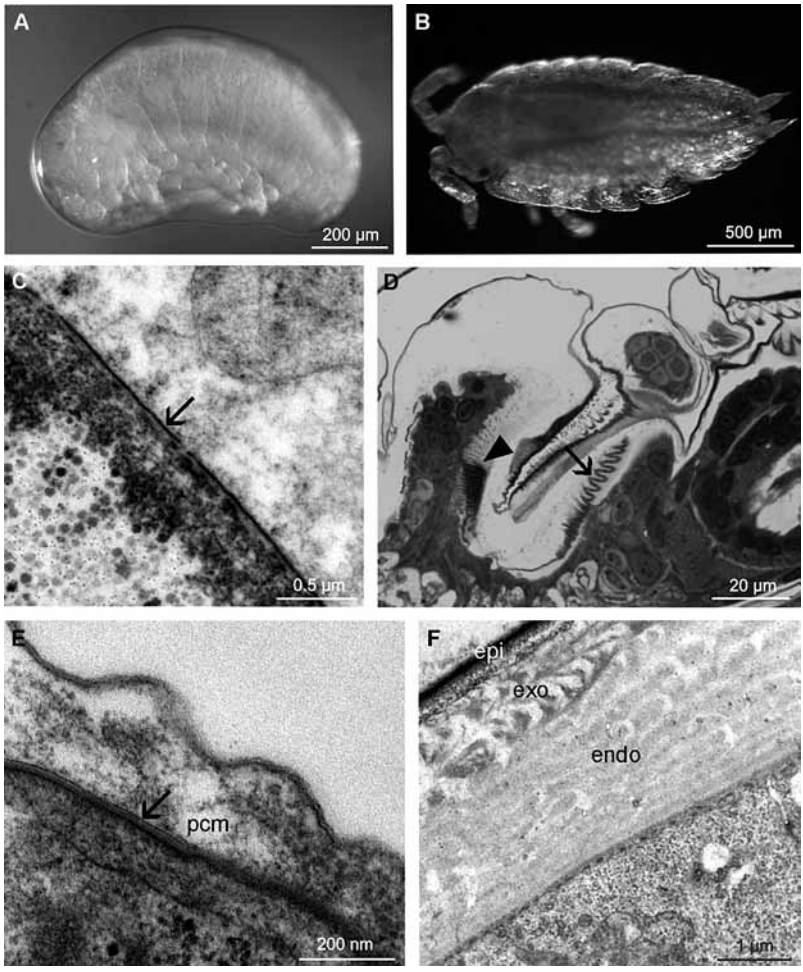

Figure 3: Morphogenesis of the gut and exoskeletal cuticle in embryos and marsupial mancas of *Porcellio scaber*. (**A**)-Late embryo of the stage 18, enclosed in the vitelline membrane. Appendages, eye and midgut glands with yolk are distinguished. (**B**)-Mid-stage marsupial manca. (**C**)-Hindgut cuticle in the late embryo is initially deposited in segments (arrow) that form a continuous layer in subsequent development. (**D**)-Primary cuticular filters (arrow) and setae of laterallia (arrowhead) in the stomach of late marsupial manca. (**E**)-The first exoskeletal cuticle is formed beneath the precuticular matrix (pcm) in the late embryo. Deposition of the multilayered epicuticle (arrow) is evidenced in the image. **(F**)-Exoskeletal cuticle of the mid-stage marsupial manca, differentiation into epicuticle (epi), exocuticle (exo) and endocuticle (endo) is evident.

Slika 3: Morfogeneza kutikule prebavnega trakta in eksoskeletne kutikule pri embrijih in marzupijskih mankah vrste *Porcellio scaber*. (A) Pozni embrij v stadiju 18, obdan z vitelinsko membrano. Razvidne so okončine, oko in prebavne žleze z rumenjakom. (B) Posnetek ličinke marzupijske manke v srednjem stadiju. (C) Pri poznem embriju se začne kutikula zadnjega črevesa nalagati v segmentih (puščica), ki se med razvojem povežejo v neprekinjen sloj. (D) Primarni kutikularni filtri (puščica) in sete (glava puščice) v želodcu pozne marzupijske manke. (E) Prva kutikula eksoskeleta se tvori pod prekutikularnim matriksom (pcm) pri poznem embriju. Na sliki je razvidna večslojna epikutikula (puščica). (F) Eksoskeletna kutikula pri srednji marzupijski manki je diferencirana v epikutikulo (epi), eksokutikulo (exo) in endokutikulo (endo).

Interactions between isopods and their microbiota

Although important, the role of isopods as decomposers in terrestrial ecosystems is mainly indirect. It includes the promotion of microbial activity by fragmentation of the substrate, increasing the number of some of the ingested microbes in their gut and the distribution of microorganisms in the terrestrial ecosystem (Zimmer 2002), while data on their direct role as decomposers remains scarce. Since the cellulolytic capability of herbivorous animals has been traditionally assigned to symbiotic microbiota, the presence of the latter has also been assumed to be in the digestive system of terrestrial isopods.

The idea on the resident microbiota being potentially involved in the digestion process was initiated by observations of filamentous bacteria attached to the inner surface of the hindgut in *P. scaber* (Drobne 1995) (Fig. 4A). At the same time, the description of pathogenesis and the presence of intracellular bacteria in the digestive glands of the same isopod species (Drobne et al. 1999b) indicated a wide array of specialized associations between the isopod hosts and bacteria. Research focused on the microbial associations of isopods had started in the group at the end of 90's, with a goal to extend our knowledge on isopod-bacteria symbiosis by microscopic observations and molecular techniques.

In collaboration with the group of Prof. Dr. Gorazd Avguštin from the Department of Animal Science at the University of Ljubljana we provided the first characterization of the resident microbiota of the gut of *P. scaber* based on 16S rRNA gene analysis (Kostanjšek et al. 2002); the filamentous bacteria attached to the gut wall were identified as commensal '*Candidatus Bacilloplasma*' (Kostanjšek et al. 2007) and the intracellular pathogen was *Rhabdochlamydia porcellionis* (Kostanjšek et al. 2004). Investigations into the latter continued in collaboration with the Department of Microbial Ecology, University of Vienna (Austria) and currently involves research into genome analysis and distribution of these pathogens in other arthropods.

Although the cellulolytic activity associated with the isopod digestive system has been assigned to endogenous cellulases, rather than to indigenous microbiota (Kostanjšek et al. 2010), our studies extended the knowledge on specialized bacterial symbionts of isopods. The descriptions of specific attachment structures of commensal *Bacilloplasma* and *R. porcellionis* as the first described chlamydia in arthropods, provide evidence that isopods may be an important, yet overlooked evolutionary playground for the development of novel bacterial groups including emerging pathogens (Corsaro and Venditti 2004). Further studies on *R. porcellionis* provided the protocol for their isolation in cell lines (Sixt et al. 2013), a detailed description of pathology in their natural host and their immune response to infection (Kostanjšek and Marolt 2015a) (Fig. 4B, C).

Recent descriptions of a heterogeneous bacterial community colonizing calcium bodies, the paired organs in the body cavity specialized for calcium storage in trichoniscid isopods represents yet another example of a unique association between woodlice and bacteria (Fig. 4E-G) (Vittori et al. 2012, Vittori et al. 2013). Despite its diversity and close affiliation to soil bacteria, the bacterial community of calcium bodies has the ability to accumulate poly-phosphate (Kostanjšek et al. 2015b). Although not fully understood, the unique symbiosis in the calcium bodies represents the first evidence of polyphosphate-accumulating bacterial symbionts in animal tissues and the first description of symbiosis confined to a specialized organ in isopod crustaceans.

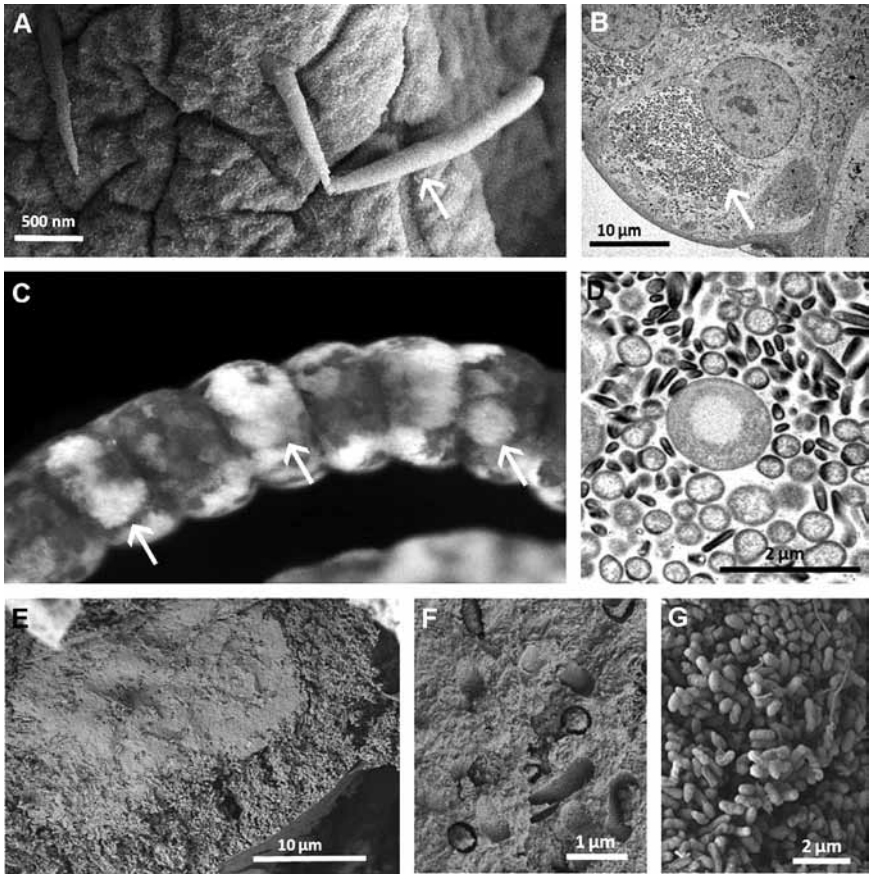

Figure 4: Associations between terrestrial isopods and bacteria. (**A)**-Scanning electron micrograph of a bacilloplasma (arrow) attached to a cuticular thorn in the hindgut of *Porcellio scaber*. (**B)**- Ultrastructure of hepatopancreatic cells of *P. scaber* infected with *R. porcellionis*. The cytoplasm of infected cells contains vacuoles (arrow) with numerous rhabdochlamydiae. (**C**)-White spots (arrows) in the hepatopancreas of *P. scaber* heavily infected with *Rhabdochlamydia porcellionis*. (**D)**-Higher magnification of rhabdoclamydiae within an infected cell. (**E)**-Scanning electron micrograph of a fractured calcium body of *Hyloniscus riparius*, containing a large bacterial population. (**F)**-Higher magnification of the mineral concretion with a calcium body of *H. riparius*, showing bacterial casts in the mineral. (**G)**-Diverse bacterial population consisting of rod-shaped, filamentous and helicoidal bacteria fills the lumen of a calcium body of *Titanethes albus*.

Slika 4: Povezave med kopenskimi enakonožci in bakterijami. (**A**)-Vrstična elektronska mikrografija bacilloplazme (puščica), pritrjene na kutikularni trn v zadnjem črevesu pri enakonožcu *Porcellio scaber*. (**B**)- Ultrastruktura celice hepatopankreasa *P. scaber*, okužene z *R. porcellionis*. Citoplazma okužene celice vsebuje vakuole (puščica) s številnimi rabdoklamidijami. (**C**)-Bele lise (puščice) v hepatopankreasu *P. scaber*, okuženim z bakterijo *Rhabdochlamydia porcellionis.* (**D**)-Večja povečava rabdoklamidij v okuženi celici. (**E**)-Vrstična elektronska mikrografija prelomljenega kalcijevega telesca pri enakonožcu *Hyloniscus riparius*, ki vsebuje številčno bakterijsko populacijo. (**F**)- Večja povečava mineralnega skupka v kalcijevem telescu *H. riparius*; v mineralu so vidni odtisi bakterij. (**G**)-Pestra bakterijska populacija, ki jo sestavljajo morfološko pestre bakterije, zapolnjuje lumen kalcijevega telesca vrste *Titanethes albus*.

Ecotoxicology

Terrestrial isopods have a long history as test organisms in ecotoxicology. This is due to their ecological relevance, their typical routes of exposure (soil and food), life history characteristics, ability to assimilate large amounts of metals, and the possibility to determine toxicity endpoints (Van Gestel 2012). According to the Thompson Reuters Web of Science™ one of the first ecotoxicity related papers on *P. scaber* was published already in 1978 reporting the accumulation of lead in these animals (Beeby 1978). In the 1980's the first papers on the assimilation of different metals in this species emerged (Hopkin et al. 1986, Dallinger and Prosi 1988, Donker et al. 1990) and ever since *P. scaber* has become a central terrestrial test organism in ecotoxicology.

Terrestrial ecotoxicology research at the Department of Biology began with the established cooperation with Steve Hopkin's group at the University of Reading (UK). As a result, the "standard laboratory test" with isopods was introduced based on studies with Zn (Drobne and Hopkin 1995, Drobne 1997). Further studies revealed that the molt frequency of isopods is influenced by the degree of Zn pollution in the food (Drobne and Štrus 1996). As molting also influences feeding and metal accumulation, a simple, brief, non-harmful identification of molt stages was proposed (Zidar et al. 1998). At that time, the majority of work at the department was focused on the assimilation of different metals in the whole body and digestive glands (Bibič et al. 1997, Zidar et al. 2003). Subsequent ecotoxicology research in the group followed the international progress in this field. The research efforts were aimed in two directions: (i) to assess the adverse effects of novel emerging pollutants and (ii) to study the effects on isopods investigated at different levels of biological organisation. In this respect, *P. scaber* has served as a central test object to investigate the effects of mercury (Hg) (Nolde et al. 2006), organophosphorus pesticides (Stanek et al. 2003), neonicotinoid pesticides (Drobne et al. 2008), natural insecticides (Zidar et al. 2012); veterinary medicines (Kolar et al. 2010, Žižek and Zidar 2013) and nanomaterials (Jemec et al. 2008, Novak et al. 2012a,b). Adverse effects of pollutants were studied on antioxidant enzyme activities and the cholinergic system (Jemec et al. 2008), cell membrane integrity (Novak et al. 2012a), lysosomal integrity (Nolde et al. 2006), whole biomolecular profile of digestive gland (Novak et al. 2013), metallothionein-like proteins (Žnidaršič et al. 2005), ultrastructure of the digestive glands (Žnidaršič et al. 2003, Lešer et al. 2008), isopod gut microbiota (Lapanje et al. 2008), and feeding behaviour (Kaschl et al. 2002, Zidar et al. 2004).

Taking a retrospective view of the achievements made during the last 20 years of intensive terrestrial ecotoxicology research, we have confirmed that *P. scaber* is indeed a suitable test model providing a great deal of information regarding the adverse effects of pollutants and we have promoted the use of this organism in ecotoxicology widely at national and international levels. We have shown that pollutants induce a number of changes on the digestive mid-gut gland (hepatopancreas): Hg was shown to increase the permeability of the lysosomal membranes (Nolde et al. 2006), Hg and pesticide imidacloprid induced the thinning of epithelium (Lapanje et al. 2008, Drobne et al. 2008), copper decreased the digestive gland cell membrane permeability (Valant and Drobne 2012), and imidacloprid and TiO_2 nanoparticles altered the activity of antioxidant enzymes (Jemec et al. 2008, Drobne et al. 2008). It has been revealed that some pollutants affect the gut bacterial community structure (Lapanje et al. 2008), and organophosphate diazinon decreases the activity of the enzyme ascetylcholinesterase in the whole body, resulting in decreased locomotory activity (Stanek et al. 2003). A strain of studies has been devoted to investigate the behavioural response of isopods to pollutants. We have commonly observed that isopods consume less polluted food when compared to the control exposure (Kaschl et al. 2002, Zidar et al. 2004, Drobne et al. 2008, Jemec et al. 2016). A number of investigations that included video tracking of foraging behaviour have revealed that isopods successfully discriminate between polluted and unpolluted food and soil (Zidar et al. 2003, 2004, 2005, 2012). The recognition of the isopods ability to detect chemicals in food or soil by using their chemoreceptors was a basis for establishing avoidance behaviour tests protocols with isopods. In the last 10 years we have been involved in stud-

ies investigating the interactions of nanomaterials with isopods. In this respect we have successfully joined the international nanotoxicity community. In the scope of this research we have shown that isopods substantially transform the nanoparticles inside the digestive tract. Namely, the dissolution of nanoparticles was substantially increased inside the animal (Golobič et al. 2012, Romih et al. 2012). We have shown that nanoparticles do not internalise in the digestive gland, but only their dissolved ions do (Novak et al. 2012b, 2013). In Fig. 5 we present some photos obtained in different ecotoxicity assays with *P. scaber*.

Figure 5: Photos obtained in different ecotoxicity assays with *P. scaber*. (**A**)-Organism during the toxicity test. (**B**)-*P. scaber* after molt probably consuming the anterior exuvium (**C**)-Cross section of hepatopancreas; large B-cells include large lipid vesicles (arrow).

Slika 5: Slike pridobljene v različnih ekotoksikoloških testiranjih s *P. scaber*. (A)- Raki med testom strupenosti. (**B**)-*P. scaber* po levitvi sprednjega dela. (**C**)-Prečni prerez prebavne žleze hepatopancras, velike B-celice imajo velike lipidne kaplje (puščica).

Systematics and evolution

The presence of morphologically diverse populations of ecologically important *A. aquaticus* in different types of freshwater habitats has challenged several authors to try to clarify the systematics of this species. In addition, the species is interesting from a phylogeographic perspective for several reasons: it is common throughout most of its range; it is present in several hydrographically separated Mediterranean drainages and all major drainages in central Europe; its current range has not been greatly affected by human impacts; it is tolerant to organic pollution and most of its range is inhabited by morphologically uniform populations of the nominotypical subspecies, while morphologically distinct subspecies with limited ranges occur in the S and SE of its range.

The first attempts at presenting racial differentiation in *A. aquaticus* were made on the basis of a small number of samples, specimens, and morphometric characters (Karaman 1952, Sket 1965a). Sket (1994) summarized the taxonomy of the species, listing as many as 10 subspecies and suggesting high probability of extensive racial diversification, especially in Slovenia. His supposition was confirmed by the results of multivariate statistical analyses of geographic variation in an extensive set of morphometric characters run on large series of populations (Prevorčnik et al. 2004). High variation in characters within the surface populations resulted in their partial overlap, while the analysed highly troglomorphic populations were clearly analized separated not only from the surface ones, but also from each other. Some inconsistencies in formerly applied classification appeared; namely, the Romanian population from sulphurous mesothermal waters had already been described as *A. a. infernus* (Turk-Prevorčnik et Blejec, 1998), while three populations from NW Dinaric karst were treated as a single subspecies (*A. a. cavernicolus* Racovitza, 1925) at the time.

To overcome the shortcomings of morphology-based taxonomy, molecular studies have been used as a promising taxonomic tool. The first studies focused on the relationships of the cave to surface populations using randomly amplified polymorphic DNA (RAPD; Verovnik et al. 2003) and nuclear and mitochondrial DNA sequences (Verovnik et al. 2004) as genetic markers. The results supported the observed morphological separation, showing that the invasions of the cave populations were spatially as well as temporally independent. Subsequently, DNA sequences were also used for the assessment

of the colonization history of the species (Verovnik et al. 2005). These analyses suggested that the European continent was colonized from the SE during late Miocene. Mitochondrial DNA still shows the pattern of several deeply subdivided clades, while much more uniform nuclear DNA sequences and geographical mixing of mitochondrial DNA groups speak in favour of extensive recent gene flow. As the original description of the nominate species was inadequate and the type material of Linné appeared to be lost, it was imperative to designate a neotype for *A. aquaticus* and provide its proper description (Verovnik et al. 2009).

Cave populations tend to be genetically isolated from nearby surface populations and thus potentially represent independent, albeit young species. The cave population from Grotta di Trebiciano/Labodnica near Trieste/Trst (lower part of the Reka River drainage, NW Italy) showed the highest degree of genetic independence among all subterranean populations. Therefore, it was described as the first cave species of the genus in Europe under the name *Asellus kosswigi* (Verovnik et al. 2009) (Fig. 6).

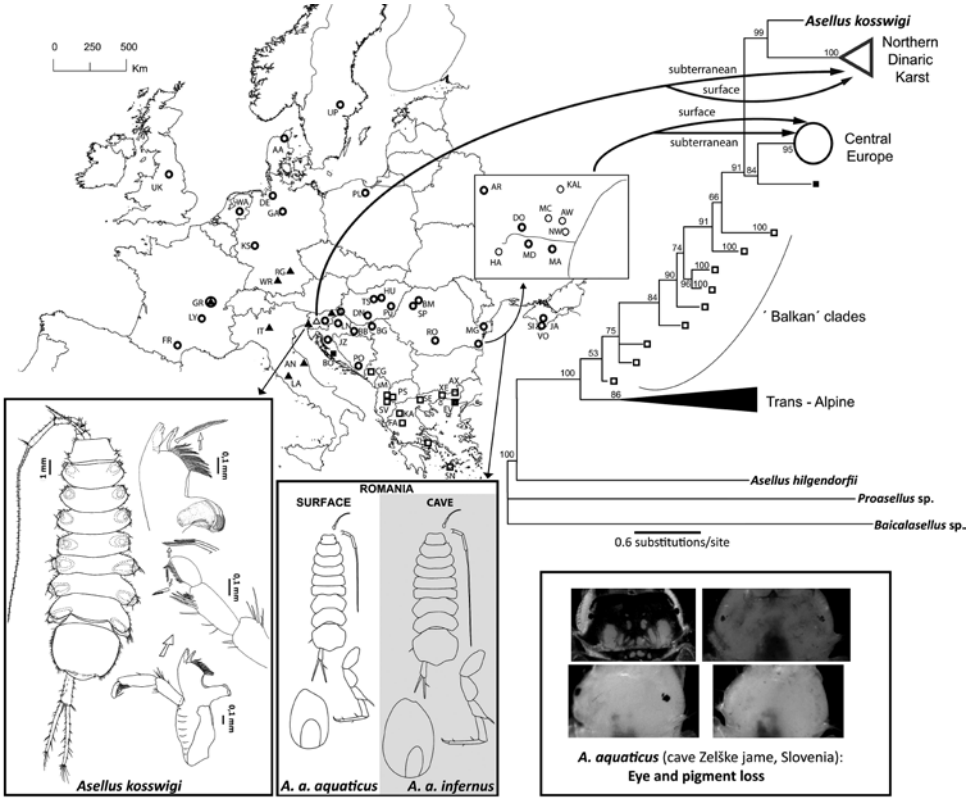

Figure 6: Systematics and evolution of *Asellus aquaticus*. The global phylogeography of the species is based on a Bayesian phylogeny of mitochondrial (COI) haplotypes (modified from Konec et al. 2015). Pictures show the recently described *A. kosswigi* from the subterranean Reka River (modified from Verovnik et al. 2009), the Romanian surface/cave ecomorph pair, and the variability of eye and pigment loss on the head in one of the Slovenian cave populations.

Slika 6: Sistematika in evolucija vodnega oslička, *Asellus aquaticus*. Globalna filogeografija vrste na podlagi Bayesove filogenije mitohondrijskega (COI) gena (prirejeno iz Konec et al. 2015). Slike prikazujejo še: nedavno opisanega troglobionta *A. kosswigi* iz reke Reke (prirejeno iz Verovnik et al. 2009), romunski površinski in jamski ekomorf ter različne stopnje izgube pigmenta in oči pri eni od slovenskih jamskih populacij.

Just recently, this species has also been discovered in the upper (Slovenian) parts of the same drainage (Konec et al. 2015). A number of other subterranean populations are known from caves in the Ljubljanica and Krka River drainages. These populations are even younger invaders into the subterranean realm than *A. kosswigi* and therefore genetically less differentiated. Their genetic structure and evolutionary history was studied using microsatellite DNA, which is more variable than mitochondrial DNA sequences. In the unique hydrological setting of the cave Planinska jama (Slovenia) with the subterranean confluence of two sinking rivers, an equally unique diversity of subterranean *Asellus* populations was discovered. Two highly troglomorphic and genetically distinct populations each dwelling in its own channel of the cave, are isolated from each other as well as from the surrounding surface populations (Konec et al. 2016). Only at the confluence they form a narrow hybrid zone, representing the first known case of secondary sympatry between two subterranean sister lineages in a cave.

In the last decade, *A. aquaticus* became one of the few invertebrate model species for the study of troglomorphic evolution. It possesses several attributes expected in such species: its cave populations have evolved many troglomorphic traits for which the direction of change is known (since they were derived from surface ancestors); trait changes can be attributed to a specific environmental factor (absence of light and reduced/absent primary productivity); their extant surface ancestors provide a dual system of both forms; they are relatively abundant in nature, ability to rear in the laboratory and they produce large numbers of easily accessible embryos (Protas and Jeffery 2012). Pairs of surface and descendant subterranean *Asellus* populations from different habitats and different geographic areas were examined to address questions of evolutionary parallelism and convergence. Surprisingly, it turned out that only one third of all changes were convergent (Konec et al. 2015), and that reduction and loss of both eyes and body pigment is caused by two or more independent genetic pathways (Protas et al. 2011).Recently, ethological observations of *Asellus* habitat selection have started in an attempt to elucidate the acquisition and maintenance of reproductive isolation between surface and cave-dwelling populations in the absence of geographical barriers.

Over decades, numerous collaborations with domestic and foreign experts led to a number of important findings and *Asellus* has certainly contributed to the recognition of the research group within the speleobiological and wider scientific community.

Conclusions and prospective view

In this paper, we have presented the research relating to two isopod model species, terrestrial *Porcellio scaber* and aquatic *Asellus aquaticus*, undertaken at the Chair of Zoology, Department of Biology during the last 45 years.

P. scaber has been successfully used as a model species in studies on functional morphology of integument and digestive system during molting and development. Within this line of research future studies will be aimed at ultrastructural and cytochemical studies of different extracellular matrices in order to establish structural and functional features of natural materials under different conditions and thus provide the basic knowledge for designing bio-inspired materials used in new technologies. Despite considerable potential in agriculture, ecology, healthcare and pest control, the current exploitation of interactions between bacteria and arthropods remains restricted to a handful of associations (Bourtzis and Miller 2003). With a wide array of specialized bacterial associations, *P. scaber* represents an appropriate and much needed model organism for studies into arthropod-microbe interactions, with a strong potential for an extension of current knowledge and a better understanding of these associations. *P. scaber* has become a central test model in terrestrial ecotoxicity studies offering an array of possibilities to investigate the effects of pollutants on several organisational levels, from single molecules to populations. We envisage that our future ecotoxicity research will continue to follow the emerging environmental threats and contribute to the understanding of potential risks to terrestrial environments.

A. aquaticus has become one of the most important organisms for the study of evolution of subterranean biodiversity and the evolution of troglomorphies. To reach this stage, substantial fundamental research on taxonomy, population

genetics, morphology and ecology was performed in the past four decades. New exciting findings are within reach in the fields of evolutionary developmental genomics, population genomics, and the biology of speciation of subterranean species. The newly revealed taxonomic complexity of what has been until recently considered as a single species, prompted the description of the first subterranean species with at least two new ones to follow. In that way, *A. aquaticus* with its descendant subterranean populations fosters the tradition of speleobiological research at the Department of Biology and contributes its share to the subterranean biodiversity of the Dinaric Karst, which is known to be the richest in the World.

The scientific studies of terrestrial and aquatic isopods at the Department of Biology have been fostered since Pavel Ličar's first reports in the early 70's. The present paper brings strong evidence on the diversity, importance and scientific potential of our research proving isopods are an excellent model organism for future research. Perhaps, even more than Professor Pavel Ličar had ever imagined.

Povzetek

V članku predstavljamo študije rakov enakonožcev, obsežne skupine višjih rakov (Malacostaraca: Peracarida: Isopoda), ki se ji na Katedri za zoologijo posvečamo že nekaj desetletij z raziskavami na področjih funkcionalne morfologije in razvojne biologije, specifičnih interakcij med gostiteljem in mikroorganizmi, ekotoksikologije ter sistematike in evolucije. V prispevku navajamo razloge za preučevanje dveh modelnih rakov enakonožcev: navadnega prašička (*Porcellio scaber*) in vodnega oslička (*Asellus aquaticus*). Poleg zgodovinskega poteka raziskav na posameznih področjih, predstavljamo tudi najpomembnejše rezultate novodobnih raziskav, njihov pomen ter načrte za prihodnost. Kopenski raki so, zaradi splošne razširjenosti, uspešnih prilagoditev življenju na kopnem, enostavnega vzdrževanja v laboratoriju in učinkovite reprodukcije odličen model za raziskave na področju funkcionalne morfologije in razvojne biologije. Pogoste levitve v zvezi z rastjo in razmnoževanjem in velika raznolikost v zgradbi eksoskeleta so priložnost za študij sinteze in razgradnje zunajceličnega matriksa in procesov biomineralizacije, predvsem dinamike kalcija v epitelijih, ki izločajo kutikulo. Eksoskelet kopenskih rakov enakonožcev je večslojni matriks iz hitina in proteinov, ki je pri amfibijskih in terestričnih skupinah v različni meri kalcificiran. Struktura, kemijska sestava in mehanske lastnosti kutikule se razlikujejo med vrstami in tudi glede na telesne regije pri isti vrsti. Z raziskavami morfogeneze prebavnega sistema in integumenta pri vrsti *P. scaber* se vključujemo v širše področje raziskav embrionalnega in postembrionalnega razvoja rakov, ki v zadnjem času hitro napreduje in obsega vse več študij tako z vidika funkcionalne morfologije kot z vidika evolucijske razvojne biologije. Na osnovi morfoloških kriterijev smo opisali 19 embrionalnih stadijev in tri stadije marzupijskih mank. Razložili smo oblikovanje ektodermalnega in endodermalnega dela prebavnega sistema. Pojasnili smo tudi ultrastrukturne značilnosti diferenciacije eksoskeletne kutikule in kutikule v črevesu med razvojem embrijev in marzupijskih mank. V nadaljevanju te raziskave nadgrajujemo s študijem diferenciacije epitelnih celic integumenta in črevesa, diferenciacije povezav z mišicami in študijem zgodnjih procesov kalcifikacije kutikule eksoskeleta. Študij navedenih vsebin je izrazito interdisciplinaren, saj povezujemo raziskave na področju anatomije, histologije, celične biologije, razvojne biologije in znanosti o materialih, kar je nujno za ustvarjanje novega znanja in njegovo uporabo v sorodnih področjih. Raziskave sekrecije in mineralizacije različnih tipov zunajceličnega matriksa med embrionalnim razvojem modelnega organizma *P. scaber* so pomembne za razumevanje vloge matriksa v diferenciaciji epitelijev prebavil in integumenta nevretenčarjev. Prisotnost raznolikih mikroorganizmov na površini kutikule prebavila je verjetno povezana s sestavo in dinamiko zunajceličnih matriksov med embrionalnim razvojem, zato raziskave nadaljujemo tudi v tej smeri. Odkrili smo številne in raznolike interakcije med *P. scaber* in mikroorganizmi v prebavilu. Raziskave interakcij med členonožci in mikroorganizmi so pomembne tudi z vidika razgradnje organske snovi in nastajanja prsti, zato so kopenski enakonožci pomemben model za ugotavljanje procesov dekompozicije. Tudi s slednjega stališča so kopenski raki *P. scaber*

pomemben osrednji model na področju kopenske ekotoksikologije. Tekom naših raziskav smo dokazali veliko število vplivov različnih vrst onesnaževal, od kovin, pesticidov, veterinarskih zdravil do nanomaterialov na organizem *P. scaber*. Vplive smo proučevali na različnih nivojih organizacije: celičnem (spremembe aktivnosti antioksidativnih encimov, holinergični sistem), tkivnem (struktura in funkcija prebavne žleze) ter prehranjevalnega vedenja živali s sledenjem in opisom.

Visoka morfološka pestrost populacij ekološko pomembnega vodnega oslička (*A. aquaticus*) je in še predstavlja taksonomski izziv. V raziskovalni skupini za speleobiologijo smo se ga lotili z morfometričnimi in molekulskimi analizami velikega števila vzorcev in osebkov. Z vsemi analizami smo dokazali jasno ločitev podzemeljskih populacij od površinskih. Še več, podzemeljske so se močno razlikovale tudi med seboj. *A. aquaticus* je izjemno primeren model za filogeografske raziskave zaradi splošne razširjenosti v medsebojno ločenih evropskih povodjih, sorazmerne odpornosti proti onesnažilom ter velike rasne diferenciacije, zlasti na jugu in jugovzhodu območja razširjenosti. Z analizo mitohondrijske in jedrne DNA smo ugotovili, da naj bi do poselitve Evrope prišlo v poznem Miocenu, podzemeljske populacije pa so podzemlje očitno poselile večkrat neodvisno. Na osnovi neskladja z dotlej veljavno klasifikacijo smo poskrbeli za dopolnjen opis nominotipske vrste in opisali prvo evropsko podzemeljsko vrsto, *A. kosswigi* iz italijanske jame Labodnica. Pred kratkim smo jo našli tudi v Sloveniji, kjer na opis čakata še dve pravi podzemeljski vrsti, ki med drugim živita v dveh rokavih Planinske jame. Ravno morfološko visoko specializirane (troglomorfne) jamske populacije, pri katerih je genetski pretok s predniškimi površinskimi populacijami zanemarljiv ali prekinjen, omogočajo poglobitev razumevanja procesov populacijske diferenciacije, speciacije in evolucije troglomorfij. Zato se je v zadnjih letih *A. aquaticus* uveljavil tudi kot ključni model v evolucijskih študijah, ki raziskujejo prilagajanje živali ob vselitvi v novo okolje. Nepričakovano se je izkazalo, da je pri osličkih le tretjina sprememb na račun življenja v podzemlju (troglomorfij) konvergenc, poleg tega pa do izgube pigmenta in oči privedeta dva različna genetska mehanizma. Pred kratkim smo se v skupini lotili tudi prvih vedenjskih poskusov, s katerimi poskušamo pojasniti nastanek reproduktivne bariere med površinskimi in podzemeljskimi populacijami. V raziskovalni skupini smo z izsledki taksonomskih, filogenetskih, filogeografskih in evolucijskih raziskav na vodnem osličku pomembno prispevali k splošnemu znanju o biodiverziteti Dinarskega Krasa, s podzemeljskim živalstvom najbogatejšega območja sveta.

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Fluorescent markers in microscopy: photophysical characteristics and applications in cell biology

Fluorescenčni označevalci v mikroskopiji: fotofizikalne značilnosti in uporaba v celični biologiji

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Izvleček: V fluorescenčni mikroskopiji bioloških vzorcev je večinoma treba strukture v celicah in tkivih ustrezno označiti z različnimi fluorescenčnimi označevalci. Tri glavne skupine fluorescenčnih označevalcev so majhni organski fluorokromi, fluorescenčni proteini in kvantne pike. Fluorescenčni označevalci se razlikujejo po fotofizikalnih lastnostih in po specifičnosti za vezavo na izbrane tarčne sestavine v vzorcu. Za označevanje izbranih struktur je treba majhne organske fluorokrome in kvantne pike običajno konjugirati s tarčno specifičnimi makromolekulami. Za označevanje s fluorescenčnimi proteini v celice ali organizem vnesemo gen za fluorescenčni protein. Napomembnejše fotofizikalne lastnosti fluorescenčnih označevalcev so vzbujevalni in izsevani spektri, Stokesov zamik, ekstinkcijski koeficient in kvantni izkoristek. Za doseganje ločljivosti pod uklonsko omejitvijo se v zadnjem času izkoriščajo posebni fluorokromi, pri katerih je fluorescenco mogoče modulirati z osvetljevanjem s svetlobo določene valovne dolžine.

Ključne besede: organski fluorokromi, fluorescenčni proteini, kvantne pike, fluorescenčna mikroskopija, označevanje

Abstract: In the fluorescence microscopy of biological specimens the structures in cells and tissues usually need to be labelled with various fluorescent markers. The three main groups of fluorescent markers are small organic fluorochromes, fluorescent proteins and quantum dots. Fluorescent markers differ according to photophysical properties and binding specificity for the selected target structures in the sample. For the labelling of specific structures with small organic fluorochromes or quantum dots it is usually necessary to conjugate them with target specific macromolecules. For the labelling with fluorescent proteins it is necessary to introduce a fluorescent protein gene into the observed cells or organism. The most important photophysical properties of fluorescent markers are absorption and emission spectra, Stokes shift, extinction coefficient and quantum yield. Currently, various super-resolution fluorescent microscopy techniques exploit special fluorochromes that enable fluorescence modulation by specific wavelength illumination, to achieve the resolution below the diffraction limit.

Keywords: organic fluorochromes, fluorescent proteins, quantum dots, fluorescence microscopy, labelling

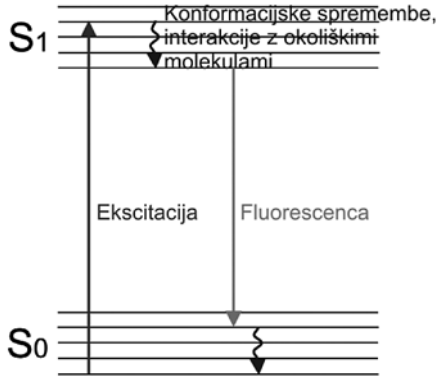
Uvod

Razvoj fluorescenčne mikroskopije je omogočil nove pristope za lokalizacijo posameznih celičnih komponent v fiksiranih in živih celicah, s čimer je fluorescenčna mikroskopija postala nepogrešljiva metoda v celični biologiji. V zadnjih desetletjih je bil dosežen velik preboj predvsem v povezavi z izboljšanjem ločljivosti fluorescenčne mikroskopije. Razvoj optičnih komponent mikroskopov in opreme za zajem in računalniško analizo slike je pripeljal do novih tehnik fluorescenčne mikroskopije super-ločljivosti, ki omogočajo prikaz struktur z ločljivostjo pod uklonsko omejitvijo (Hell in Wichmann 1994, Gustafsson 2000, Betzig in sod. 2006, Rust in sod. 2006). Ker je v fluorescenčni mikroskopiji za prikaz določene celične strukture potrebno označevanje te z določenim fluorescenčnim označevalcem, je bil poleg razvoja mikroskopov pomemben tudi razvoj že poznanih in novih fluorescenčnih označevalcev. Trenutno so v fluorescenčni mikroskopiji v uporabi tri glavne skupine fluorescenčnih označevalcev: majhni organski fluorokromi, fluorescenčni proteini in kvantne pike. Izmed vseh treh skupin fluorescenčnih označevalcev so najdlje v uporabi majhni organski fluorokromi. Na voljo je širok nabor teh molekul, vendar so večinoma derivati nekaj osnovnih heterocikličnih spojin (Lavis in Raines 2008, Terai in Nagano 2013). Fluorescenčni proteini so v glavnem derivati osnovnega zelenega fluorescenčnega proteina, ki je bil izoliran iz meduze vrste *Aequeorea victoria* (Shimomura in sod. 1962, Tsien 1998). Kvantne pike pa so polprevodniški nanokristali s posebno lastnostjo, da je valovna dolžina oddane fluorescenčne svetlobe odvisna od njihove velikosti (Medintz in sod. 2005). Poleg drugih lastnosti, je za uporabnost fluorescenčnih snovi v mikroskopiji pomembno, da je označevanje z njimi specifično. Specifičnost označevanja s fluorescenčnimi proteini se zagotovi z vnosom gena za fuzijski protein, v katerem je fluorescenčni protein vezan na tarčni protein. Kljub temu je pred poskusi potrebno preveriti ali je fuzijski protein funkcionalen in ali se v celici nahaja na običajnem mestu za tarčni protein. Majhne organske fluorokrome in kvantne pike za specifično označevanje običajno konjugirajo z ustrezno makromolekulo, ki zagotovi specifičnost vezave na opazovano celično komponento. Vzporedno z razvojem različnih tehnik fluorescenčne mikroskopije super-ločljivosti poteka tudi razvoj fluorescenčnih snovi s posebnimi fotokemičnimi lastnostmi. Te tehnike namreč za doseganje ločljivosti pod uklonsko omejitvijo izkoriščajo specifične odzive fluorescenčnih snovi na osvetljevanje s svetlobo določene valovne dolžine, pogosto pri visoki intenziteti. Z modifikacijami tako poskušajo pridobiti fluorescenčne snovi, pri katerih lahko fluorescenco moduliramo z osvetljevanjem s svetlobo določene valovne dolžine (Fernandez-Suarez in Ting 2008, Uno in sod. 2015).

Fluorescenca in z njo povezane lastnosti fluorescenčnih snovi

Za fluorescenčne molekule oziroma fluorokrome je značilen specifičen odziv na osvetljevanje. Zanje je značilen pojav fluorescence pri katerem molekula absorbira svetlobo določene valovne dolžine in nato izseva svetlobo nižje energije, torej daljše valovne dolžine. V povezavi s fluorescenco imajo različne fluorescenčne snovi specifične fotofizikalne lastnosti. Dve ključni lastnosti sta vzbujevalni in izsevani spekter. Vzbujevalni spekter je območje valovnih dolžin, s katerimi lahko vzbudimo določeno fluorescenčno molekulo. Foton z ustrezno valovno dolžino lahko povzroči prehod zunanjega elektrona fluorescenčne molekule v orbitalo višje energije in s tem vzbudi fluorescenčno molekulo iz osnovnega stanja S_0 v vzbujeno stanje, npr. v S_1 (Sl. 1). Ker fluorescenčna molekula ob vzbujanju lahko preide na različne vibracijske nivoje stanja S_1 ali celo v višja vzbujena stanja, je določeno fluorescenčno molekulo mogoče vzbujati s svetlobo v določenem razponu valovnih dolžin. Od tega razpona, ki je odvisen od dovoljenih energijskih stanj, je odvisna širina vzbujevalnega spektra. Ob prehodu iz vzbujenega stanja S_1 nazaj v osnovno stanje S_0 fluorescenčna molekula odda foton z izsevano valovno dolžino. Izsevani spekter nam pove, katere valovne dolžine svetlobe lahko fluorescenčna molekula odda ob tem prehodu. Pred emisijo fluorescenčne svetlobe se del energije različnih vibracijskih nivojev vzbujenega stanja S_1 vedno sprosti zaradi konformacijskih sprememb znotraj fluorescenčne molekule in interakcij z okoliškimi molekulami.

Zato je izsevana valovna dolžina vedno daljša od vzbujevalne, profil izsevanega spektra pa je neodvisen od uporabljene vzbujevalne valovne dolžine. Kljub temu pa molekula ob sproščanju v osnovno stanje lahko preide na različne vibracijske nivoje osnovnega stanja S_0. Zaradi tega ima tudi oddana svetloba valovne dolžine v določenem razponu, kar se odraža v širini izsevanega spektra (Murphy 2001, Lichtman in Conchello 2005, Johnson in Spence 2010). Razliko med vzbujevalno in izsevano valovno dolžino imenujemo Stokesov zamik. Fluorescenčne molekule se zelo razlikujejo po vrednosti Stokesovega zamika. Prednost fluorokromov z večjim Stokesovim zamikom je, da v fluorescenčnem mikroskopu lažje ločimo njihovo vzbujevalno in izsevano svetlobo. Intenziteta fluorescence določene fluorescenčne molekule je odvisna od njenega ekstinkcijskega koeficienta in kvantnega izkoristka. Ekstinkcijski koeficient pove koliko svetlobe lahko fluorescenčna molekula absorbira pri določeni valovni dolžini vzbujevalne svetlobe. Kvantni izkoristek pa pove koliko fotonov lahko molekula odda s fluorescenco, če absorbira en foton (Murphy 2001, Lavis in Raines 2008, Johnson in Spence 2010). Pri uporabi fluorokromov je treba upoštevati tudi bledenje njihove fluorescence, ki je izguba fluorescence zaradi s fotoni povzročenih kemičnih poškodb in kovalentnih modifikacij molekul fluorokroma. Fotofizikalne lastnosti fluorokromov so odvisne tudi od kemijskega okolja v katerem je fluorokrom. Na fotofizikalne lastnosti tako vplivajo pH, ionska jakost, polarnost topila, koncentracija O_2, prisotnost molekul, ki utišajo fluorescenco in drugi dejavniki (Murphy 2001).



Slika 1: Osnovna shema energijskih stanj molekule in prehodov elektronov med njimi. Ob vzbujanju fluorescence zunanji elektron molekule preide iz osnovnega stanja S_0 v vzbujeno stanje S_1. Energija različnih vibracijskih nivojev stanja S_1 se porazgubi s konformacijskimi spremembami znotraj molekule in interakcijami z okoliškimi molekulami. Ob prehodu elektrona iz vzbujenega stanja S_1 nazaj v osnovno stanje S_0 molekula odda fluorescenčno svetlobo.

Figure 1: Basic scheme of the energy states of a molecule and electron transitions between them. During the excitation of fluorescence an outer electron of a molecule moves form the ground state S_0 to the excited state S_1. Energy of different vibrational levels of state S_1 is dissipated through conformational changes within a molecule and interactions with surrounding molecules. During the transition from the excited state S_1 back to the ground state S_0 a molecule emits fluorescent light.

Majhni organski fluorokromi

Majhni organski fluorokromi so večinoma aromatske ali heterociklične spojine. Zaradi konjugiranih dvojnih vezi lahko že fotoni vidnega spektra elektromagnetnega valovanja z relativno nizko energijo sprožijo njihov prehod v vzbujeno stanje. V splošnem velja, da več kot ima molekula konjugiranih dvojnih vezi, nižja energija je potrebna za vzbujanje fluorescence (Lichtman in Conchello 2005). Prvi identificirani organski fluorokrom je bil kinin sulfat, ki ob osvetljevanju z

ultravijolično svetlobo fluorescira modro. Kasneje so bili odkriti ali pridobljeni z modificiranjem obstoječih številni novi organski fluorokromi, ki lahko oddajajo fluorescenčno svetlobo drugih valovnih dolžin. Organski fluorokromi, ki so na voljo danes, pokrivajo praktično celoten vidni spekter elektromagnetnega valovanja. V fluorescenčni mikroskopiji so najpogosteje v uporabi različni derivati fluoresceina in rodamina, cianini ter barvila BODIPY (boron-dipyrromethene). Proizvajalci fluorescenčnih barvil ponujajo derivate teh osnovnih fluorescenčnih molekul z izboljšanimi lastnostmi pod različnimi imeni, ki predstavljajo njihove blagovne znamke. Takšen primer so na primer barvila Alexa Fluor® (Molecular Probes, Inc.) (Johnson in Spence 2010, Terai in Nagano 2013).

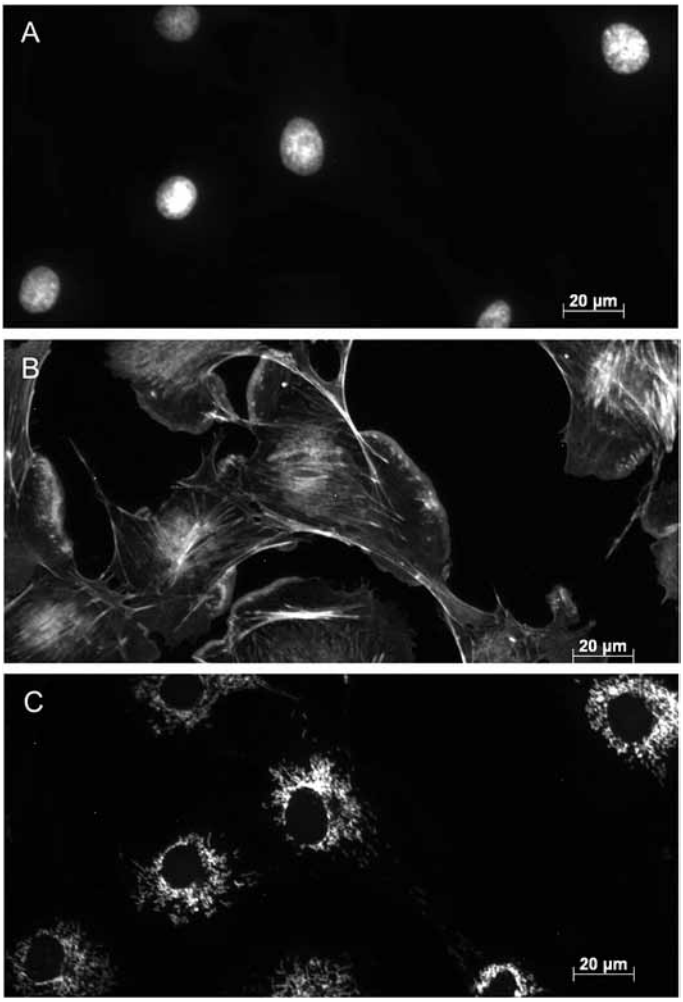
V fluorescenčni mikroskopiji lahko opazujemo naravno prisotne endogene organske fluorescenčne molekule (avtofluorescenca), ali pa opazovano strukturo v vzorcu označimo z neko sintetično fluorescenčno organsko molekulo (sekundarna fluorescenca) (Sl. 2). Če je v vzorcu s fluorokromi, ki fluorescirajo v različnih barvah, označenih več struktur, je mogoče posnete slike posameznih struktur sestaviti v skupno sliko (Dodatna Sl. 1). Na voljo so sintetični organski fluorokromi za različne načine uporabe. Prva skupina so fluorokromi, ki jih za uporabo konjugiramo s tarčno specifičnim ligandom, kar nam omogoča specifično lokalizacijo izbranih molekul v vzorcu. Druga skupina so fluorokromi, ki se specifično vežejo na nukleinske kisline. Tretja skupina so nekonjugirani fluorokromi, ki se lahko koncentrirajo v različnih organelih na podlagi fizioloških procesov in kemijskega okolja v določenem organelu. V četrto skupino spadajo fluorescenčne molekule, ki se uporabljajo kot senzorji za specifične ione.

Endogene fluorescenčne organske molekule

Med fluorescenčne organske molekule, ki so v bioloških vzorcih naravno prisotne, spadajo med drugim aromatske aminokisline (fenilalanin, tirozin, triptofan), reducirani nikotinamidni kofaktorji (NADH, NADPH) in flavini. Za vzbujanje teh molekul je potrebna večinoma ultravijolična svetloba. Njihova fluorescenca je v območju med ultravijoličnim in zelenim delom vidnega spektra. Endogene fluorescenčne molekule so vzrok za avtofluorescenco, ki v fluorescenčni mikroskopiji pogosto moti zajem signala iz fluorescenčno označenih struktur, ki jih opazujemo. Temu se lahko do določene mere izognemo, če za označevanje izberemo fluorescenčno barvilo, ki ima vzbujevalni in izsevani maksimum v rdečem delu vidnega spektra oziroma izven območja avtofluorescence (Lavis in Raines 2008) ali pa na različne načine avtofluorescenco čim bolj zmanjšamo.

Majhni organski fluorokromi konjugirani s tarčno specifičnimi ligandi

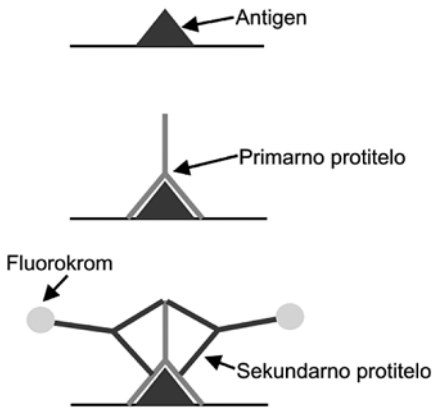
Večina majhnih organskih fluorokromov v splošnem ne izkazuje specifičnosti za vezavo na določene komponente v biološkem vzorcu. Da lahko z njimi specifično označimo neko celično komponento jih je treba konjugirati z nekim tarčno specifičnim ligandom, običajno s protitelesom. Fluorescenčno označena protitelesa se nato uporabljajo v imunofluorescenčni mikroskopiji za označevanje specifičnih biomolekul v vzorcu. Na ta način se najpogosteje označujejo specifični proteini (Sl. 3). Druga možnost je konjugacija fluorescenčne molekule z enoverižno protismerno DNA za specifičen gen ali drugo nukleotidno zaporedje. Takšne fluorescenčno označene oligonukleotidne sonde so uporabne pri fluorescenčni *in situ* hibridizaciji (FISH), s katero lahko spremljamo gensko ekspresijo ali lokaliziramo specifičen gen na kromosomu ali v jedru (Suzuki in sod. 2007). Za konjugacijo s tarčno specifičnimi makromolekulami se izmed majhnih organskih fluorokromov najpogosteje uporabljajo različni derivati fluoresceina (Dodatna sl. 2A), kot je na primer fluorescein izotiocianat (FITC). Fluorescein absorbira modro svetlobo in fluorescira zeleno svetlobo. Težava pri njegovi uporabi je relativno hitro bledenje fluorescence in občutljivost na nizek pH (Lavis in Raines 2008, Johnson in Spence 2010). Poleg derivatov fluoresceina so za konjugacijo z makromolekulami pogosto v uporabi derivati rodamina, kot je na primer tetrametilrodamin (Dodatna sl. 2B) v obliki reaktivnega izotiocianata (TRITC) ali karboksilne kisline (TAMRA). Tetrametilrodamin absorbira zeleno svetlobo in fluorescira oranžno svetlobo.



Slika 2: Primer fluorescenčnega označevanja celičnih jeder, citoskeleta in mitohondrijev v endotelnih celicah. A – S fluorescenčnim barvilom DAPI (4',6-diamidino-2-fenilindol) označena jedra. DAPI se specifično veže na DNA. **B** – Aktinski filamenti označeni s fluorescenčnim barvilom Alexa Fluor® 488. Fluorescenčno barvilo je konjugirano s faloidinom, ki se specifično veže na aktinske filamente. **C** – Mitohondriji označeni z barvilom MitoTracker® Red CMXRos, ki se koncentrira v mitohondijih. Celične komponente so označene na preparatu celic goveje pljučne arterije.

Figure 2: An example of the fluorescent labelling of cell nuclei, cytoskeleton and mitochondria in endothelial cells. A – Cell nuclei labelled with fluorescent dye DAPI (4',6-diamidino-2-phenylindole), which specifically binds to the DNA. **B** – Actin filaments labelled with fluorescent dye Alexa Fluor® 488. Fluorescent dye is conjugated to phalloidin, which specifically binds to the actin filaments. **C** – Mitochondria labelled with fluorescent dye MitoTracker® Red CMXRos, which concentrates in the mitochondria. Cell components are labeled in the bovine lung artery cell specimen.

V primerjavi s fluoresceinom je bolj fotostabilen, njegova fluorescenca pa je neodvisna od pH (Lavis in Raines 2008, Johnson in Spence 2010). Za označevanje protiteles, nukleinskih kislin, lipidov in drugih bioloških molekul so pogosto v uporabi cianini, kot so na primer CyDye barvila. To so kationske molekule sestavljene iz dveh heterocikličnih podenot povezanih s polienskо verigo (Dodatna sl. 2D). Številka v imenu CyDye barvil nam pove število ogljikovih atomov v polienski verigi. S podaljševanjem polienske verige se absorpcijski in izsevani spekter pomikata proti daljšim valovnim dolžinam. Cy®3 tako absorbira zeleno svetlobo in fluorescira rumeno svetlobo, Cy®5 absorbira in fluorescira rdečo svetlobo, Cy®7 pa absorbira in fluorescira v bližini infrardečega dela elektromagnetnega spektra. V infrardečem delu spektra se izboljša zaznava signala zaradi manj avtofluorescence, vendar imajo cianini z dolgimi polienskimi verigami bistveno slabši kvantni izkoristek. Težava cianinov je njihova slaba fotostabilnost (Wang in sod. 2006, Lavis in Raines 2008). Kot nadomestna barvila za nekatere starejše organske fluorokrome se uporabljajo barvila BODIPY. BODIPY FL (Dodatna sl. 2C) ima absorbcijski in izsevani spekter podoben fluoresceinu, BODIPY TMR pa tetrametilrodaminu. To so nepolarne lipofilne molekule, zaradi česar njihovi konjugati lažje vstopajo v celice kot konjugati organskih fluorokromov z nabojem (Johnson in Spence 2010). Relativno nova skupina majhnih organskih fluorokromov so barvila Alexa Fluor®. Po kemijski zgradbi so to različne molekule, ki so jih pridobili s sulfonacijo različnih organskih fluorokromov. Sulfonske skupine dajejo barvilom Alexa Fluor® negativen naboj, zaradi česar so bolj topna v vodi. Poleg tega so zelo fotostabilna s fluorescenco neodvisno od pH. Zaradi boljših lastnosti se pogosto uporabljajo kot alternativa starejšim fluorescenčnim barvilom. Alexa Fluor® 488 se tako lahko uporablja kot alternativa fluoresceinu, namesto barvila Cy®3 in tetrametilrodamina pa se lahko uporabljata barvili Alexa Fluor® 546 in Alexa Fluor® 555 (Wang in sod. 2006, Johnson in Spence 2010).



Slika 3: Shematski prikaz imunooznačevanja s specifičnimi primarnimi protitelesi in sekundarnimi protitelesi, ki so konjugirana z organskim fluorokromom. Na izbrani antigen se najprej vežejo zanj specifična primarna protitelesa. Vezana primarna protitelesa označimo s sekundarnimi protitelesi konjugiranimi s fluorokromom. Ker se na eno primarno protitelo lahko veže več sekundarnih ob tem pride do ojačenja signala.

Figure 3: Schematic visualization of immunolabeling with the organic fluorochrome conjugated secondary antibodies. First, the specific primary antibodies bind to selected antigen. Bound primary antibodies are labeled with fluorochrome conjugated secondary antibodies. The signal is amplified, because more than one secondary antibodies can bind to the primary antibody.

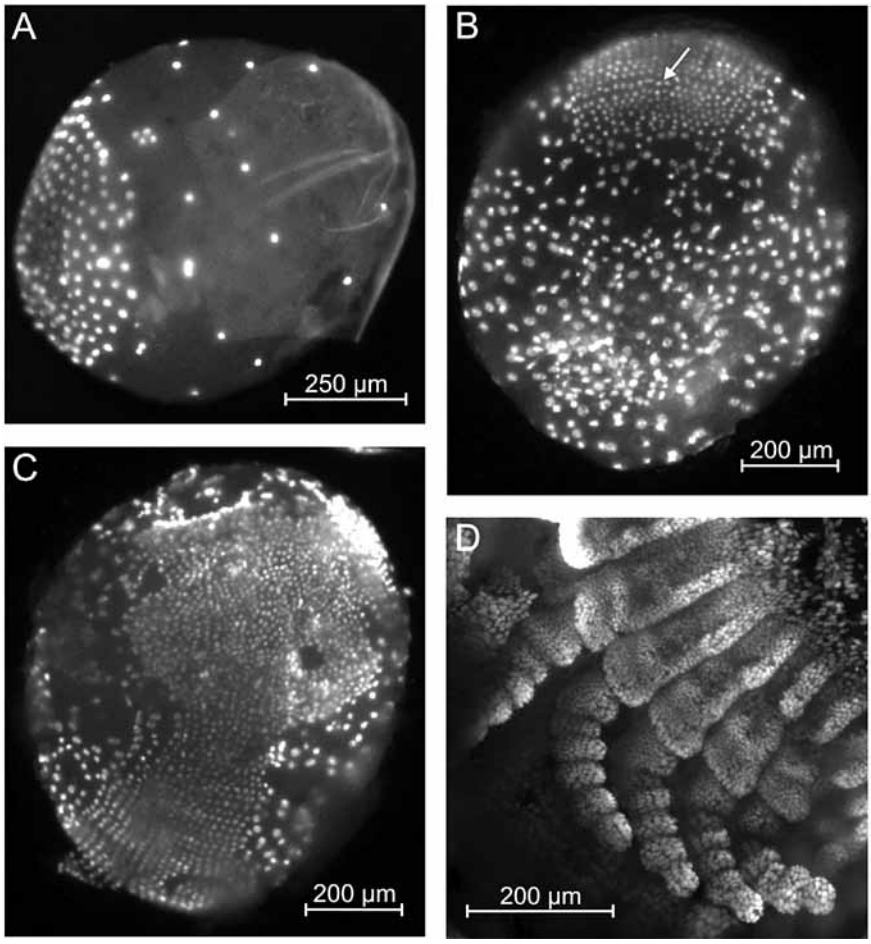
Fluorescenčna barvila za nukleinske kisline

Določeni majhni organski fluorokromi se specifično vežejo na nukleinske kisline. Vezava je lahko posledica vrivanja (interkalacije) fluorokromov med bazne pare nukleinskih kislin ali pa vezave v mali ali veliki žleb dvojne vijačnice DNA (Johnson in Spence 2010). Z vrivanjem med bazne pare se na nukleinske kisline vežejo etidijev bromid, etidijev homodimer, propidijev jodid in akridin oranžno. Etidijev bromid in propidijev jodid sta strukturno podobni molekuli, ki absorbirata zeleno in fluorescirata rdečo svetlobo. Ker ne moreta prehajati skozi celične membrane sta uporabni za označevanje mrtvih celic. Akridin oranžno lahko prehaja skozi celične membrane. Ob vezavi na DNA sta njegov absorpcijski in izsevani maksimum v zelenem delu vidnega spektra, ob vezavi na RNA pa je absorpcijski maksimum v modrem delu spektra, izsevani pa v rdečem. Poleg označevanja jeder je akridin oranžno uporabno za označevanje kompartmentov s kislo vsebino, kot so na primer lizosomi (Johnson in Spence 2010). Uporablja se tudi za lokalizacijo kislih sestavin zunajceličnih matriksov in je zelo primerno za barvanje različnih slojev kutikule členonožcev (Marlowe and Dillaman 1995). Barvilo DAPI in barvila Hoechst se vežejo v mali žleb dvojne vijačnice DNA. V fluorescenčni mikroskopiji se najpogosteje uporabljajo za označevanje celičnih jeder (Dodatna sl. 3). Barvila Hoechst lahko prehajajo skozi celične membrane in so tako uporabna za označevanje živih celic, medtem ko je DAPI primeren le za označevanje fiksiranih celic (Lavis in Raines 2008; Johnson in Spence 2010). DAPI fluorescira v modri barvi, za vzbujanje njegove fluorescence pa je potrebna UV svetloba. Označitev celičnih jeder z barvilom DAPI je pogosto uporabljena metoda za sledenje dinamike celic med embrionalnim razvojem (Sl. 4). Z označevanjem z barvilom DAPI dobimo podatke o številu in razporeditvi celičnih jeder v embriju, kar je posebej uporabno za spremljanje zgodnjih razvojnih faz embrionalnega razvoja nevretenčarskih in vretenčarskih organizmov (Milatovič in sod. 2010, Sandell in sod. 2012, Mittman in Wolff 2012, Mittmann insod. 2014). Barvila Hoechst podobno kot DAPI absorbirajo v UV delu spektra in fluorescirajo v modri barvi. Za razliko od barvila DAPI je fluorescenca barvil Hoechst utišana ob vezavi na DNA, ki vsebuje 5-bromo-2'-deoksiuridin, zaradi česar so uporabna v analizi celičnega cikla (Lavis in Raines 2008, Johnson in Spence 2010).

Nekonjugirani organski fluorokromi specifični za organele

Nekateri majhni organski fluorokromi se lahko koncentrirajo v specifičnih celičnih organelih. Z njimi je mogoče označevati npr. mitohondrije, lizosome, endoplazemski retikulum in Golgijev aparat. Vsi lahko prehajajo skozi celične membrane in vstopajo v celice. Tako za razliko od fluorescenčno označenih protiteles omogočajo označevanje celičnih organelov v živih celicah (Johnson in Spence 2010). Za označevanje mitohondrijev se uporabljata kationski barvili Rodamin 123 in tetrametilrodamin (Johnson in Spence 2010). Specifičen privzem določenih kationskih barvil v mitohondrije je odvisen od membranskega potenciala na mitohondrijskih membranah (Darzynkiewicz in sod. 1982). Težava pri uporabi teh barvil je v tem, da se ob fiksaciji sperejo iz celic. Namesto njih je mogoče uporabiti barvila MitoTracker®, ki vsebujejo reaktivno klorometilno skupino, s katero se kovalentno vežejo na tiolne skupine proteinov v mitohondrijskem matriksu. Obstajajo različna barvila MitoTracker®, ki fluorescirajo v različnih barvah od zelene do dolgovalovno rdeče (Johnson in Spence 2010). Za označevanje lizosomov se lahko uporablja kationsko barvilo akridin oranžno, vendar je njegova specifičnost slaba. Boljšo specifičnost imajo barvila LysoTracker®. To so različni organski fluorokromi vezani na šibko bazo. Njihova specifičnost temelji na protonaciji pri nizkem pH v lizosomih. Na voljo je več barvil LysoTracker, ki oddajajo fluorescenčno svetlobo različnih barv od modre do dolgovalovno rdeče (Johnson in Spence 2010). Endoplazemski retikulum je možno fluorescenčno označevati s kratko-verižnimi karbocianini, kot sta $DiOC_6(3)$ in $DiOC_5(3)$. To sta kationski lipofilni barvili, ki se vežeta na znotrajcelične membrane in sta uporabni za označevanje mitohondrijske membrane in membrane endoplazemskega retikuluma. Z vezavo barvil BODIPY FL in BODIPY TR na sulfonilureo so pridobili barvili ER-Tracker™ Green in ER-Tracker™ Red, ki imata večjo specifičnost za vezavo na endoplazemski retikulum in nista

strupeni za celice. Preko sulfoniluree se vežeta na receptorje za sulfonilureo povezane z od ATP odvisnimi K^+ kanali, ki so značilni za membrane endoplazemskega retikuluma. (Johnson in Spence 2010). Za označevanje Golgijevega aparata se uporabljata barvili NBD C_6 ceramid in BODIPY FL C_5 ceramid. To sta organska fluorokroma vezana na molekulo ceramida, ki se aktivno transportirata v Golgijev aparat (Johnson in Spence 2010).



Slika 4: Prikaz uporabe fluorescenčnega barvila DAPI za označitev celičnih jeder v embrijih (*Porcellio scaber*). Vizualizacija razporeditve celičnih jeder v embrijih v različnih razvojnih fazah nam omogoča spremljanje nekaterih morfoloških vidikov embrionalnega razvoja. **A** – Večina jeder je agregiranih na enem polu embrija, v območju zarodnega diska. To je značilnost zgodnjih embrijev v fazi S2. **B** – Zgodnji embrij v fazi S3, vidna je ureditev ektoteloblastov v vrsto (puščica). **C** – Zgodnji embrij v fazi S5, v tej fazi poteka podaljševanje zarodnega pasu. **D** – Srednji embrij, vidna je segmentacija okončin in oblikovanje tergitov.

Figure 4: Demonstration of the fluorescent dye DAPI application to label cell nuclei in embryos (*Porcellio scaber*). Visualisation of the cell nuclei arrangement in embryos of different developmental stages allows us to track some of the morphological aspects of embryonic development. **A** – Most of the nuclei are aggregated at one pole of the embryo, in the area of germ disc. This is a characteristic of the early embryos in stage S2. **B** – Early embryo in stage S3, arrangement of the ectoteloblasts into a row (arrow) is visible. **C** – Early embryo in stage S5, during this stage the elongation of the germ band takes place. **D** – Mid embryo, the segmentation of appendages and the formation of tergites are visible.

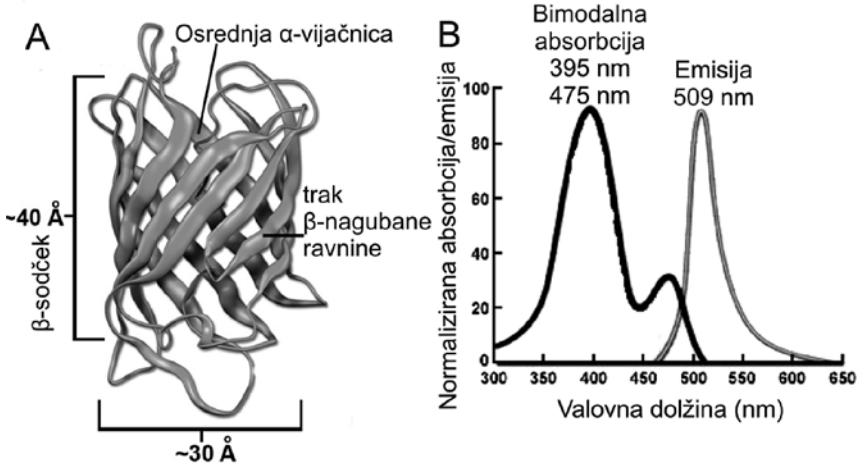
Fluorescenčni senzorji za specifične ione

Organske fluorokrome, katerih spektralne lastnosti se spremenijo ob vezavi določenega iona, je mogoče uporabljati za spremljanje lokacije in koncentracije tega iona v celici. Lahko se spremeni intenziteta fluorescence, lahko pa ob vezavi pride do spremembe absorpcijskega ali izsevanega spektra. Za detekcijo kovinskih ionov so to večinoma fluorescenčne molekule, ki poleg fluorescenčnega dela vsebujejo še kelatorski del. Na kelatorski del se veže specifični kovinski ion, ob tem pa se spremenijo spektralne lastnosti molekule (Terai in Nagano 2013). Za detekcijo Ca^{2+} so na voljo številni fluorescenčni senzorji. Večina ima za vezavo kalcija kelatorsko skupino BAPTA (Dodatna sl. 2E), ki je bila razvita iz kelatorja EGTA. EGTA ima sicer visoko specifičnost za vezavo Ca^{2+} glede na Mg^{2+}, vendar ga je pri fiziološkem pH malo v neprotonirani obliki, ki lahko veže Ca^{2+}. S kemičnimi modifikacijami so razvili kelator BAPTA, ki dobro veže Ca^{2+} tudi pri fiziološkem pH in je neobčutljiv na manjše spremembe pH (Terai in Nagano 2013). Zaradi različnih fluorescenčnih delov v Ca^{2+} senzorjih obstaja širok nabor teh molekul, ki lahko absorbirajo in fluorescirajo svetlobo različnih valovnih dolžin. Ob vezavi Ca^{2+} se pri nekaterih fluorescenčnih senzorjih, kot so na primer fura-2, indo-1 in Fura Red, spremenijo absorpcijski in izsevani spektri. Pri drugih, kot so na primer fluo-3, fluo-4, rhod-2, Calcium Orange in Calcium Crimson, pa se poveča intenziteta fluorescence (Johnson in Spence 2010). Za detekcijo K^+ se uporabljata fluorescenčna senzorja PBFI in TAC-Red, za detekcijo Na^+ senzorji SBFI, CoroNa Green in CoroNa Red (Terai in Nagano 2013), za lokalizacijo Zn^{2+} pa na primer indikatorji TSQ, Zinquin (Dodatna sl. 4), ZnAF-2, FluoZin-3, FuraZin, RhodZin-3 (Zalewski in sod. 1994, Snitsarev in sod. 2001, Kikuchi in sod. 2004, Kay 2005, Nowakovski in sod. 2015). Prvi odkriti fluorescenčni senzor za Zn^{2+} je bil TSQ. Njegov derivat Zinquin lahko prehaja skozi celične membrane in je uporaben za vizualizacijo Zn^{2+} v živih celicah (Kikuchi in sod. 2004, Kay 2005). Različni ZnAF senzorji, kot je na primer ZnAF-2, imajo za detekcijo Zn^{2+} kelatorsko skupino TPEN. Kelatorske skupine senzorjev FluoZin-3, FuraZin in RhodZin-3 so pridobili z modifikacijami kelatorja BAPTA. FluoZin-3 je zaradi velike občutljivosti uporaben za spremljanje sproščanja Zn^{2+} iz celic (Kikuchi in sod. 2004, Kay in sod. 2005).

Fluorescenčni proteini

Za razliko od sintetičnih organskih fluorokromov in kvantnih pik, kjer za označevanje uporabljamo določeno eksogeno fluorescenčno snov, pri fluorescenčnih proteinih za označevanje izkoriščamo endogeno izražen fluorescenčni protein. Gen zanj s pomočjo tehnik genskega inženiringa vnesemo v celico ali organizem. Vnos je mogoč v katerokoli celico ali organizem, saj za sintezo in zvijanje fluorescenčnih proteinov ter formiranje njihovega fluorofora niso potrebni specifični encimi ali kofaktorji. Da se vneseni gen lahko izraža, mora biti pod vplivom promotorja za izbrani specifični gen. Tako se fluorescenčni protein izraža skupaj z izbranim proteinom. Fluorescenčni protein je lahko tudi fizično vezan na tarčni protein, če je bil vnesen gen za fuzijski protein. S tem je zagotovljena specifičnost označevanja, zaradi česar fluorescenčnih proteinov ni treba konjugirati s tarčno specifičnimi makromolekulami. Označevanje s fluorescenčnimi proteini je tako manj invazivno, kar je njihova glavna prednost pri mikroskopiji živih bioloških vzorcev. Fluorescenčni proteini omogočajo vizualizacijo regulacije genske ekspresije pod vplivom promotorja tarčnega gena, označevanje tarčnih proteinov, opazovanje interakcij med proteini in spremljanje njihove dinamike (Chudakov in sod. 2005). Fluorescenčni proteini z različnimi absorpcijskimi in izsevanimi spektri ob uporabi ustreznih vzbujevalnih in zapornih filtrov omogočajo hkratno označevanje več struktur na istem vzorcu v različnih barvah. Občutljivost fluorescence nekaterih fluorescenčnih proteinov na pH in koncentracijo halidnih ionov je mogoče izkoriščati za spremljanje znotrajceličnega okolja. Za spremljanje znotrajceličnega okolja in encimskih aktivnosti so na voljo tudi senzorji, ki temeljijo na pojavu resonančnega prenosa energije s fluorescenco – FRET (fluorescence resonance energy transfer). Gre za prenos energije med donorjem in akceptorjem, ki poteka le na zelo kratke razdalje. Senzorji so običajno fuzijski proteini modrozelenega in rumenega fluorescenčnega

proteina. Običajno sta pri senzorjih za zaznavanje določene snovi v celici oba proteina dovolj blizu za pojav FRET le ob vezavi specifične snovi, pri senzorjih za proteazno aktivnost pa ob aktivnosti encima pride do ločitve obeh proteinov in prekinitve FRET med njima. Pojav FRET se izkorišča tudi za spremljanje interakcij med proteini. Za spremljanje dinamike proteinov je v uporabi tehnika FRAP (fluorescence recovery after photobleaching). Pri tej tehniki fluorescenco proteina na določenem področju najprej zbledimo z osvetljevanjem z visoko jakostjo vzbujevalne svetlobe in nato spremljamo, kako hitro se fluorescenca na tem področju spet pojavi, kar je odvisno od mobilnosti proteina (Johnson in Straight 2013). Osnova področja uporabe fluorescenčnih proteinov v fluorescenčni mikroskopiji je bilo odkritje zelenega fluorescenčnega proteina (green fluorescent protein: GFP) (Shimomura in sod. 1962), uspešen vnos in izražanje gena v celicah drugih organizmov (Chalfie in sod. 1994) in razvoj novih različic proteina z različnimi fotokemičnimi lastnostmi (Tsien 1998). Za te dosežke so navedeni avtorji leta 2008 prejeli Nobelovo nagrado za kemijo (https://www.nobelprize.org/nobel_prizes/chemistry/laureates/2008/). Shimomura je GFP izoliral iz meduze *Aequorea victoria* in tudi identificiral njegov kromofor (Shimomura 1979), povzetek poteka raziskav je opisal v članku 'The discovery of aequorin and green fluorescent protein' (Shimomura 2005). Pri meduzi A. victoria GFP absorbira modro svetlobo, ki jo oddaja luminiscenčni protein ekvorin, sam pa zato oddaja zeleno fluorescenčno svetlobo. Pri koralnjakih (Anthozoa) so kasneje odkrili fluorescenčne proteine, ki fluorescirajo pri daljših valovnih dolžinah (Chudakov in sod. 2005). Tridimenzionalna struktura GFP iz *A. victoria* je β-sodček iz 11 trakov β-nagubane ravnine, na sredini katerega je osrednja α-vijačnica (Sl. 5A). Osrednja α-vijačnica vsebuje fluorofor, ki fluorescira. Fluorofor ob prisotnosti kisika nastane s ciklizacijo aminokislinskih ostankov serina, tirozina in glicina na mestih od 65 do 67 (Tsien 1998, Shaner in sod. 2007). Spektralne lastnosti fluorescenčnih proteinov so v glavnem odvisne



Slika 5: Osnovni zeleni fluorescenčni protein GFP. **A** – Zgradba zelenega fluorescenčnega proteina. GFP je β-sodček z osrednjo α-vijačnico, ki vsebuje fluorofor (Prirejeno z dovoljenjem Company of Biologists: Journal of Cell Science (Shaner in sod. 2007)). **B** – Absorbcijski in izsevani spekter osnovnega zelenega fluorescenčnega proteina. Absorbcijski spekter ima dva vrhova pri 395 in 475 nm.

Figure 5: Original wild-type fluorescent protein GFP. **A** – Structure of the green fluorescent protein. GFP is a β-barrel with central α-helix, which bears the fluorophore (Adapted with permission from Company of Biologists: Journal of Cell Science (Shaner in sod. 2007)). **B** – Absorption and emission spectra of the original green fluorescent protein. Absorption spectrum has two peaks at 395 and 475 nm.

od teh treh aminokislinskih ostankov in od kemijskega okolja, ki obdaja osrednjo α-vijačnico in je odvisno od zgradbe β-sodčka (Shaner in sod. 2007, Stepanenko in sod. 2011). Z zamenjavami aminokislin na mestu kromofora in v β-sodčku, so tako pridobili širok nabor fluorescenčnih proteinov, ki pokrivajo širok del vidnega spektra elektromagnetnega valovanja. Poleg spreminjanja spektralnih lastnosti poskušajo z modifikacijami obstoječih fluorescenčnih proteinov povečati intenzivnost njihove fluorescence, izboljšati njihovo zvijanje in formiranje fluorofora pri 37 °C ter zmanjšati njihovo tendenco za dimerizacijo in oligomerizacijo (Zhang in sod. 2002, Lippincott-Schwartz in Patterson 2003, Shaner in sod. 2005, Shaner in sod. 2007). Predvsem pri rumenih fluorescenčnih proteinih poskušajo izboljšati fotostabilnost ter zmanjšati občutljivost na nizek pH in spremembe v koncentraciji kloridnih ionov (Zhang in sod. 2002, Shaner in sod. 2005). V zadnjem času poteka razvoj predvsem v smeri iskanja novih fluorescenčnih proteinov, pri katerih je z vzbujevalno svetlobo možno spreminjati njihove spektralne lastnosti. To so fotoaktivacijski fluorescenčni proteini, ki so uporabni predvsem pri tehnikah fluorescenčne mikroskopije super-ločljivosti, kot sta na primer PALM (photo-activation localization microscopy) (Betzig in sod. 2006) in STORM (stochastic optical reconstruction microscopy) (Rust in sod. 2006). Druga smer trenutnega razvoja so fluorescenčni proteini, ki lahko oddajajo dolgovalovno rdečo fluorescenčno svetlobo. Ti so uporabni v mikroskopiji debelih vzorcev tkiv in dvo-fotonski mikroskopiji (Stepanenko in sod. 2011).

Fluorescenčni proteini iz meduze Aequorea victoria

Fluorescenčne proteine pridobljene z modifikacijami osnovnega zelenega fluorescenčnega proteina iz meduze *A. victoria* lahko glede na njihove spektralne lastnosti razdelimo v štiri skupine: zeleni fluorescenčni proteini, modri fluorescenčni proteini, modrozeleni fluorescenčni proteini in rumeni fluorescenčni proteini (Shaner in sod. 2007). Prednosti osnovnega zelenega fluorescenčnega proteina (GFP) sta dobra fotostabilnost in intenzivna fluorescenca. Težavo pri uporabi predstavlja kompleksen absorpcijski spekter z dvema maksimumoma pri 395 in 475 nm (Sl. 5B). Dodatna težava je tendenca GFP-ja po združevanju v dimere (Tsien 1998). Izboljšani zeleni fluorescenčni protein (EGFP – enhanced green fluorescent protein) ima absorpcijski spekter z enim maksimumom in manjšo tendenco za dimerizacijo (Lippincott-Schwartz in Patterson 2003). Modri fluorescenčni proteini so bili pridobljeni z zamenjavo tirozina na mestu 66 s histidinom (Dodatna sl. 5A). Posledica je premik absorpcijskega in izsevanega spektra proti krajšim valovnim dolžinam v modro območje vidnega spektra. Težavi pri modrih fluorescenčnih proteinih sta nizka intenziteta fluorescence in njeno hitro bledenje (Lippincott-Schwarz in Patterson 2003). Absorpcijski in izsevani spektri modrozelenih fluorescenčnih proteinov se nahajajo med spektri zelenih in modrih fluorescenčnih proteinov. Pridobili so jih z zamenjavo tirozina na mestu 66 s triptofanom (Dodatna sl. 5B). V primerjavi z modrimi fluorescenčnimi proteini so svetlejši in bolj fotostabilni (Lippincott-Schwarz in Patterson 2003). Njihovi vzbujevalni in izsevani spektri so široki s po dvema maksimumoma (Tsien 1998, Shaner in sod. 2007). Modrozeleni fluorescenčni protein mTFP1 ima v fluoroforu na mestu 66 tirozin namesto triptofana. Njegova vzbujevalna in izsevana spektra sta v primerjavi z ostalimi modrozelenimi fluorescenčnimi proteini ožja in s po enim maksimumom. Modrozeleni fluorescenčni proteini se pogosto uporabljajo kot FRET donorji skupaj z rumenimi fluorescenčnimi proteini (Shaner in sod. 2007). Pri rumenih fluorescenčnih proteinih je premik absorpcijskega in izsevanega spektra proti daljšim valovnim dolžinam posledica zamenjave treonina na mestu 203 v β-sodčku blizu fluorofora s tirozinom (Dodatna sl. 5D). Zaradi interakcij med π-sistemoma fluorofora in tirozina je energija vzbujenega stanja nižja, posledično pa sta vzbujevalni in izsevani spekter premaknjena proti daljšim valovnim dolžinam (Tsien 1998). Fluorescenca rumenih fluorescenčnih proteinov je v primerjavi z drugimi fluorescenčnimi proteini ena od najsvetlejših, vendar precej odvisna od pH. Od rumenih fluorescenčnih proteinov je še vedno pogosto v uporabi EYFP (enhanced yellow fluorescent protein). Fluorescenca EYFP je močno odvisna od pH in je že pri vrednostih pod 7 močno utišana. To je lahko tudi prednost, saj se posledično lahko EYFP uporablja kot senzor za pH (Tsien 1998, Shaner in sod. 2007).

Fluorescenčni proteini ožigalkarjev iz skupine koralnjakov (Cnidaria: Anthozoa)

Poskusi, da bi iz fluorescenčnih proteinov iz vrste *A. victoria* pridobili fluorescenčne proteine z izsevanimi spektri v oranžnem in rdečem območju vidnega spektra, so bili večinoma neuspešni. Iz koral in morskih vetrnic iz skupine Anthozoa so izolirali nove fluorescenčne proteine, ki oddajajo oranžno in rdečo fluorescenco. Dva primera teh proteinov sta DsRed iz korale rodu *Discosoma* in HcRed iz morske vetrnice *Heteractis crispa* (Lippincott-Schwarz in Patterson 2003). Za večino oranžnih in rdečih fluorescenčnih proteinov je značilno, da med formiranjem njihovega fluorofora preidejo skozi vmesno stanje, v katerem fluorescirajo zeleno. Pri nekaterih rdečih fluorescenčnih proteinih pretvorba zelenega fluorofora v rdečega poteče avtomatično, pri drugih pa je za to potrebno osvetljevanje z UV svetlobo. Rdeča fluorescenca in potek formiranja fluorofora pri rdečih fluorescenčnih proteinih sta odvisna predvsem od aminokislinskega ostanka na mestu 65 (Verkhusha in Lukyanov 2004, Stepanenko in sod. 2011). Ker rdeča svetloba prodira globlje skozi tkiva, so ti fluorescenčni proteini uporabni v mikroskopiji debelih vzorcev. Uporaba rdečih fluorescenčnih proteinov je smiselna tudi zaradi manj avtofluorescence v rdečem območju vidnega spektra (Shaner in sod. 2007). Fluorescenčni protein DsRed fluorescira v oranžni barvi, njegova uporabnost pa je omejena s počasnim formiranjem njegovega fluorofora, dolgotrajnim vmesnim zelenim stanjem in obvezno tetramerno obliko. Iz DsRed so pridobili prvi monomerni rdeči fluorescenčni protein mRFP, ki fluorescira v rdeči barvi. Iz proteina DsRed je bil pridobljen tudi fluorescenčni protein dTomato, ki je dimerni fluorescenčni protein z oranžno fluorescenco in velja za enega najsvetlejših rdečih fluorescenčnih proteinov (Shaner in sod. 2007). Iz fluorescenčnega protein mRFP so pridobili rdeča fluorescenčna proteina mCherry in mStrawberry. Fluorescenčni proteini mPlum, Katushka in mKate fluorescirajo dolgovalovno rdečo svetlobo (Shaner in sod. 2007).

Kvantne pike

Kvantne pike so polprevodniški nanokristali, velikosti od 1 do 10 nm. V mikroskopiji in drugih bioloških aplikacijah so uporabne zaradi svoje fluorescence. Posebnost kvantnih pik je, da je pri enaki sestavi valovna dolžina njihove fluorescence odvisna od njihove velikosti (Stanisavljevic 2015). V bioloških aplikacijah so najpogosteje v uporabi kvantne pike z jedrom iz CdSe, ki je obdano s tankim slojem ZnS (Sl. 6). Sloj ZnS zaščiti jedro pred oksidacijo, prepreči prehajanje kadmija in selena v okoliški medij in izboljša optične lastnosti kvantne pike. Kvantne pike z jedrom iz CdSe ali CdTe oddajajo fluorescenčno svetlobo vidnega

Slika 6: Zgradba kvantne pike. Kvantne pike za uporabo v fluorescenčni mikroskopiji so najpogosteje sestavljene iz jedra iz CdSe, ki fluorescira, sloja ZnS, ki prepreči prehajanje kadmija in selena v okoliški medij in izboljša optične lastnosti kvantne pike, ter organskega plašča, ki je potreben za topnost kvantnih pik v vodi in vezavo tarčno specifičnih makromolekul (Prirejeno z dovoljenjem Macmillan Publishers Ltd: Nature Methods (Giepmans in sod. 2005)).

Figure 6: Quantum dot structure. Quantum dots used in fluorescence microscopy are most often composed of a CdSe core, which fluoresces, a layer of ZnS, which prevents leakage of cadmium and selenium into the surrounding medium and improves optical properties of a quantum dot, and an organic coat, which makes quantum dots water soluble and allows addition of the target specific macromolecules (Adapted with permission from Macmillan Publishers Ltd: Nature Methods (Giepmans et al. 2005)).

dela elektromagnetnega spektra. Obstajajo pa tudi kvantne pike sestavljene iz drugih elementov (ZnS, CdS, ZnSe, PbSe), ki fluorescirajo v UV in infrardečem delu elektromagnetnega spektra (Medintz in sod. 2005). Za uporabo v bioloških vzorcih je potrebno na plašč iz ZnS dodati še organske plašče, da so kvantne pike topne v vodnih raztopinah in da omogočimo vezavo tarčno specifičnih makromolekul na njihovo površino (Medintz in sod. 2005, Resch-Genger in sod. 2008, Johnson in Spence 2010). Zaradi dodatnih organskih plaščev in tarčno specifičnih makromolekul na površini je končna velikost kvantne pike pripravljene za označevanje okrog 20 nm, kar je bistveno več kot pri majhnih organskih fluorescenčnih molekulah in približno v rangu fluorescenčnih proteinov (Johnson in Spence 2010).

Do pojava fluorescence pri kvantni piki pride po absorpciji fotona, kar povzroči prehod elektrona iz valenčnega v prevodni pas. Pri tem nastane eksciton oziroma par elektronske vrzeli in elektrona. Kvantna pika lahko absorbira fotone različnih valovnih dolžin, katerih energija je višja od energijske vrzeli med valenčnim in prevodnim pasom. Posledica tega je širok absorpcijski spekter kvantnih pik. Nastali ekscitoni imajo različno visoko energijo in se sprostijo na nivo energijske vrzeli med valenčnim in prevodnim pasom. Ob ponovni združitvi elektrona in elektronske vrzeli pride do emisije fotona, ki ima energijo enako energijski vrzeli med valenčnim in prevodnim pasom. Vsi oddani fotoni imajo zato približno enako energijo. Posledica so ozki izsevani spektri kvantnih pik. Zaradi majhnosti kvantnih pik je par elektronske vrzeli in elektrona omejen na razdaljo manjšo od Bohrovega radija. Z manjšanjem premera kvantne pike je potrebna vse višja energija za omejitev ekscitona in posledično je višja tudi energija s fluorescenco oddanih fotonov. Z manjšanjem premera kvantne pike se tako krajša valovna dolžina fluorescenčne svetlobe, ki jo ta lahko oddaja (Johnson in Spence 2010).

Kvantne pike se po nekaterih spektralnih lastnostih bistveno razlikujejo od organskih fluorescenčnih molekul. Zaradi teh specifičnih lastnosti so za določene biološke aplikacije bolj uporabne kot organski fluorokromi. Za kvantne pike je značilen širok absorpcijski spekter, pri katerem je absorpcija večja pri krajših valovnih dolžinah. Njihovi izsevani spektri so ozki in odvisni od velikosti kvantnih pik. Manjši kot je premer kvantne pike, krajša je valovna dolžina njene fluorescence (Dodatna sl. 6). Stokesovi zamiki med absorpcijskimi in izsevanimi spektri so veliki in so običajno med 300 in 400 nm. Zaradi teh lastnosti so kvantne pike zelo primerne za večbarvno označevanje vzorcev. Pri tem se za označevanje različnih struktur ali molekul uporabijo kvantne pike različnih velikosti. Fluorescenco vseh kvantnih pik v vzorcu vzbujamo s svetlobo enotne valovne dolžine, kvantne pike različnih velikosti pa pri tem fluorescirajo v različnih barvah. Zaradi velikih Stokesovih zamikov ne pride do prekrivanja med vzbujevalno in fluorescenčno svetlobo. Ravno tako ni prekrivanja med fluorescenco različnih kvantnih pik zaradi njihovih ozkih izsevanih spektrov (Resch-Genger in sod. 2008, Johnson in Spence 2010, Stanisavljevic in sod. 2015). V primerjavi z organskimi fluorokromi imajo kvantne pike od 10 do 100-krat višje ekstinkcijske koeficiente, njihovi kvantni izkoristki pa so primerljivi s tistimi pri organskih fluorescenčnih molekulah. Njihova fluorescenca je tako načeloma intenzivnejša kot pri organskih fluorokromih. Kvantni izkoristki so visoki tudi pri kvantnih pikah, ki oddajajo fluorescenčno svetlobo v bližini infrardečega dela spektra, medtem ko so kvantni izkoristki organskih fluorokromov, ki fluorescirajo v tem delu spektra, načeloma nizki (Resch-Genger in sod. 2008, Johnson in Spence 2010, Stanisavljevic in sod. 2015). Zaradi intenzivne fluorescence kvantnih pik v območju dolgovalovne rdeče svetlobe in njihove učinkovite dvo-fotonske absorbcije so kvantne pike zelo primerne tudi za uporabo v mikroskopiji debelih vzorcev tkiv (Medintz in sod. 2005, Resch-Genger in sod. 2008). Poleg svetle fluorescence je za kvantne pike značilna tudi velika odpornost proti bledenju fluorescence (Johnson in Spence 2010, Stanisavljevic in sod. 2015). To omogoča njihovo uporabo v tehnikah, ki zahtevajo dolgotrajno osvetljevanje vzorca (Medintz in sod. 2005), kot sta na primer sledenje endocitotskih procesov in spremljanje dinamike proteinov na celični površini (Jaiswal in sod. 2003). Kvantne pike lahko služijo tudi kot učinkoviti FRET donorji. S spreminjanjem velikosti kvantnih pik je namreč možno prilagoditi njihov izsevani spekter absorpcijskemu spektru FRET akceptorja (Medintz in sod. 2005, Johnson in Spence 2010). Kvantne pike so elektronsko goste, zato jih lahko dobro

razločimo tudi z elektronskim mikroskopom. To pa skupaj z njihovimi fluorescenčnimi lastnostmi omogoča uporabo v korelativni mikroskopiji, ko isti objekt ali dogodek v celici opazujemo tako s fluorescenčno kot z elektronsko mikroskopijo (Johson in Spence 2010). Vnos kvantnih pik v žive celice je otežen zaradi njihove velikosti. Kvantne pike lahko vnesemo z mikroinjeciranjem, elektroporacijo ali z endocitozo, ki je nespecifična ali pa receptorsko posredovana (Menditz in sod. 2005, Resch-Genger in sod. 2008). *'In vivo'* se tako kvantne pike na nivoju celic v glavnem uporabljajo za vizualizacijo in sledenje dinamike membranskih receptorjev in spremljanje endocitotskih procesov. Na nivoju celotnih organizmov se uporabljajo kot kontrastno sredstvo za vizualizacijo krvožilnega in limfnega sistema, za sledenje s kvantnimi pikami napolnjenih celic v organizmih in za vizualizacijo tumorjev (Jaiswal in sod. 2003, Smith in sod. 2008).

Fluorescenčne snovi v tehnikah fluorescenčne mikroskopije super-ločljivosti

V mikroskopiji je prostorska ločljivost zaradi uklona omejena na približno polovico valovne dolžine elektromagnetnega valovanja s katerim osvetljujemo preparat (Abbe 1873). Za vidno svetlobo je uklonska omejitev približno 200 nm. Pri opazovanju točkastega objekta s premerom pod uklonsko omejitvijo se ta vedno preslika v disk s končnim premerom enakim vrednosti uklonske omejitve. Pri tehnikah fluorescenčne mikroskopije super-ločljivosti se uklonski omejitvi lahko izognemo s pomočjo prostorske ali časovne modulacije prehajanja fluorokromov med njihovim svetlim in temnim stanjem. V grobem lahko te tehnike razdelimo v dve skupini: deterministične in stohastične tehnike (Hell 2007, Fernandez-Suarez in Ting 2008, Heilemann in sod. 2009, Huang in sod. 2009, Schermelleh in sod. 2010, Agrawal in sod. 2013, Uno in sod. 2015). Za razvoj super-ločljivostne fluorescenčne mikroskopije, za razvoj metod STED (Stimulated Emission Depletion, Mikroskopija z vzbujenim praznjenjem emisije) in PALM, je bila leta 2014 podeljena Nobelova nagrada za kemijo (https://www.nobelprize.org/nobel_prizes/chemistry/laureates/2014/). Pri razvoju mikroskopije STED in pri njeni vpeljavi v raziskave na področju celične fiziologije so sodelovali tudi raziskovalci iz Slovenije, prototip mikroskopa STED je nameščen na Medicinski fakulteti Univerze v Ljubljani (Jorgačevski in sod. 2011, Kreft in Jorgačevski 2014, Guček in sod. 2016).

Pri deterministični tehnikah se uporablja prostorsko specifičen vzorec osvetljevanja, ki vzbuja fluorescenco celotnega nabora fluorescenčnih molekul na področju žarišča, a hkrati dopušča fluorescenco le molekulam v centru žarišča osvetljevanja, ki ima premer manjši od uklonske omejitve. Tak primer je tehnika STED, pri kateri vzorec osvetljujemo z dvema laserskima žarkoma visoke intenzitete. Vzbujanju fluorescence z vzbujevalnim žarkom nemudoma sledi osvetljevanje z žarkom STED, ki je v preseku obročaste oblike s točko ničelne intenzitete v sredini in visoko intenziteto na robu. Žarek STED molekule na robu osvetljevanja vrne iz vzbujenega nazaj v osnovno stanje preko stimulirane emisije. Zaradi tega fluorescirajo le molekule v zelo ozkem območju, kjer je intenziteta žarka STED zelo nizka, oz. skoraj enaka nič. Z vrstičnim pomikanjem po celotnem vzorcu je možno dobiti sliko z ločljivostjo nekaj deset nanometrov (Hell in Wichmann 1994, Klar in sod. 2000, Fernandez-Suarez in Ting 2008, Heilemann in sod. 2009, Huang in sod. 2009, Schermelleh in sod. 2010, Agrawal in sod. 2013, Uno in sod. 2015). Pri izbiri ustreznega fluorescenčnega označevalca v mikroskopiji STED je pomembno, da valovna dolžina laserja STED ne sovpada z vzbujevalnim spektrom označevalca. V takšnem primeru bi namreč namesto stimulirane emisije prišlo do dodatnega vzbujanja fluorescence. Zaradi visokih jakosti predvsem žarka STED mora biti fluorescenčni označevalec tudi fotostabilen (Schermelleh in sod. 2010). Pri mikroskopiji STED se lahko uporabljajo običajni organski fluorokromi in fluorescenčni proteini, kot so na primer barvila ATTO in DY ter zeleni in rumeni fluorescenčni proteini. Kasneje so na podlagi tehnike STED razvili različne metode RESOLFT (Reversible Saturable Optical Linear Fluorescence Transitions) pri katerih se uporabljajo takšni fluorokromi, ki lahko preklapljajo med fluorescenčnim in temnim stanjem. Pri tej tehniki fluorokromov izven žariščne točke ne utišamo s stimulirano emisijo, ampak jih preklopimo v njihovo temno stanje, za kar je potrebna bistveno nižja energija laserskega žarka.

Fluorokromi za RESOLFT morajo tako hitro in reverzibilno preklapljati med obema stanjema. Izmed fluorescenčnih proteinov so bili v teh tehnikah uspešno uporabljeni proteini FP595, Dronpa-3, rsEGFP, rsEGFP2, mGeos-X, Padron, Kohinoor in Dreiklang (Fernandez-Suarez in Ting 2008, Heilemann in sod. 2009, Uno in sod. 2015).

Druga skupina tehnik fluorescenčne mikroskopije super-ločljivosti so stohastične tehnike. Pri teh tehnikah se ob istem času vzbuja fluorescenca zelo majhnega deleža molekul fluorokroma v vzorcu. Ker fluorescira zelo malo molekul, je možnost, da bi fluorescirali dve molekuli medsebojno oddaljeni manj od uklonske omejitve, zelo majhna. Za posamezne molekule se iz statistične razporeditve fotonov zajetih iz njih lahko natančno izračuna njihove pozicije. Na ta način se več tisočkrat ponovi vzbujanje zelo majhnega števila naključno razporejenih molekul fluorokroma in določevanje njihovih pozicij. Iz pridobljenih pozicij molekul je mogoče rekonstruirati celotno sliko z ločljivostjo nekaj nanometrov. Primeri stohastičnih tehnik fluorescenčne mikroskopije super-ločljivosti so PALM (Photo-Activated Localization Microscopy), FPALM (Fluorescence Photo-Activation Localization Microscopy) in STORM (Stochastic Optical Reconstruction Microscopy) (Fernandez-Suarez in Ting 2008, Heilemann in sod. 2009, Huang in sod. 2009, Schermelleh in sod. 2010, Agrawal in sod. 2013, Uno in sod. 2015). Pri stohastičnih tehnikah je pomembno, da je možno na nek način nadzorovati, koliko molekul fluorokroma v vzorcu fluorescira hkrati. Za to se uporabljajo različni fluorokromi, ki jih je mogoče ireverzibilno fotoaktivirati ali reverzibilno preklapljati med njihovim fluorescenčnim in temnim stanjem. Ob tem mora biti stopnja bledenja fluorescence ali deaktivacije v ravnovesju s stopnjo aktivacije. Pod tem pogojem v vzorcu hkrati fluorescira le majhno število molekul fluorokroma (Fernandez-Suarez in Ting 2008). Za natančno lokalizacijo posameznih fluorescenčnih molekul mora biti iz njih možno zajeti dovolj fotonov, preden fluorescenca zbledi ali se deaktivira (Uno in sod. 2015). Prva skupina fluorescenčnih označevalcev za uporabo v stohastičnih tehnikah fluorescenčne mikroskopije super-ločljivost so fluorescenčni proteini, ki jih je s svetlobo določene valovne dolžine možno ireverzibilno fotoaktivirati. Takšni so na primer fluorescenčni proteini PA-GFP, PA-RFP1, PAmCherry1in PATagRFP (Fernandez-Suarez in Ting 2008, Uno in sod. 2015). Druga skupina so fluorescenčni proteini, ki ob osvetljevanju s svetlobo določene valovne dolžine ireverzibilno spremenijo barvo svoje fluorescence. Takšni so na primer fluorescenčni proteini PS-FP kaede, KikGR, mEosFP, Dendra2, PS-CFP2 in PSmOrange (Fernandez-Suarez in Ting 2008, Uno in sod. 2015). Tretja skupina so fluorescenčni proteini, ki lahko reverzibilno preklapljajo med fluorescenčnim in temnim stanjem. Pri teh proteinih se fluorescenca aktivira z osvetljevanjem s svetlobo ene valovne dolžine in deaktivira z osvetljevanjem s svetlobo druge valovne dolžine. Fluorescenco ene molekule fluorescenčnega proteina je tako možno zajeti večkrat. Od teh fluorescenčnih proteinov so v stohastičnih tehnikah uporabni proteini FP595, Dronpa, rsEGFP, mGeosX in Dreiklang (Fernandez-Suarez in Ting 2008, Uno in sod. 2015). Za označevanje se pri stohastičnih tehnikah uporabljajo tudi različni majhni organski fluorokromi. Določene izmed teh je možno ireverzibilno aktivirati. Pri teh je na fluorokrom vezana določena kemijska skupina s katero je utišana fluorescenca fluorokroma. Ob osvetljevanju z UV svetlobo se ta skupina odcepi od fluorokroma, ki prične fluorescirati. Nekateri majhni organski fluorokromi lahko reverzibilno preklapljajo med fluorescenčnim in temnim stanjem. Ob osvetljevanju Rodamina B z UV svetlobo ta prične prehodno svetlo fluorescirati. Po določenem času zaradi termičnih procesov preide nazaj v ne-fluorescenčno stanje (Fernandez-Suarez in Ting 2008, Uno in sod. 2015).

Zaključek

Ob širokem naboru fluorescenčnih označevalcev je pomembno, da znamo izbrati pravega za naš namen uporabe. V prvi vrsti je pri izbiri treba upoštevati vzbujevalne in izsevane spektre fluorescenčnega označevalca, ki ga želimo uporabiti. Ti morajo namreč ustrezati vzbujevalnim in zapornim filtrom oziroma vzbujevalnemu laserju fluorescenčnega mikroskopa, ki nam je na voljo. Spektralne lastnosti fluorokroma je treba upoštevati tudi glede na tkivo, ki ga želimo opazovati. Za označevanje na debelih vzorcih

tkiv z intenzivno avtofluorescenco so primernejše fluorescenčne snovi, ki absorbirajo in fluorescirajo svetlobo v rdečem delu vidnega spektra. Če na enem vzorcu hkrati označujemo več struktur v različnih barvah, se morajo spektri uporabljenih fluorokromov čim manj prekrivati. Zelo pomembna lastnost fluorokromov je tudi njihova fotostabilnost oziroma odpornost proti bledenju fluorescence. Fotostabilnost je pomembna predvsem pri aplikacijah, pri katerih zajem slike traja dlje časa, na primer pri zajemu optičnih rezin za 3D rekonstrukcije, oziroma pri katerih se uporabljajo visoke jakosti osvetljevanja, na primer pri tehniki STED. Pri izbiri ustreznega fluorokroma je zelo pomembno tudi ali opazujemo žive ali fiksirane preparate. Za označevanje živih preparatov so tako najprimernejši fluorescenčni proteini, medtem ko za označevanje fiksiranih preparatov njihova uporaba ni smiselna zaradi relativno težavnega vnosa njihovih genov v organizme. V zadnjem času v povezavi s tehnikami fluorescenčne mikroskopije super-ločljivosti na pomenu pridobivajo fluorokromi, ki jih je mogoče fotoaktivirati ali preklapljati med fluorescenčnim in temnim stanjem. Poleg različnih organskih fluorokromov se vse več uporabljajo tudi različni fluorescenčni anorganski nanodelci, kot so na primer kvantne pike. Te imajo posebej velik potencial v različnih tehnikah korelativne mikroskopije.

Summary

Fluorescence microscopy comprises a versatile array of techniques and is an essential method in cell biology research and applications. During the last decades great improvement in spatial and temporal resolution has been achieved with the development of fluorescence microscopes' optical components and equipment for computer image analysis. Simultaneously, various fluorescent markers has been developed, which enable specific labelling of structures and chemical compounds in cells and tissues. Fluorescent substances absorb light of specific wavelength, which elicits their transition into the excited state. During relaxation back to the ground state they emit light of a longer wavelength in a process called fluorescence. Fluorescence is characterized by the photophysical properties of a fluorescent substance, including excitation and emission spectra, Stokes shift, extinction coefficient and quantum yield. In this article we review the photophysical properties and different applications of the three main groups of fluorescent markers used in fluorescence microscopy: small organic fluorochromes, fluorescent proteins and quantum dots. The final part of the article is dedicated to special fluorescent markers used in various super-resolution fluorescence microscopy techniques.

Small organic fluorochromes are most often aromatic or heterocyclic compounds. Some of them are endogenous to the biological samples and produce autofluorescence. Predominately small organic fluorochromes are used as fluorescent labels in different modes of application. Organic fluorochromes conjugated with target specific ligands such as antibodies or single strand anti-sense DNA are used in immunofluorescent labelling and fluorescence *in situ* hybridization, respectively. Some small organic fluorochromes specifically bind to nucleic acids and are often used to label cell nuclei. Various small organic fluorochromes concentrate in some cell organelles on the basis of physiological processes or chemical properties specific for such organelles. Small organic fluorochromes are used also as sensors for specific ions. Molecules of such fluorochomes usually contain a chelator moiety, which binds specific ions. This binding causes a change in the spectral properties of the fluorescent part of the molecule.

Labelling with fluorescent proteins employs endogenously expressed fluorescent protein, thus the labelling is less invasive and appropriate especially for imaging of the living systems. A gene for fluorescent protein can be introduced into a cell or organism, where it is expressed. No specific enzymes or cofactors are necessary for folding and fluorophore formation. Fluorescent proteins are used for visualization of selected protein expression, dynamics and localization within the cell and analysis of interactions between proteins. Most fluorescent proteins were acquired by mutagenesis of the wild-type green fluorescent protein isolated from a jellyfish *Aequorea victoria*. Blue, cyan and yellow fluorescent proteins were acquired by substitutions of the amino acid residues of fluorophore and in the β-barrel region

near fluorophore. Proteins that fluoresce in the orange and red part of the visible spectrum were isolated from various species of Anthozoa. Various modifications of fluorescent proteins are aimed to increase their brightness, improve their folding and fluorophore formation and reduce their tendency for dimerization and oligomerization.

Quantum dots are fluorescent semiconductor nanocrystals. A unique characteristic of quantum dots is the size-dependence of their emission wavelength. Quantum dots of smaller diameter fluoresce at shorter wavelengths and vice versa. In biological applications quantum dots with CdSe core and ZnS shell are the most common. The surface of quantum dots is covered by additional organic coats, which make them water soluble and allow conjugation of target specific macromolecules. Quantum dots have broad excitation spectra and narrow, size-dependent emission spectra. Thus quantum dots of different sizes are useful for the labelling of multiple structures in different colours in the same specimen. In comparison with the small organic fluorochromes quantum dots exhibit brighter fluorescence and superior photostability. They are also useful in correlative microscopy because of their electron density.

Super-resolution fluorescent microscopy techniques overcome the diffraction limit by spatial or temporal modulation of the fluorophores' transitions between their dark and bright states. In deterministic super-resolution techniques a spatially specific pattern of illumination is used to excite fluorescence of an ensemble of fluorescent molecules in the sample, but which at the same time allows only the molecules in the centre of illumination to fluoresce. Spot where the molecules are allowed to fluoresce has a diameter smaller than the diffraction limit and by scanning this spot across the sample a complete super-resolution image is acquired. In STED (Stimulated Emission Depletion) microscopy molecules located out of the illumination centre are transferred back to the ground state by stimulated emission. This is accomplished by illumination with red-shifted STED beam of zero intensity at the focal centre and high intensity at the periphery. Fluorescent markers in STED microscopy are the usual small organic fluorochromes and fluorescent proteins. Still it is very important that their excitation spectra do not overlap with the wavelength of the STED beam, and that they are photostable because of the high intensity of STED beam. In RESOLFT (Reversible Saturable Optical Linear Fluorescence Transitions) methods photoswitchable fluororescent markers are used. In contrast to the STED microscopy, fluorescent molecules are not quenched by stimulated emission, but switched to their dark state instead, which requires much lower laser intensities. In stochastic super-resolution techniques the exact locations of individual molecules are determined and merged into a complete super-resolution image. This requires that the images of individual molecules do not overlap. Temporal modulation of the fluorescent molecules is thus required that allows only a very low number of fluorescent molecules in the sample to fluoresce simultaneously at any given time. By the illumination with light of specific wavelength only a small fraction of all photoactivatable/photoswitchable fluorescent molecules in the sample are activated/switched on, their locations determined and then deactivated/switched off. The whole cycle is repeated a few thousand times, each time activating/switching on a random fraction of fluorescent molecules in the sample. The whole super-resolution image can be reconstructed from the acquired positions of all individual fluorescent molecules in the sample.

Avtorji fotografij

Slike 2A, 2B, 2C in Dodatna slika 1: Urban Bogataj in Nada Žnidaršič; Slike 4A, 4B, 4C in 4D: Maša Milatovič; Dodatna slika 3A: Polona Mrak; Dodatni sliki 3B in 4: Nada Žnidaršič.

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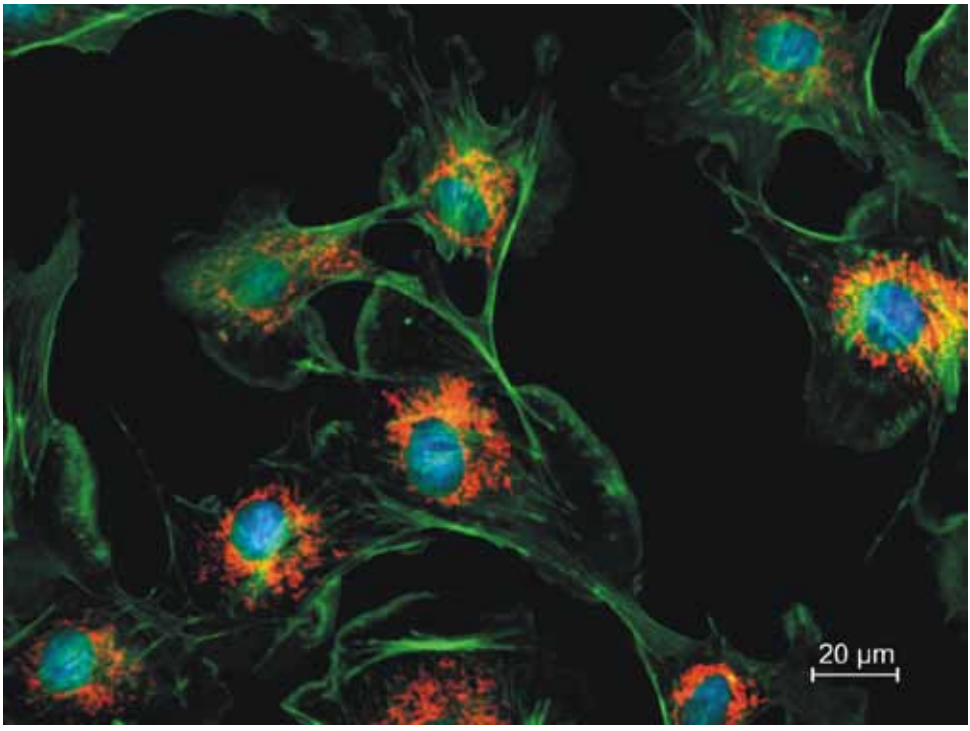
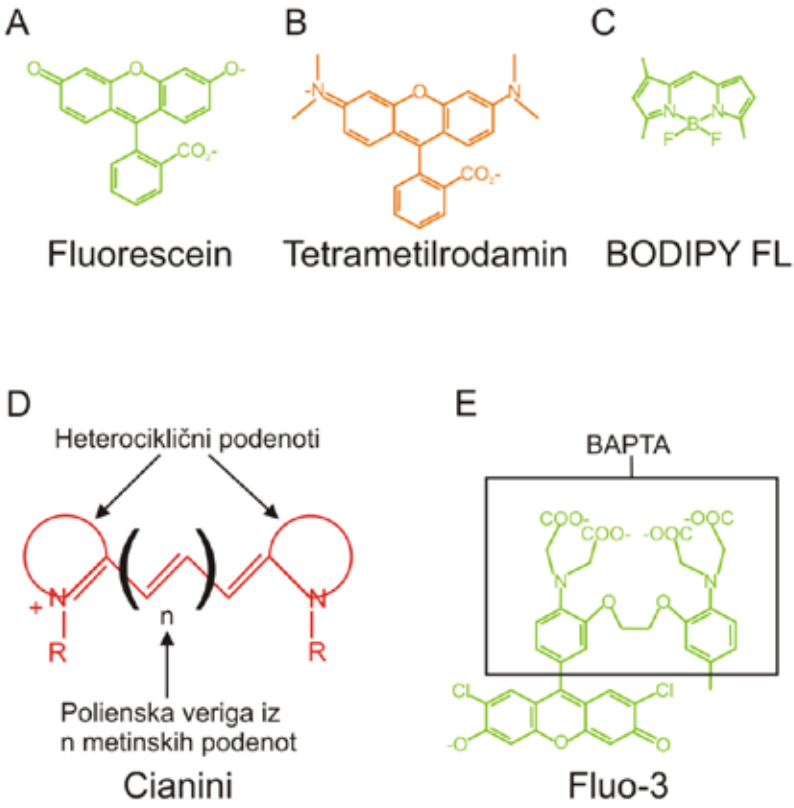
Dodatne slike

Supplementary figures



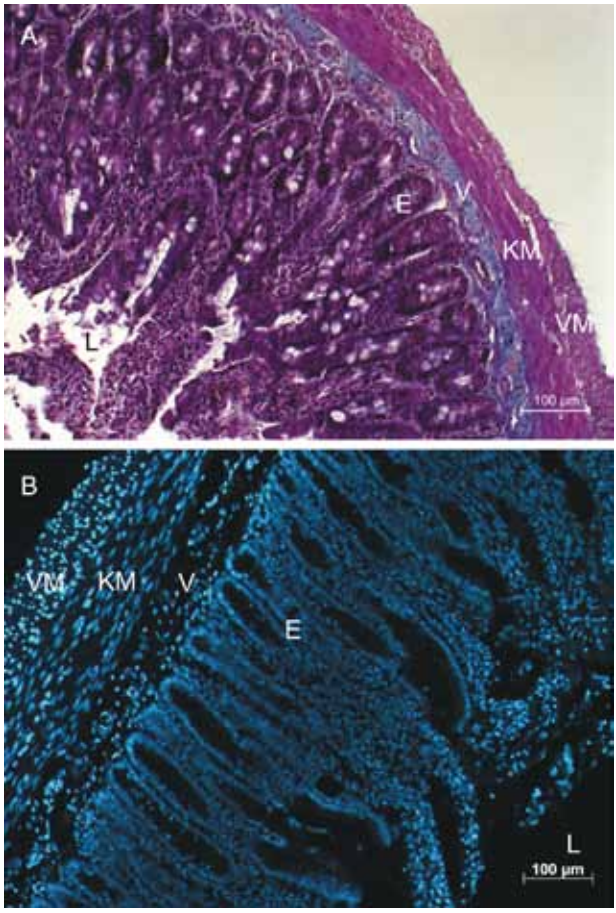
Dodatna slika 1: Sestavljena slika fluorescenčno označenih celičnih jeder, aktinskega citoskeleta in mito hondrijev v endotelnih celicah. Sliko istega področja na preparatu smo zajeli trikrat, vsakič z ustrezno kombinacijo vzbujevalnih in zapornih filtrov za posamezen fluorokrom. Dobljene slike celičnih jeder, aktinskega citoskeleta in mitohondrijev smo v programu za obdelavo in analizo mikroskopskih slik (Fiji) sestavili v enotno sliko.

Supplementary figure 1: Merged image of the fluorescently labelled cell nuclei, actin filaments and mitochondria in endothelial cells. The image of the same area in the sample was acquired three times, each time with the appropriate combination of excitation and emission filters for individual fluorochrome. Acquired images were merged with the software for processing and analysis of microscopic images (Fiji).



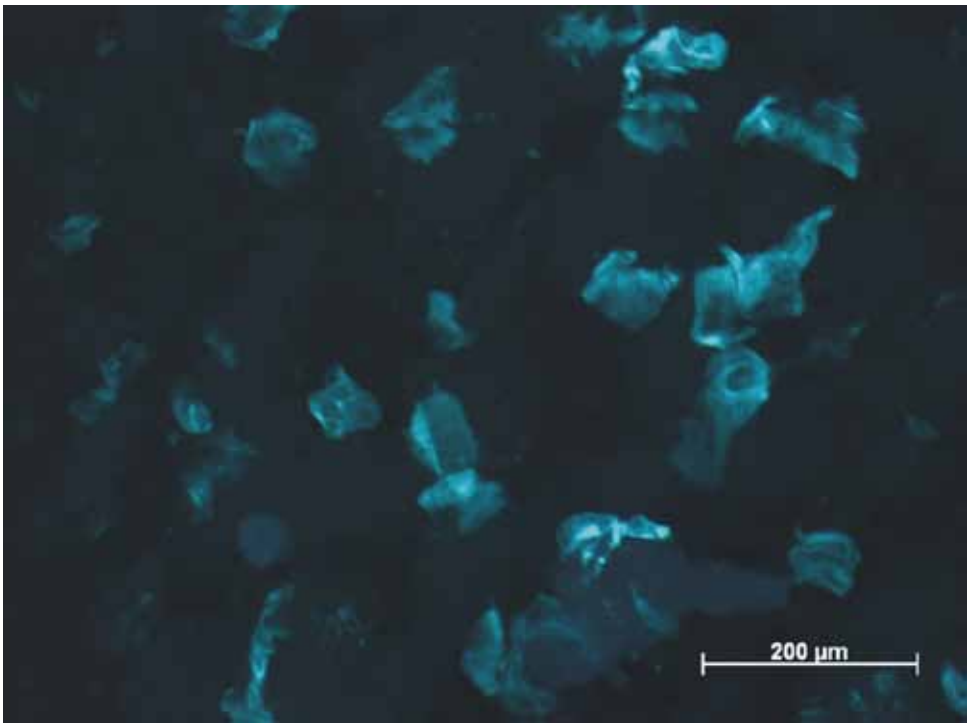
Dodatna slika 2: Strukturne formule majhnih organskih fluorokromov. Majhni organski fluorokromi so večinoma heterociklične in aromatske molekule, ki vsebujejo konjugirane dvojne vezi. **A** – Strukturna formula fluoresceina. **B** – Strukturna formula tetrametilrodamina. **C** – Strukturna formula fluorescenčnega barvila BODIPY FL. **D** – Poenostavljena strukturna formula cianinov. **E** – Strukturna formula fluorescenčnega Ca^{2+} senzorja fluo-3 z označeno kelatorsko skupino BAPTA.

Supplementary figure 2: Structure formulas of small organic fluorochromes. Small organic fluorochromes are mostly heterocyclic and aromatic molecules, which contain conjugated double bonds. **A** – Structural formula of fluorescein. **B** – Structural formula of tetramethylrhodamine. **C** – Structural formula of the fluorescent dye BODIPY FL. **D** – Simplified structural formula of cyanines. **E** – Structural formula of the fluorescent Ca^{2+} sensor fluo-3 with its chelator moiety BAPTA designated.



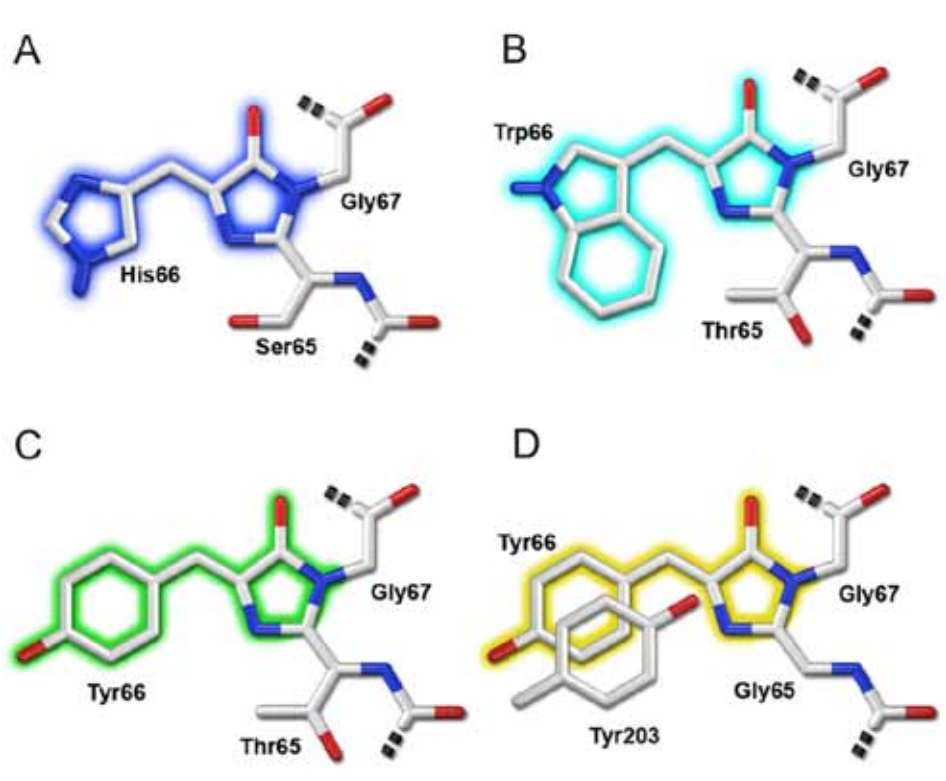
Dodatna slika 3: Primerjava diferencialnega histološkega barvanja in uporabe barvila DAPI na rezinah istega vzorca (prečni prerez črevesa sesalca). Črevesno steno sestavljajo različna tkiva; epitelno tkivo (E) sluznice pokriva črevesne resice, podsluznica je predvsem iz vezivnega tkiva (V), mišično plast tvorita dva sloja: notranje krožno mišičje (KM) in zunanje vzdolžno mišičje (VM). L: lumen (svetlina) črevesa. **A** – Diferencialno Massonovo trikromno barvanje je predvsem primerno za razločevanje posameznih vrst tkiv v vzorcu in za prikaz splošne histološke zgradbe. Vezivno tkivo v črevesni steni je obarvano modro zaradi modrega obarvanja kolagena. Rdeče/vijolično sta obarvani epitelno in mišično tkivo. **B** – Fluorescenčno označena jedra celic z barvilom DAPI. Zelo razločno je vidna razporeditev in oblika jeder ter različna orientacija jeder v krožnem in vzdolžnem sloju mišic črevesne stene.

Supplementary figure 3: A comparison of a differential histological staining and DAPI application on sections of the same specimen (cross section of mammalian intestine). Intestinal wall is composed of various tissues; epithelial tissue (E) of mucosa covers the intestinal villi, submucosa is mainly composed of connective tissue (V), muscular layer consists of inner circular muscles (KM) and outer longitudinal muscles (VM). L: intestinal lumen. **A** – Differential Masson's trichrome stain is useful to differentiate individual tissues in a sample and to visualize general histological structure. Connective tissue is stained blue because of the blue stained collagen. Epithelial and muscle tissues are stained red/violet. **B** – Fluorescently labelled cell nuclei with dye DAPI. The distribution and shape of nuclei is clearly distinguishable. In the circular and longitudinal muscle layers different orientations of nuclei are visible.



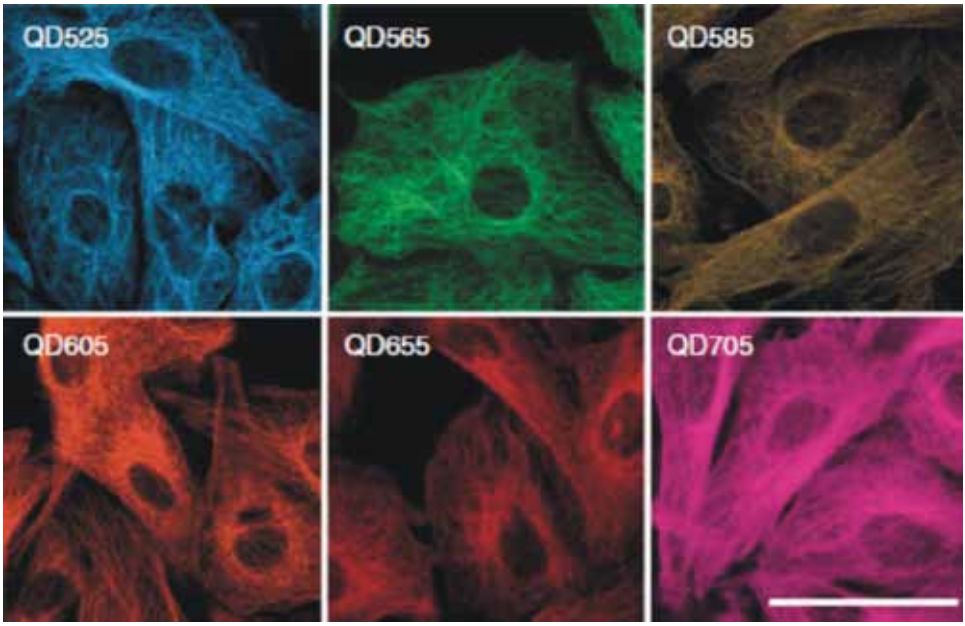
Dodatna Slika 4: Lokalizacija cinka v hepatopankreasnem tkivu (mečkanec) s fluorescenčnim označevalcem Zinquin. V tkivu so vidne fluorescenčno označene celice med nefluorescenčnimi, kar nakazuje, da vsaj nekatere celice vsebujejo veliko dostopnega cinka.

Supplementary figure 4: Zinquin staining of squashed hepatopancreatic tissue for labile zinc detection. Cells displaying intense fluorescent signal are interspersed among non-fluorescent cells, which suggests that at least certain cells in the tissue contain abundant pool of labile zinc.



Dodatna slika 5: Zgradba fluoroforov različnih proteinov. **A –** Fluorofor modrega fluorescenčnega proteina. **B** – Fluorofor modrozelenega fluorescenčnega proteina. **C** – Fluorofor zelenega fluorescenčnega proteina. **D** – Fluorofor rumenega fluorescenčnega proteina (Prirejeno z dovoljenjem Company of Biologists: Journal of Cell Science (Shaner in sod. 2007)).

Supplementary figure 5: Fluorophore structures of various fluorescent proteins. **A** – Blue fluorescent protein fluorophore. **B** – Cyan fluorescent protein fluorophore. **C** – Green fluorescent protein fluorophore. **D** – Yellow fluorescent protein fluorophore (Adapted with permission from Company of Biologists: Journal of Cell Science (Shaner in sod. 2007)).



Dodatna slika 6: Primer uporabe kvantnih pik v celični biologiji. Imunooznačevanje mikrotubulov fibroblastov v kulturi s kvantnimi pikami različnih velikosti. Mikrotubuli so označeni s primarnimi protitelesi proti α-tubulinu, sekundarnimi protitelesi konjugiranimi z biotinom in kvantnimi pikami konjugiranimi s streptavidinom. Fluorescenco QD525, QD565, QD585, QD605 in QD655 so vzbujali z dvo-fotonsko ekscitacijo pri 800 nm. Fluorescenco QD 705 so vzbujali z eno-fotonsko ekscitacijo pri 488 nm. Številke v imenih kvantnih pik povejo valovno dolžino njihove fluorescence (Prirejeno z dovoljenjem Macmillan Publishers Ltd: Nature Methods (Giepmans in sod. 2005)).

Supplementary figure 6: Example of an application of quantum dots in cell biology. Immunolabeling of microtubules in cultured fibroblasts with quantum dots of different sizes. Microtubules are labelled with primary antibodies against the α-tubulin, biotin conjugated secondary antibodies and streptavidin conjugated quantum dots. For the excitation of fluorescence of QD525, QD565, QD585, QD605 and QD655 two-photon excitation at 800 nm was used. Fluorescence of QD 705 was excited with single-photon excitation at 488 nm. Numbers in the names of quantum dots corresponds to the wavelength of their fluorescence (Adapted with permission from Macmillan Publishers Ltd: Nature Methods (Giepmans et al. 2005)).

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The presence of bacteria in calcium bodies of the terrestrial isopods *Androniscus roseus* and *Haplophthalmus mengei*

Prisotnost bakterij v kalcijevih telescih kopenskih enakonožcev *Androniscus roseus* in *Haplophthalmus mengei*

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Izvleček: Pri kopenskih enakonožcih iz družine Trichoniscidae so prisotna kalcijeva telesca, epitelne vrečke, v katerih se kopičijo kalcijevi minerali. Predhodne raziskave so pokazale, da sta pri vrstah *Titanethes albus* in *Hyloniscus riparius* prisotna po dva para teh organov. Pri *T. albus* so vsa kalcijeva telesca napolnjena z bakterijami, pri *H. riparius* pa vsebuje bakterije le posteriorni par. V tej raziskavi smo preučili ultrastrukturo kalcijevih telesc pri vrstah *Androniscus roseus* in *Haplophthalmus mengei*. Pri obeh vrstah sta prisotna po dva para kalcijevih telesc, ki vsebujejo paličaste bakterije. Mineraliziran matriks z bakterijami je obdan z enoslojnim epitelijem z nagubano apikalno plazmalemo, ki jo prekriva tanka ovojnica, ki je plast elektronsko gostega matriksa. Rezultati potrjujejo, da je ultrastruktura kalcijevih telesc z bakterijami splošna značilnost predstavnikov družine Trichoniscidae. Kalcijeva telesca brez bakterij, ki funkcionalno nadomeščajo kalcijeve sternalne depozite, so bila dosedaj opisana zgolj pri predstavniku rodu *Hyloniscus*.

Ključne besede: Trichoniscidae, biomineralizacija, simbioza, raki

Abstract: Terrestrial isopods of the family Trichonscidae possess calcium bodies, epithelial sacs that accumulate calcium minerals. Previous studies have demonstrated that two pairs of these organs are present in the species *Titanethes albus* and *Hyloniscus riparius*. In *T. albus*, all calcium bodies are filled with bacteria, whereas only the posterior pair of calcium bodies contains bacteria in *H. riparius*. In the present work we studied the ultrastructure of calcium bodies in *Androniscus roseus* and *Haplophthalmus mengei*. Two pairs of calcium bodies containing rod-shaped bacteria are present in both species. The bacteria-containing mineralized matrix is enclosed in a simple epithelium with a folded apical plasma membrane, which is covered by a thin, electron dense envelope. Our results show that the presence of bacteria is a general feature of trichoniscid calcium bodies, which are ultrastructurally similar. A combination of bacteria-containing calcium bodies and calcium bodies lacking bacteria has only been found in the genus *Hyloniscus*, in which bacteria-free calcium bodies likely functionally replace sternal $CaCO_3$ deposits.

Keywords: Trichoniscidae, biomineralization, symbiosis, crustacean

Uvod

Kutikula rakov je mineraliziran eksoskelet, ki ga raki redno obnavljajo v procesu levitve. Pred levitvijo raki kalcijev karbonat pogosto kopičijo v začasnih mineraliziranih strukturah v prebavilu ali prostoru med staro ter nastajajočo novo kutikulo (Luquet 2012). Kopenski enakonožci (Oniscidea) pred levitvijo minerale praviloma kopičijo v sternalnih depozitih, kjer se nalagajo v obliki granul amorfnega kalcijevega karbonata (Zidar in sod. 1998, Štrus in Blejec 2001, Ziegler in sod. 2005). Pri kopenskih enekonožcih iz družine Trichoniscidae so znana tudi kalcijeva telesca, posebne strukuture, v katerih se kopičijo kalcijevi minerali (Verhoeff 1926, Méhely 1932, Ziegler 2003, Vittori in sod. 2012a,b). Do sedaj so bili ti organi raziskani na mikroskopskem nivoju pri vrstah *Titanethes albus* ter *Hyloniscus riparius* (Vittori in sod. 2012b, 2013). Pri obeh vrstah sta prisotna po dva para kalcijevih telesc v telesni votlini posteriornih členov pereona. Histološke in ultrastrukturne raziskave dveh parov kalcijevih telesc pri vrsti *Titanethes albus* so pokazale, da gre za epitelijske vrečke, ki vsebujejo številčno populacijo bakterij v zunajceličnem matriksu, mineraliziranem z apatitom. Med pripravo na levitev se med bakterijami in epitelijem oblikuje dodatna plast mineraliziranega matriksa, ki vsebuje amorfni kalcijev karbonat ter amorfni kalcijev fosfat in se po levitvi razgradi (Vittori in sod. 2012b). Pri vrsti *H. riparius* so bakterije prisotne zgolj v posteriornem paru kalcijevih telesc, v anteriornem paru pa niso bile opažene. V posteriornih kalcijevih telescih te vrste sta sestava in dinamika mineraliziranega matriksa, ki vsebuje kalcijev fosfat, med pripravo na levitev podobna kot pri vrsti *T. albus*, medtem ko se v anteriornih kalcijevih telescih, kjer bakterij ni, kopiči razmeroma čist kalcijev karbonat (Vittori in sod. 2013). Po levitvi žival resorbira začasno naložene minerale tako v anteriornih kot v posteriornih kalcijevih telescih. Pri tem je *Hyloniscus* izjemen tudi z vidika odsotnosti sternalnih depozitov kalcija, ki so sicer splošna značilnost kopenskih enakonožcev in se pojavljajo tudi pri trichoniscidih, npr. pri vrsti *T. albus* (Vittori in sod. 2012a). Anteriorna kalcijeva telesca pri *H. riparius* verjetno funkcionalno nadomeščajo sternalne depozite $CaCO_3$.

V tej raziskavi smo preučili histološke in ultrastrukturne značilnosti kalcijevih telesc še pri dveh vrstah trichoniscidov. *Androniscus roseus* je predstavnik poddružine Trichoniscinae, v katero uvrščajo tudi obe do sedaj preučeni vrsti, *Haplophthalmus mengei* pa je uvrščen v poddružino Haplophthalminae. Predstavniki obeh vrst kopenskih enakonožcev so veliki nekaj milimetrov in živijo v prsti. Želeli smo ugotoviti, ali so bakterije splošno prisotne v kalcijevih telescih družine Trichoniscidae in ali so anteriorna kalcijeva telesca brez bakterij, kakršna najedemo znotraj rodu *Hyloniscus*, prisotna tudi pri drugih vrstah.

Material in metode

Uporabljeni enakonožci

V raziskavi smo preučili osebke vrst ***Androniscus roseus*** in ***Haplophthalmus mengei***, ki smo jih nabrali v Središču ob Dravi. Uporabili smo spolno zrele osebke ***H. mengei*** v velikostnem razredu od 2,5 mm od 3 mm in pri ***A. roseus*** v velikostnem razredu od 3 mm do 5 mm. Živali smo žive prenesli vlaboratorij, kjer smo jih fiksirali.

Svetlobna mikroskopija

Za prikaz položaja in števila kalcijevih telesc smo živali fiksirali v 70% etanolu. Osebkom smo odstranili noge, jih prepojili z glicerolom čez noč in pripravili sveže preparate v glicerolu na objektnih steklih z utorom. Za prikaz položaja kalcijevih telesc smo izkoristili avtofluorescenco tkiva ob vzbujanju z ultravijolično svetlobo. Mikrografije smo zajeli s fluorescenčnim mikroskopom AxioImager Z1 (Zeiss), opremljenim z digitalno kamero AxioCam MRm (Zeiss).

Presevna elektronska mikroskopija

Za presevno elektronsko mikroskopijo smo tkiva fiksirali v mešanici 2,5% glutaraldehida in 2% paraformaldehida v 0,1 M kakodilatnem pufru (pH = 7,3). Po spiranju fiksativa smo material dekalcinirali v 10% vodni raztopini EDTA čez noč in postfiksirali z 1% vodno raztopino OsO_4. Fiksativ smo sprali s tremi menjavami deionizirane vode. Tkiva smo nato dehidrirali v naraščajoči vrsti

etanola in vklopili v umetno smolo Spurr (SPI).

Poltanke rezine (500 nm) tkiv smo zbrali na objektnih steklih in jih obarvali z mešanico barvil Azur II in metilenskega modrega (Richardson 1960). Ultratanke rezine (70 nm) kalcijevih telesc smo prenesli na bakrene mrežice ter jih kontrastirali z 1% uranil acetatom in 0,5% svinčevim citratom. Rezine smo opazovali s presevnim elektronskim mikroskopom CM 100 (Philips). Mikrografije smo zajeli s kamero 792 BioScan (Gatan).

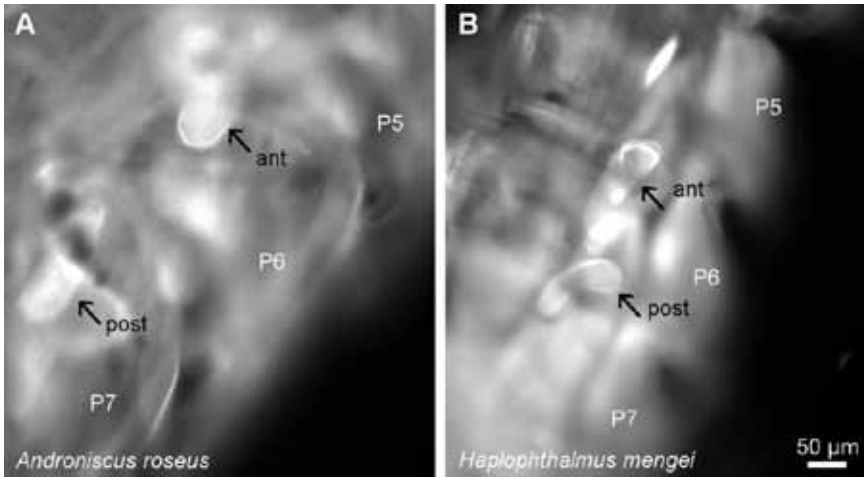
Vrstična elektronska mikroskopija

Za vrstično elektronsko mikroskopijo smo tkiva fiksirali enako kot za presevno elektronsko mikroskopijo. Po postfiksaciji z 1% vodno raztopino OsO_4 in spiranju fiksativa smo material dehidrirali v naraščajoči koncentracijski vrsti etanola. Nato smo ga najprej prenesli v izopropanol in potem v ksilen ter vklopili v parafin. Pripravili smo 10 µm debele parafinske rezine, ki smo jih zbrali na krovnih steklih in deparafinizirali s ksilenom. Tega smo nato nadomestili najprej z izopropanolom ter potem z absolutnim etanolom in posušili v heksametildisilazanu (HMDS). Posušene vzorce smo pritrdili na medeninaste nosilce in jih naprašili s 14 nm platine. Mikrografije smo zajeli z vrstičnim elektronskim mikroskopom na poljsko emisijo JSM-7500F (JEOL).

Rezultati

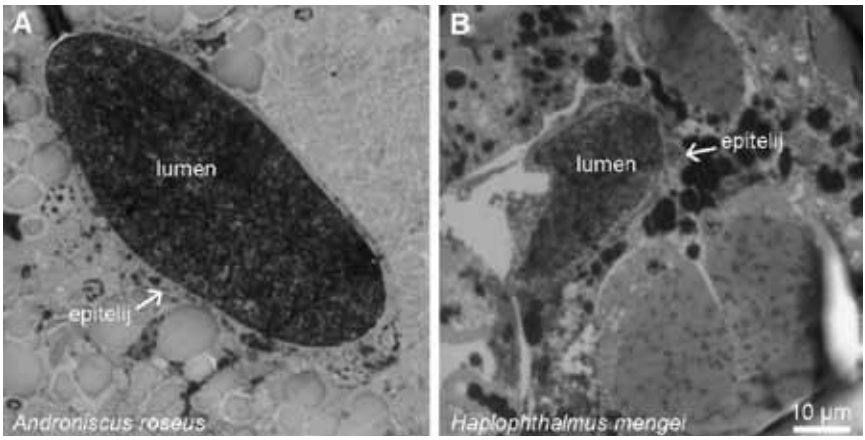
Položaj kalcijevih telesc in histološke značilnosti

Pri obeh vrstah enakonožcev sta prisotna po dva para kalcijevih telesc ledvičaste oblike v posteriornih členih pereona (slika 1). Anteriorni par kalcijevih telesc leži na meji med 5. in 6. pereonitom, posteriorni par pa na meji med 6. in 7. pereonitom. Vsako od kalcijevih telesc pri 5 mm dolgem osebku *A. roseus* meri v dolžino približno150 µm in v širino okoli 50 µm (slika 1A). Oblika kalcijevih telesc pri *H. mengei* je podobna (slika 1B). Pri 3 mm dolgih osebkih so kalcijeva telesca dolga približno 130 µm s premerom približno 30 µm. Pri obeh vrstah gre za vrečaste strukture, ki se nahajajo ventrolateralno v telesni votlini in jih obdaja enoslojni kubični epitelij, debeline med 2 µm in 5 µm (slika 2). Notranjost (lumen) kalcijevih telesc je napolnjena z bakterijami, ki so s svetlobno mikroskopijo vidne kot zrnat material (slika 2).



Slika 1: Položaj in oblika kalcijevih telesc pri *Androniscus roseus* **(A)** in *Haplophthalmus mengei* **(B).** P5 = pereonit 5; P6 = pereonit 6; P7 = pereonit 7; ant = anteriorno kalcijevo telesce; post = posteriorno kalcijevo telesce.

Figure 1: The position and shape of calcium bodies in *Androniscus roseus* **(A)** and *Haplophthalmus mengei* **(B).** P5 = pereonite 5; P6 = pereonite 6; P7 = pereonite 7; ant = anterior calcium body; post = posterior calcium body.



Slika 2: Poltanke rezine kalcijevih telesc pri *Androniscus roseus* **(A)** in *Haplophthalmus mengei* **(B)**. Steno telesca tvori enoslojen epitelij, lumen telesca pa je zapolnjen z bakterijami.

Figure 2: Semithin sections of calcium bodies in *Androniscus roseus* **(A)** and *Haplophthalmus mengei* **(B)**. The wall of a calcium body is formed by a simple epithelium and its lumen is filled with bacteria. Epitelij = epithelium; lumen = calcium body lumen.

Ultrastruktura kalcijevih telesc

Kalcijeva telesca obeh vrst imajo podobno ultrastrukturo (slika 3). Obdaja jih enoslojni epitelij, z apikalno površino usmerjen proti lumnu kalcijevega telesca (slika 3A,B). Apikalna plazmalema celic epitelija oblikuje številne prstaste izrastke debele okoli 300 nm in je prekrita s približno 40 nm debelo elektronsko gosto ovojnico (slika 3C). V lumnu je prisotno tudi večje število elektronsko gostih lamin (slika 3D). Kljub dekalcinaciji tkiv, ki je olajšala pripravo rezin, so v matriksu pogosto še vedno prisotni elektronsko gosti kristali minerala (slika 3C). Celice epitelija imajo dobro razvit zrnati endoplazemski retikulum in vsebujejo številne mitohondrije ter elektronsko goste vezikle (slika 3A,B). V lumnu kalcijevih telesc se nahaja mineraliziran zunajcelični matriks, ki vsebuje številne bakterije (slika 4A, B).

Zunanja morfologija bakterij

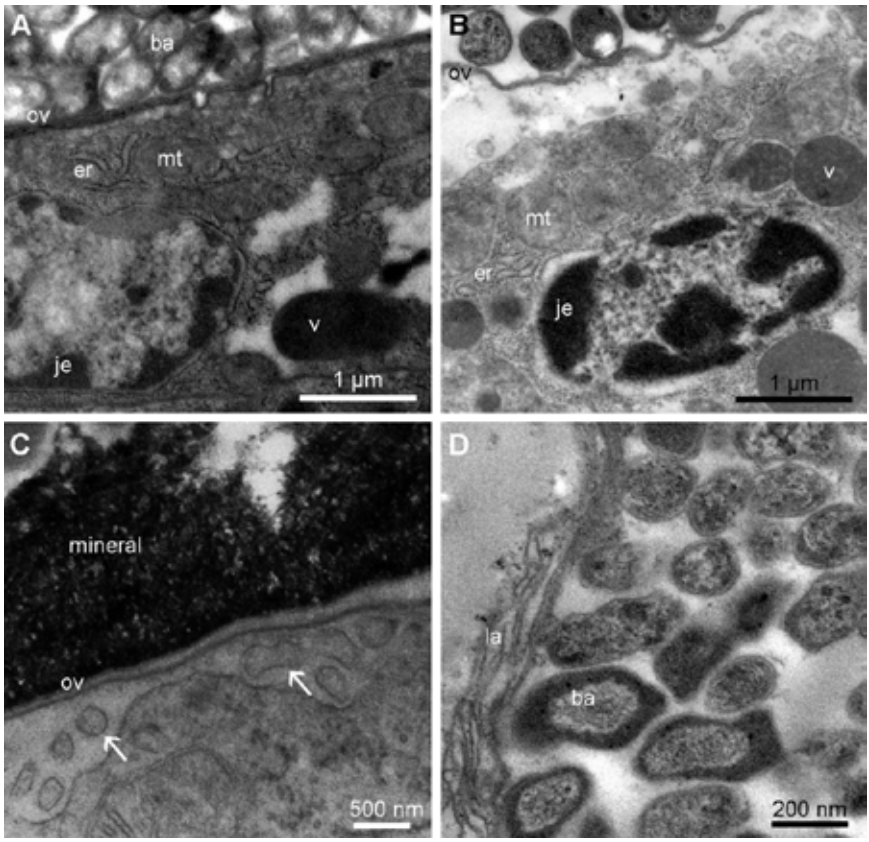
Vrstična elektronska mikroskopija kalcijevih telesc je pokazala veliko gostoto bakterij v zunajceličnem matriksu v lumnu kalcijevih telesc pri obeh enakonožcih (slika 4C,D). Z uporabo vrstične elektronske mikroskopije smo lahko opisali morfologijo bakterij in pokazali, da gre za drobne paličaste bakterije, dolge okoli 1 µm in debele okoli 0,4 µm.

Diskusija

Ugotovili smo, da sta pri vrstah *A. roseus* in *H. mengei* prisotna po dva para razmeroma majhnih kalcijevih telesc, ki ultrastrukturno spominjajo na že opisana kalcijeva telesca jamske vrste *T. albus* (Vittori in sod. 2012b). Naši rezultati so pokazali, da so bakterije prisotne v obeh parih kalcijevih telesc pri obeh preučenih vrstah. V kalcijevih telescih je bakterijska populacija številčna in povsem zapolnjuje njihov lumen.

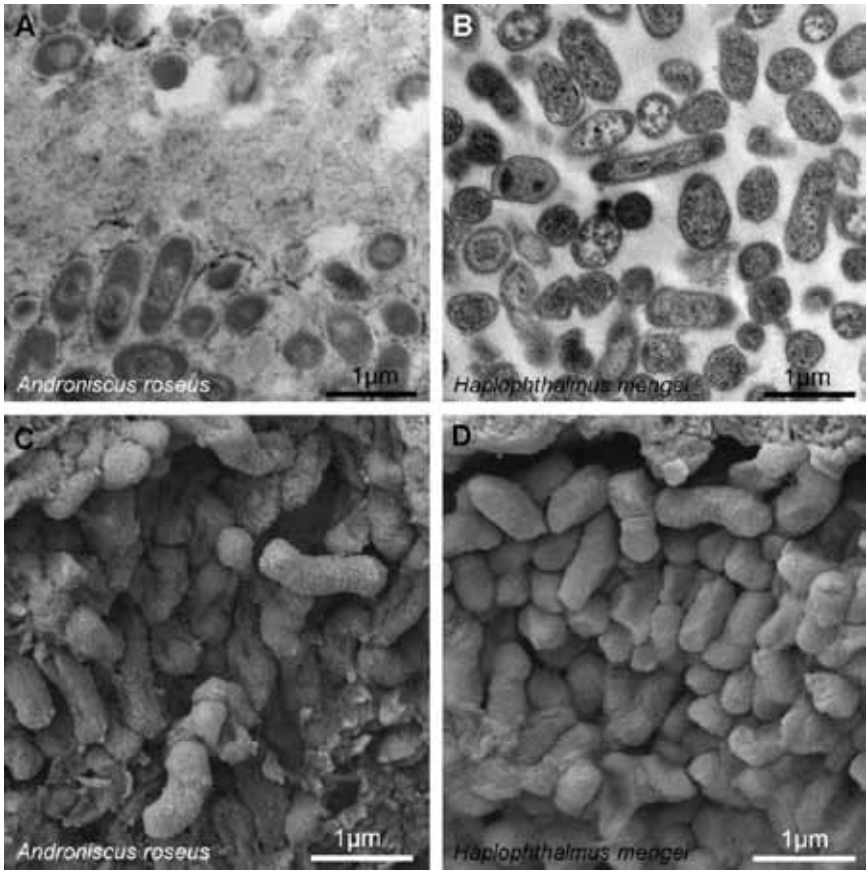
Položaj kalcijevih telesc pri vrstah *A. roseus* in *H. mengei* lahko primerjamo z drugimi do sedaj preučenimi trichoniscidi iz rodov *Titanethes* in *Hyloniscus* (slika 5). Pri rodu *Titanethes* anteriorna kalcijeva telesca segajo od meje med 5. in 6. pereonitom do sredine 7. pereonita, posteriorna kalcijeva telesca pa se začnejo na meji med 6. in 7. pereonitom ter se nadaljujejo do začetka pleona (Vittori in sod. 2012b). Pri vrsti *H. riparius* se posteriorna kalcijeva telesca nahajajo na meji med 6. in 7. pereonitom kot pri drugih trichoniscidih, anteriorni par kalcijevih telesc pa se začne na meji med 5. in 6. pereonitom ter sega v anteriorni smeri

v tretji pereonit (Vittori in sod. 2013). Pri vrsti *H. riparius* so bakterije prisotne zgolj v posteriornih kalcijevih telescih (Vittori in sod. 2013). Pri drugih do sedaj preučenih trichoniscidih napolnjujejo bakterije oba para kalcijevih telesc.



Slika 3: Ultrastruktura kalcijevih telesc. Epitelij kalcijevega telesca pri *Androniscus roseus* **(A)** in *Haplophthalmus mengei* **(B)** ima nagubano apikalno plazmalemo (zgoraj), ki je prekrita z elektronsko gosto ovojnico (ov). Celice epitelija imajo dobro razvit zrnati endoplazemski retikulum (er), v citoplazmi pa so prisotni številni mitohondriji (mt) in elektronsko gosti vezikli (v). V lumnu (zgoraj) so vidne bakterije (ba). Pri večji povečavi apikalnega dela celice pri *A. roseus* **(C)** so vidni prstasti izvihki apikalne plazmaleme (puščice). V lumnu organa pri *H. mengei* **(D)** so vidne tanke lamine (la); je = jedro.

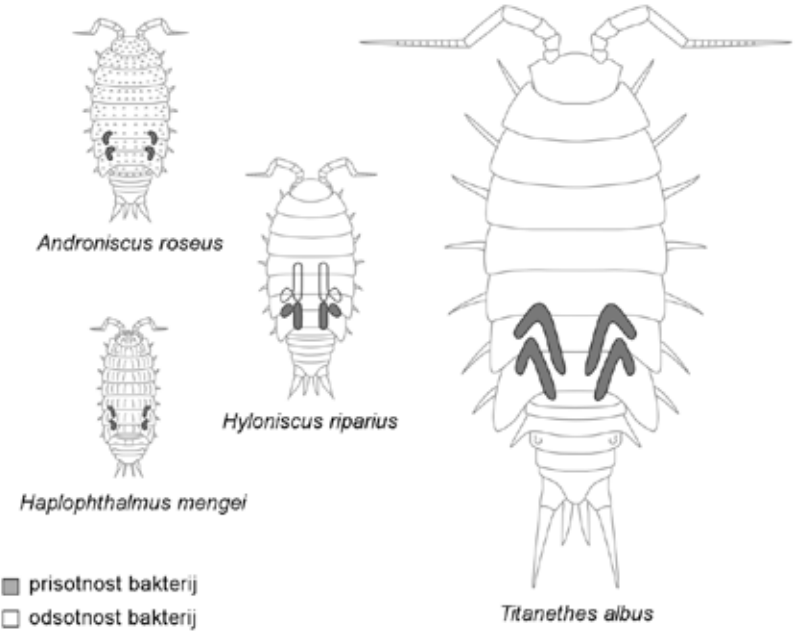
Figure 3: Ultrastructure of calcium bodies. The calcium body epithelium in *Androniscus roseus* **(A)** and *Haplophthalmus mengei* **(B)** has a folded apical membrane (towards the top of the image), which is covered with an electron dense envelope (ov). Epithelial cells have a well developed rough endoplasmic reticulum (er) and numerous mitochondria (mt) and electron dense vesicles (v) in their cytoplasm. Bacteria (ba) are present in the lumen. Higher magnification of the apical cell surface in *A. roseus* **(C)** shows the finger-like extensions of the plasma membrane (arrows). Thin lamina (la) are present in the lumen of the organ, shown here in *H. mengei* **(D)**; je = nucleus.



Slika 4: Bakterije v kalcijevih telescih. Presevna elektronska mikroskopija je pokazala prisotnost številnih bakterij v lumnu kalcijevih telesc pri *Andronicus roseus* **(A)** in *Haplophthalmus mengei* **(B)**. Z vrstično elektronsko mikroskopijo je vidno, da gre tako pri *A. roseus* **(C)** kot pri *H. mengei* **(D)** za paličaste bakterije.

Figure 4: Bacteria in calcium bodies. Transmission electron microscopy demonstrated the presence of numerous bacteria in the lumen of calcium bodies in *Andronicus roseus* **(A)** and *Haplophthalmus mengei* **(B)**. Scanning electron microscopy showed that these bacteria are rod-shaped in *A. roseus* **(C)** as well as in *H. mengei* **(D)**.

Pokazali smo, da bakterije naseljujejo kalcijeva telesca tako pri poddružini Haplophthalminae kot pri poddružini Trichoniscinae. Prisotnost bakterij v kalcijevih telescih je torej v družini Trichoniscidae splošna značilnost in praviloma naseljujejo oba para teh organov. Kombinacija posteriornega para kalcijevih telesc, napolnjenega z bakterijami, ter anteriornega para, v katerem bakterij ni, je bila opažena izključno pri vrsti *H. riparius*, zato gre najverjetneje za izpeljano lastnost te vrste oz. njenega ožjega sorodstva. V prihodnosti bi bilo pomembno ugotoviti, če so kalcijeva telesca prisotna tudi pri drugih predstavnikih skupine Synocheta, ki vključuje trihoniscide (Schmidt 2008). O podobnih organih je pri kopenskih enakonožcih izven družine Trichoniscidae poročal Méhely pri rodovih *Ligidium* in *Mesoniscus* (Méhely 1932), ni pa opravil histološke analize, zato ne moremo z gotovostjo trditi, ali gre v teh primerih res za kalcijeva telesca.



Slika 5: Prisotnost bakterij v kalcijevih telescih do sedaj preučenih trihoniscidov. Kalcijeva telesca se praviloma nahajajo posteriorno v pereonu. Pri vrstah *Androniscus roseus*, *Haplophthalmus mengei* in *Titanethes albus* so bakterije prisotne v obeh parih kalcijevih telesc (sivo polnilo). Pri *Hyloniscus riparius* so bakterije prisotne v posteriornem paru kalcijevih telesc, v anteriornem pa ne (belo polnilo).

Figure 5: The presence of bacteria in calcium bodies of trichoniscids studied to date. Calcium bodies are located posteriorly in the pereon. In *Androniscus roseus*, *Haplophthalmus mengei* and *Titanethes albus*, bacteria are present in both pairs of calcium bodies (gray fill). In *Hyloniscus riparius*, bacteria are only present in the posterior pair of calcium bodies and not in the anterior pair (white fill).

V predhodnih raziskavah smo ugotovili, da so bakterije pri vrsti *T. albus* prisotne pri vseh osebkih in s spremljanjem živali v kulturi pokazali, da te ne kažejo bolezenskih znakov in normalno zaključijo levitveni cikel, zato najverjetneje ne gre za patogene bakterije (Vittori in sod. 2012). Podatki o sestavi mineralov v kalcijevih telescih vrste *H. riparius*, pri kateri so prisotna tako kalcijeva telesca z bakterijami kot kalcijeva telesca brez njih, kažejo na udeleženost bakterij v presnovi kalcijevega fosfata. V posteriornih kalcijevih telescih vrste *H. riparius*, v katerih so prisotne bakterije, se namreč kopiči pretežno kalcijev fosfat, medtem ko se v anteriornih kalcijevih telescih, v katerih ni bakterij, kopiči kemijsko razmeroma čist kalcijev karbonat (Ziegler 2003, Vittori in sod. 2013).

O vrstni pestrosti bakterij v kalcijevih telescih *A. roseus* in *H. mengei* težko sodimo. Da je v posameznih kalcijevih telescih prisotna pestra združba bakterij, smo predhodno pokazali pri vrsti *T. albus*. Pri tej vrsti gre za različne vrste bakterij, ki so sicer prisotne v prsti in so zmožne akumulacije polifosfata (Kostanjšek in sod. 2015). To govori v prid predvidevanju, da so bakterije v kalcijevih telescih udeležene v metabolizmu fosfata.

Ultrastruktura kalcijevih telesc, ki vsebujejo bakterije, je precej podobna pri različnih predstavnikih družine Trichoniscidae. Enoslojni epitelij, ki tvori steno takšnih kalcijevih telesc, ima apikalno plazmalemo nagubano v prstaste izrastke. Epitelij anteriornih kalcijevih telesc vrste *H. riparius*, ki ne vsebujejo bakterij, nima opazno nagubane apikalne plazmaleme, kar nakazuje na

možnost, da je povečana apikalna površina epitelija kalcijevih telesc povezana s prisotnostjo bakterij.

Pri vseh preučenih vrstah je apikalna površina epitelija kalcijevih telesc prekrita z elektronsko gostim matriksom, ki tvori zunajcelično ovojnico debeline nekaj deset nanometrov in je ultrastrukturno podoben epikutikuli (Vittori in sod. 2013). Predhodne raziskave pri vrsti *H. riparius* so pokazale, da med pripravo na levitev epitelij kalcijevih telesc sintetizira novo ovojnico, elektronsko goste lamine v lumnu pa so zelo verjetno ovojnice, ki so nastale v predhodnih levitvenih ciklih (Vittori in sod. 2013).

Povzetek

Kopenski raki enakonožci kopičijo kalcijeve soli med pripravo na levitev v začasnih depozitih mineralov. Večina kopenskih enakonožcev kopiči $CaCO_3$ v obliki sternalnih depozitov v prostoru med starim in nastajajočim novim eksoskeletom. Pri predstavnikih družine Trichoniscidae so prisotna kalcijeva telesca kot dodatne strukture, v katerih kopičijo mineral. Te strukture smo predhodno preučili pri jamskem enakonožcu *Titanethes albus* ter pri njegovem površinskem sorodniku *Hyloniscus riparius*. Ugotovili smo, da imata obe vrsti po dva para kalcijevih telesc. Gre za epitelne vrečke, ki vsebujejo mineraliziran matriks. Pri *T. albus* so v obeh parih kalcijevih telesc prisotne številne bakterije, medtem ko so pri *H. riparius* te prisotne zgolj v posteriornem paru kalcijevih telesc, ki vsebuje kalcijev fosfat, ni jih pa v anteriornem paru, ki vsebuje kalcijev karbonat.

V tej raziskavi smo preučili položaj, histološke značilnosti in ultrastrukturo kalcijevih telesc še dveh predstavnikov družine Trichoniscidae, ki živita v prsti. *Androniscus roseus* je predstavnik poddružine Trichoniscinae, *Haplophthalmus mengei* pa poddružine Haplophthalminae. Tudi pri teh dveh vrstah sta prisotna po dva para kalcijevih telesc, ki vsebujejo bakterije. Njihove histološke in ultrastrukturne značilnosti so podobne kot pri kalcijevih telescih *T. albus*. Steno kalcijevih telesc tvori enoslojen kubični epitelij, z apikalno površino usmerjeno proti lumnu. Apikalna plazmalema celic epitelija je nagubana v prstaste izrastke. Apikalno je epitelij prekrit z elektronsko gostim matriksom, ki tvori ovojnico. Bakterije v kalcijevih telescih so paličaste in napolnjujejo njihov lumen pri obeh vrstah.

Zaključimo lahko, da je prisotnost bakterij najverjetneje splošna značilnost kalcijevih telesc družine Trichoniscidae. Bakterije so praviloma prisotne v obeh parih kalcijevih telesc, kombinacija para kalcijevih telesc z bakterijami in para brez bakterij pa je najverjetneje prisotna le pri predstavnikih rodu *Hyloniscus* ali pri njegovih ožjih sorodnikih. Morebitna funkcija bakterij v kalcijevih telescih še ni znana, verjetno pa so udeležene v metabolizem fosfata. Epiteliji kalcijevih telesc, ki vsebujejo bakterije, imajo podobne ultrastrukturne značilnosti in nagubano apikalno plazmalemo, kar je lahko povezano z intenzivnim transportom ali izločanjem.

Summary

Terrestrial isopods accumulate calcium minerals in transient mineral deposits during their preparation for molt. In most terrestrial isopods, minerals are accumulated as sternal $CaCO_3$ deposits in the space between the old exoskeleton and the newly forming one. In representatives of the family Trichoniscidae, calcium bodies are present as additional structures in which mineral is accumulated. These structures have previously been analyzed in the cavernicolous isopod *Titanethes albus* and its epigean relative *Hyloniscus riparius*. It has been established that these species possess two pairs of calcium bodies. These structures are epithelial sacs enclosing a mineralized matrix. In *T. albus*, bacteria are consistently present in both pairs of calcium bodies, whereas in *H. riparius*, they populate only the posterior pair, which contains calcium phosphate, whereas the anterior pair of calcium bodies, which lack bacteria, contains calcium carbonate.

In this work, we analyzed the position, histology and ultrastructure of calcium bodies in two further endogean representatives of the family Trichoniscidae: *Androniscus roseus* (subfamily Trichoniscinae), and *Haplophthalmus mengei* (subfamily Haplophthalminae). These two species also possess two pairs of calcium bodies, all of which contain bacteria. Histologically and ultrastructurally, these structures are similar to those found in *T. albus*. Their walls are formed

by a simple cubic epithelium, the apical side of which faces the lumen containing bacteria. The apical plasma membrane of epithelial cells is folded and forms finger-like extensions. Apically, the epithelium is lined by an electron dense envelope. Bacteria present within the calcium bodies are rod shaped in both species and fill the entire lumen of these structures.

We can conclude that the presence of bacteria is a general feature of calcium bodies in the family Trichoniscidae. Furthermore, bacteria are generally present in both pairs of calcium bodies, making the combination of bacteria-containing and bacteria-free calcium bodies a feature likely unique to *Hyloniscus* and possibly its close relatives. Although the possible function of bacteria in these structures remains unclear, it is likely that they contribute to the metabolism of phosphate. The epithelia of bacteria-containing calcium bodies of all species examined to date share common ultrastructural features, such as a folded apical membrane, which may be linked to the maintenance of a large bacterial population.

Zahvale

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Distribution of epilithic diatoms in the Savinja River flowing through an urban landscape

Razširjenost epilitskih diatomej v reki Savinji, ki teče skozi mestno krajino

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Abstract: The catchment area in urban and agricultural landscapes is greatly influenced by human activities that reflect also in physical and chemical characteristics of water as well as in species diversity in waterbodies. The diversity and the species composition of epilithic diatom communities in the Savinja River, as well as basic environmental parameters were analysed. Sampling sites were selected in reaches subjected to different influences from the catchment area and with different physical and chemical characteristics. Samples were collected at the site where the Savinja River enters the urban area of the town Celje, at the end of urban landscape and downstream of the Celje waste water treatment plant outflow. The most common and dominant diatom species in the periphyton community was *Achnanthes biasolettiana.* Other common diatom taxa that were found in all samples and at least in one sample exceeded relative abundance of 10% were *Nitzschia fonticola, Amphora pediculus* and *Nitzschia dissipata.* The results of the redundancy analyses (RDA) revealed that the variance of the epilithic diatom community was explained by O_2 saturation (35%) and saprobic index (33% of TVE). Diatom species richness was positively correlated with O_2 saturation. Shannon-Wiener diversity index was positively correlated with saprobic index values based on all algae and trophic index calculated on the base of diatoms indicating a relatively low organic matter and nutrient input into the river system. The results showed that no significant changes in epilithic diatom species composition and no negative impacts on diversity of epilithic diatom community in the Savinja River were detected on its flow through the urban landscape. Moreover, changes between the seasons were more evident than changes between sampling sites, confirming the importance of sampling date for monitoring.

Keywords: Diatoms, microphytobenthos, periphyton, environmental factors, torrential river

Izvleček: Prispevno območje v mestnih in kmetijskih krajinah je pod močnim človekovim vplivom, kar se odraža v fizikalnih in kemijskih lastnostih kot tudi v raznolikosti vrst v vodnih telesih. Analizirali smo raznolikost in vrstno sestavo epilitonskih združb diatomej v reki Savinji, kot tudi osnovne okoljske parametre. Vzorčna mesta smo izbrali na odsekih, ki so izpostavljeni različnim vplivom iz prispevnega območja in z različnimi fizikalnimi in kemijskimi lastnostmi. Vzorci so bili nabrani

na mestu, kjer reka vstopi v urbano območje mesta Celje, na koncu mestne krajine in dolvodno od iztoka centralne čistilne naprave Celje. Najpogostejša in prevladujoča vrsta kremenastih alg v perifitonski združbi je *Achnanthes biasolettiana*. Drugi pogosti taksoni diatomej, ki so bili najdeni v vseh vzorcih in so vsaj v enem vzorcu presegli 10 %, so bili: *Nitzschia fonticola, Amphora pediculus* in *Nitzschia dissipata*. Rezultati redundančne analize (RDA), so pokazali, da variabilnost epilitskih združb diatomej lahko pojasnimo z nasičenostjo vode s kisikom (35 %) in s saprobnim indeksom (33 %). Vrstna pestrost diatomej je bila v pozitivni korelaciji z nasičenostjo s O_2. Shannon--Wiener indeks je v pozitivni korelaciji z vrednostjo saprobnega indeksa, ki je izračunan na podlagi združbe vseh alg in z vrednostjo trofičnega indeksa, izračunanega na osnovi združbe kremenastih alg, kar kaže na relativno nizko vsebnost organskih snovi in hranil v rečnem sistemu. Glede na naše rezultate, nismo zaznali opaznih sprememb v vrstni sestavi in negativnih vplivov na raznolikost epilitske združbe diatomej iz reke Savinje pri njenem toku skozi mestno krajino. Poleg tega smo ugotovili, da so spremembe med sezonami bolj očitne, kot spremembe med vzorčnimi mesti, kar potrjuje pomembnost datuma vzorčenja pri monitoringu.

Ključne besede: diatomeje, mikrofitobentos, perifiton, okoljski dejavniki, hudourniška reka

Introduction

Monitoring of ecological status of aquatic ecosystems is essential for the estimation of human influence on aquatic environment as well as for the evaluation of aquatic environment management efficiency. Benthic diatoms are one of the biological quality elements used for the assessment of ecological status according to the European Water Framework Directive (WFD) (2000/60/EC). Benthic diatoms are used for the calculation of different metrics such as trophic and saprobic indices, measuring the extent of human impact to the rivers and lakes (emission of nutrients and dissolved organic matter, respectively). Diatoms are frequently used for the evaluation of the ecological status of running waters (Virtanen et al. 2011, Kelly et al. 2012), since they are various, dominant in phytobenthos, and since the ecological preferences of several diatom species are well known (Beltrami et al. 2012). Habitat and species diversity of biotic communities in running waters are strongly influenced by the properties of the catchment area, land use and pollution sources.

Diatoms are a taxonomically diverse group of organisms with high sensitivity to chemical stressors (Martínez De Fabricius et al. 2003, Frankovich et al. 2006, Almeida and Feio 2012, Várbíró et al. 2012), and vary spatially and temporally (Passy 2007, Soininen 2007). The relationships between diatoms and environmental variables were shown by many authors (Passy 2007, Soininen 2007, Lange et al. 2011, Virtanen and Soininen 2012, Toman et al. 2014).

Diversity and abundance of diatoms are controlled by environmental factors like nutrients, temperature, light intensity, grazing pressure, substrate stability and discharge (Izagirre and Elosegi 2005). Major environmental determinants for diatom distribution in streams, as reported by Soininen (2007) are pH, conductivity, total phosphorus, temperature, alkalinity, altitude, nitrates, calcium, biological oxygen demand (BOD), chlorophyll *a* and substrate type. Lange et al. (2011) found that light availability, nutrient concentrations and grazing pressure determined the stream diatom community composition. Biggs and Close (1989) suggested that disturbances such as spates reduce the effect of grazing pressure, because re-colonization of invertebrates is usually slow compared to periphyton growth.

In depth studies dealing with the relationships between environmental parameters and algal communities in Slovenia have been performed only in extreme environments (Krivograd Klemenčič et al. 2010, Krivograd Klemenčič and Toman 2010), while the distribution of diatoms along the environmental gradients in running waters

of Slovenia is poorly known. Similar study as is present one on environmental effects on diatom communities in rivers in Slovenia was conducted for Kamniška Bistrica River (Toman et al. 2014) together with a preliminary study on the Savinja River (Koren 2009, Čatorič 2013).

On the studied section the Savinja River flows through the urban landscape of the town Celje and is subjected to numerous influences from the catchment area i.e. emissions from different kinds of industry, intensive agriculture and Celje wastewater treatment plant.

The main goal of this research was to investigate the influence of various environmental and temporal factors on the species composition and diversity of epilithic diatom assemblages and possible longitudinal changes. We hypothesized that there will be greater differences in species composition and diversity of epilithic diatom assemblages between the seasons than between the sampling sites due to general degradation of environment within the research area. We also hypothesized that the loading of the river will be higher at the sites downstream of the major part of the settled area and emissions of the wastewater than at the reference site upstream the urban landscape of Celje.

Materials and methods

Study area

The Savinja River is a left tributary of the Sava River and an important part of the Danube catchment area, collecting water from the southern belt of the limestone Alps. The length of the river is 101 km, drainage area 1848 km^2 and the average (monthly) discharge near the sampling site S3 is from 25 to 56 m^3 s^{-1} (data available on: http://www.arso.gov.si/vode/podatki/amp/H6200_g_1.html) Hydrological conditions in the Savinja River are extremely variable; at the highest water levels the flow can increase more than 300- fold compared to the basal flow. The source (GKX: 140697, GKY: 472458) is at an altitude of 734 m a.s.l. The riverbed in the studied reach consists of different types of rock (sandstones, conglomerates), but limestone and magnesium limestone (dolomite) of Middle to Early Jurassic age is the most common rock type (Buser 2009). The catchment area in the middle and lower Savinja valley is characterized by agricultural land, farms and numerous settlements.

Samples were taken at three sampling sites (Fig. 1). The sites S1 was chosen as a reference site in this study, since it is situated on the Savinja River before it enters into densely populated urban landscape of the town Celje. Sites S2 and S3 where the influences of human activities was to be detected are downstream of the urban area of Celje with all its emissions. The distance from the upper sampling site S1 to the lowest site S3 is approximately 10 km. The catchment between S1 and S2 is the urban area of the town Celje with population over 40.000 inhabitants, where an urban landscape can be found with different types of settlements and different kinds of industry, all of which are potential sources of inorganic and organic compounds (Fig. 1). The sampling site S3 is downstream the Celje wastewater treatment plant, which influences the river with its effluent.

Sampling and laboratory analyses

Samples were taken in different seasons in the years 2011 and 2012. The sample from the beginning of September is considered a summer sample due to low discharges and high water temperatures, which reached up to 23 °C (Sampling site S2). The sample from the beginning of December represents winter sample, since there was a longer period of relatively cold weather. The sample from the end of March is a typical spring sample. Epilithic diatoms in the periphyton communities were sampled from stones (φ = 6-20 cm) at each site, by scraping and brushing off the stone surface (5 cm^2 per stone). Samples for diatom identification and for biomass determination were preserved in 4% formaldehyde, samples for chlorophyll *a* analyses were stored at 4 °C till the next day. For taxonomic identification of diatoms all samples were diluted with distilled water to 50 mL, homogenized with a magnetic stirrer and subsamples were treated with concentrated nitric acid (HNO_3). Permanent slides of diatom frustules were prepared using the high refraction mountant Naphrax®. Diatom taxa were identified and counted using an Olympus CX41 light microscope with an oil-immersion objective at a magnification of

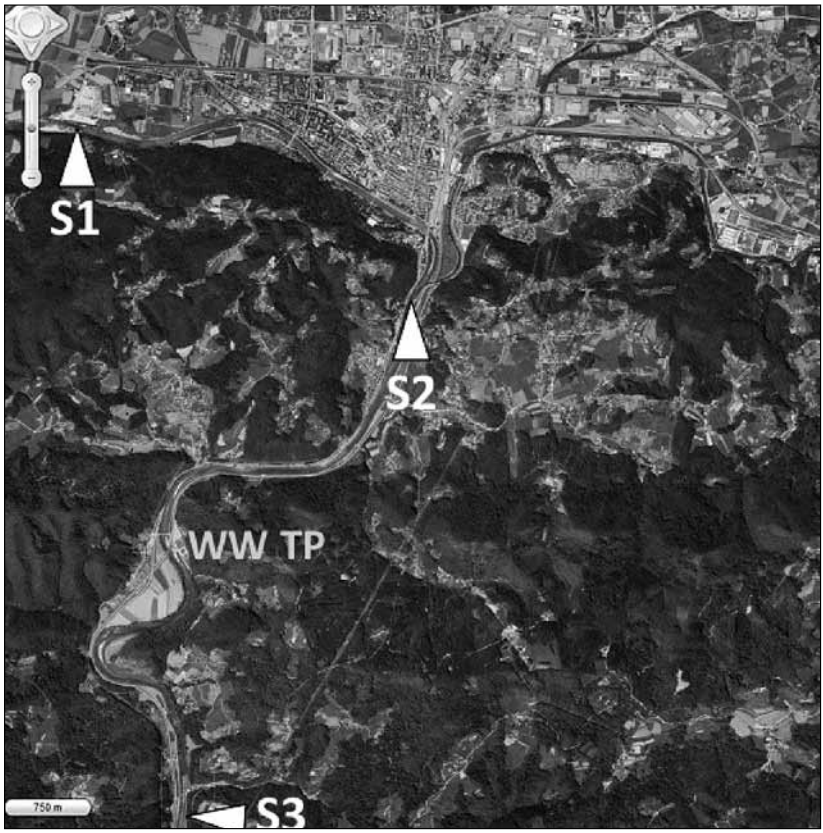

Figure 1: Map showing the location of the sampling sites S1, S2 and S3 along the river Savinja (WW TP = Celje wastewater treatment plant). (Source: Geopedia 2016)

Slika 1: Zemljevid, ki prikazuje lokacijo vzorčnih mest S1, S2 in S3 ob Savinji (WW TP = Celjska centralna čistilna naprava). (vir: Geopedia 2016)

1000×, taxonomy followed Kramer and Lange-Bertalot (1986-1991).

The proportion of diatoms and species composition of cyanobacteria and other algal groups were analysed with the same light microscope at a magnification of 400×. Cyanobacteria and other non-diatom algae were identified using Hindák et al. (1978), Biggs and Kilroy (2000), and Komárek and Anagnostidis (2002). Proportions of diatom taxa among all other algae taxa were obtained by further division of their total share according to the proportions of around 500 counted frustules at a magnification of 1000×.

At each sampling site selected physical and chemical parameters were measured at the same time as the samples were collected. Temperature, pH, O_2 concentration, O_2 saturation, and conductivity were measured using the portable multi-meter PCD 650 (Eutech Instruments, Singapore). Water depth and current velocity above the selected stones were measured as well. The cover of inorganic and organic substrate was estimated according to the AQEM (2002) protocol (see Tab. 1).

Table 1: The structure of organic and inorganic substrates at the sampling sites (S1-S3). Mean coverage (%) of each fraction of the substrate; CPOM - coarse particulate organic matter, FPOM - fine particulate organic matter.
Tabela 1: Deleži različnih tipov organskega in anorganskega substrata na posameznih vzorčnih mestih (S1-S3). Navedeni so povprečni deleži (%) pokrovnosti posameznega tipa substrata; CPOM – večji delci organskih snovi, FPOM – drobni delci organskih snovi.

	S1	S2	S3
filamentous algae	67	53	75
submerged macrophytes	.	.	1
xylal	2	4	3
CPOM	.	2	3
FPOM	5	27	17
megalithal	5	.	40
macrolithal	5	5	20
mesolithal	50	20	20
microlithal	40	60	20
psammal	.	10	.
argyllal	.	5	.

Water samples were analyzed in the laboratory for the concentration of nitrates and soluble reactive phosphorous (SRP). Chlorophyll *a* content was measured spectrophotometrically according to the method described in (Urbanič and Toman, 2003). Concentration of nitrates were measured with the Na-salicylate method (Monteiro et al. 2003), while the soluble reactive phosphorus (SRP) was measured using the $SnCl_2$ method (APHA 1998). Periphyton biomass was also determined as dry weight (at 105 °C) of the biofilm covering the sampled stones scratched from another 5 cm^2 rectangle on each stone. Results of these measurements are given in Table 2.

Table 2: Measured environmental variables on sampling sites S1, S2 and S3 including mean, minimum and maximum values.
Tabela 2: Izmerjene vrednosti okoljskih spremenljivk na merilnih mestih S1, S2 in S3, vključno s povprečnimi, minimalnimi in maksimalnimi vrednostmi.

	S1			S2			S3		
	Mean	Min.	Max.	Mean	Min.	Max.	Mean	Min.	Max.
Temperature (°C)	11.8	4.5	21.0	13.2	5.5	23.2	13.1	5.3	22.8
pH	8.2	8.1	8.3	8.2	8.1	8.3	8.3	8.1	8.5
conductivity (µS/cm)	379	375	384	683	630	738	462	458	469
O_2 (mg/L)	12.6	11.7	14.2	13.1	12.3	12.5	13.1	12.0	14.8
O_2 saturation (%)	115	106	130	123	113	142	123	116	138
nitrate (mg/L)	5.2	3.9	6.0	5.1	4.8	5.5	5.8	4.8	6.6
current velocity (m/s)	0.55	0.42	0.63	0.57	0.46	0.77	0.32	0.28	0.39
dry mass of periphyton (mg/cm^2)	5.3	3.3	6.4	4.2	1.7	7.6	7.6	7.1	8.8
Chlorophyll a (mg/m^2)	5.2	4.0	5.9	3.5	2.3	4.2	7.6	6.5	9.1

Data analyses

Relative abundance (percentage values) of the diatom taxa were calculated for each sample. The Shannon-Wiener (S-W) diversity index was used to estimate diatom diversity and the saprobic index (SI) was calculated using saprobic (s_i) and indicator values (G_i) according to Kosi et al. (2006) to determine water quality using the following formula:

$$SI = \frac{\sum_{i=1}^{n}(h_i \times G_i \times s_i)}{\sum_{1=1}^{n}(h_i \times G_i)}$$

(h_i – abundance of the taxon i;
n – number of taxa)

The trophic index was calculated in the same manner as saprobic, however the trophic and indicator values according to Rott (1999) were found in Kosi et al. (2006). The cluster analyses were performed with the program Syn-Tax (Podani 2001) to establish the similarity between diatom communities from different sampling sites/seasons. As a method of linkage, unweighted pair group method with arithmetic mean (UPGMA) was used and the Sørensen index served as a similarity measure for the creation of a dendrogram.

Detrended correspondence analysis (DCA) was applied to the diatom percentage data to explore the patterns of species changes and biological species turnover (the gradient length). The eigenvalue for the first DCA axis was < 0.4 (0.35, while gradient length was 1.70 SD (standard deviation units of species turnover) and indicated linearity (ter Braak and Verdonschot 1995) and therefore redundancy analysis (RDA) was chosen to explore the relationships between diatom assemblages and explanatory variables.

Separate RDAs for smaller groups of all studied environmental and temporal variables were performed to test the significance of their influence on the variation of species composition. Forward selection of explanatory variables was used to provide a ranking of the relative importance of the specific variables and to avoid co-linearity. Unrestricted Monte Carlo test with 499 permutations was used to test the statistical significance of the variables and canonical axes. A series of RDAs were done with subsets of statistically significant variables ($p < 0.05$) and the proportions of variance explained by these variables were calculated. Ordination of the samples according to the species composition was made using DCA and most important environmental parameters were passively projected on the ordination diagram. The whole set of analyses was performed using CANOCO 4.5 (ter Braak and Šmilauer 2002).

Relationships between the diatom diversity and environmental factors were explored with Spearman correlation coefficients in SPSS version 17.

Results

Distribution of diatoms and diversity of diatom assemblages

A total of 50 diatom taxa were identified in the samples. Almost all of them that is 45 taxa were found at the sampling site S2, in the regulated channel, downstream the urban area. The highest number of diatom species in a single sample (37) was found in summer samples at second and third site (Tab. 3), and the lowest (30) at the upper sampling site in all seasons. Two species occurred with a high share in all seasons and at all sampling sites, namely *Achnanthes biasolettiana* (18-46%) and *Nitzschia fonticola* (4-14%). The similarity of diatom assemblages is presented in Fig. 2, which indicates that about three quarters of taxa were common to all samples (Tab. 3). The dendrogram (Fig. 2) showed that two clusters are formed according to seasons. The left subgroup uniting the summer samples from all three locations (S1_P, S3_P and S2_P) shows a higher similarity of the samples within seasons than sites. Moreover, the cluster in the middle unites winter samples (S1_Z, S2_Z and S3_Z) from all three sites.

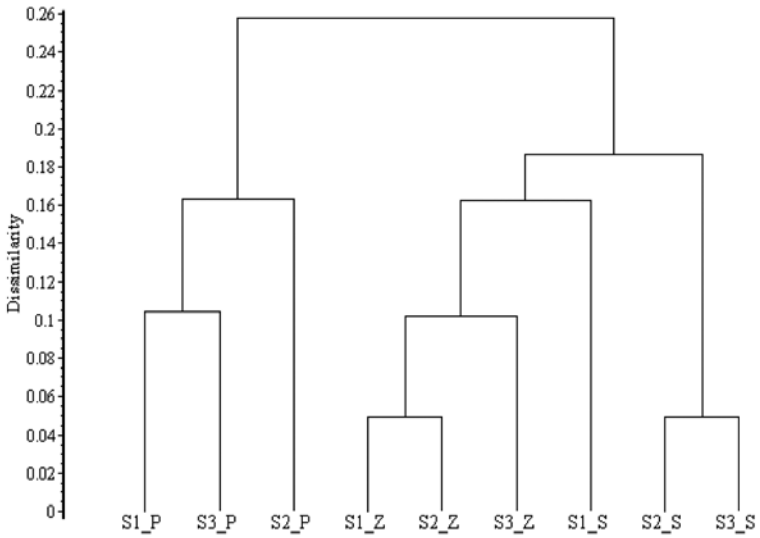

Figure 2: Dendrogram showing the similarity of diatom communities in different sampling sites (S1-S3) in different seasons/months (P = summer, Z = winter, S = spring).

Slika 2: Dendrogram, ki kaže podobnost združb kremenastih alg na različnih vzorčnih mestih (S1-S3) v različnih letnih časih / mesecih (P = poletna, Z = zimska, S = spomladanska).

In the samples from the upper site (S1) 38 diatom taxa were found all together (Tab. 3). The most abundant species (≥ 5%) were: *Achnanthes biasolettiana*, *Nitzschia fonticola, Nitzschia dissipata, Navicula capitatoradiata, Cymbella affinis* and *Navicula menisculus*.

The total number of diatom taxa was the highest at site S2 downstream of the town Celje where 45 diatom species were found. The highest number per sample was in the summer (Tab. 3). The majority of co-dominant species were the same as in site S1: *Achnanthes biasolettiana*, *Nitzschia fonticola, Nitzschia dissipata, Navicula capitatoradiata, Cymbella affinis,* but here additional taxa occurred with high abundance: *Amphora pediculus, Gomphonema angustatum* and *Cymbella minuta*.

The site S3 which is in the regulated channel like site S2 exhibited the most diverse diatom assemblage in December when S-W index reached the value 4.1 (Tab. 3). Ten taxa reached ≥ 5% of the relative abundance. *Achnanthes biasolettiana* occurred with a high share in all seasons. Other common diatom species included: *Amphora pediculus, Nitzschia fonticola, Navicula capitatoradiata, Cymbella affinis, Navicula menisculus, N. reichardtiana, Diatoma vulgaris, Cocconeis placentula.*

Table 3: Number of diatom taxa, Shannon-Wiener (S-W) diversity index, saprobic indices, the percentage (%) of diatoms in phytobenthos and relative abundances (%) of common diatom species on sampling sites (S1-S3) in different seasons/months (S – September/P – summer; D – December/Z – winter; M – March/S - spring). Abundances reaching at least 5% are in bold; +, species present with relative abundance < 1%; . , not detected

Tabela 3: Število taksonov diatomej, Shannon-Wiener (SW) indeks raznolikosti, saprobni indeksi, delež kremenastih alg v fitobentosu in relativne abundance (%) pogostih diatomej na vzorčnih mestih (S1-S3) v različnih letnih časih / mesecih (S – september/P – poletni; D – december/Z – zimski; M – marec/S - spomladanski). Abundance, ki dosegajo vsaj 5% so v krepkem tisku; +,: vrste so prisotne z <1%; . takson/vrsta ni zaznan/a.

sampling site	S1			S2			S3		
Nr. of taxa per site	38			45			44		
month	S	D	M	S	D	M	S	D	M
season	P	Z	S	P	Z	S	P	Z	S
Nr. of taxa per sample	30	30	30	37	31	31	37	33	30
S-W diversity index	3.6	3.0	2.5	3.3	3.5	3.9	3.9	4.1	3.0
Saprobic index (all algae)	1.72	1.74	1.69	1.67	1.82	1.92	1.78	1.87	1.78
Saprobic index (diatoms)	1.66	1.73	1.64	1.60	1.82	1.92	1.77	1.88	1.79
Trophic index (diatoms)	2.4	2.0	1.7	2.1	2.1	2.1	2.5	2.7	2.2
% of diatoms in phytobenthos	75	77	81	80	82	89	77	87	79
% of diatoms taxa									
Achnanthes biasolettiana	**26**	**46**	**57**	**40**	**29**	**18**	**19**	**19**	**37**
Nitzschia fonticola	**9**	**14**	**13**	4	**11**	**14**	**6**	**10**	**9**
Amphora pediculus	+	+	**5**	+	+	**9**	**8**	**10**	**26**
Nitzschia dissipata	1	**12**	2	+	**19**	**7**	1	**6**	2
Navicula capitatoradiata	**17**	+	+	**12**	.	.	**20**	+	.
Cymbella affinis	**11**	4	+	**14**	1	.	**10**	+	.
Navicula menisculus	**8**	+	+	**5**	2	2	**6**	3	2
Gomphonema angustatum	+	+	2	1	**5**	**13**	+	.	2
Cymbella minuta	+	2	4	+	3	**6**	1	4	2
Gomphonema olivaceum	+	3	1	+	3	4	+	4	1
Diatoma vulgaris	1	2	2	3	2	1	1	**6**	+
Navicula reinchardtiana	.	3	1	.	**5**	2	.	**8**	+
Cymbella silesiaca	3	+	4	1	+	3	+	2	2
Navicula lanceolata	.	2	+	+	4	3	+	**5**	2
Cocconeis placentula	4	+	+	3	+	+	**6**	+	+
Fragillaria capucina vaucheria	2	2	+	1	3	2	1	2	+
Rhoicosphaenia abbreviata	2	+	+	2	+	+	2	3	3
Nitzschia palea	3	+	2	2	+	+	3	+	+
Cymbella sinuata	+	+	2	1	2	4	1	.	2
Navicula gregaria	.	1	+	.	+	3	.	**5**	2
Gomphonema parvulum	2	+	.	3	1	+	2	+	+
Surirella brebissonii	+	+	+	.	2	2	+	4	+
Cocconeis pediculus	2	+	+	+	+	+	2	1	1
Navicula tripunctata	3	+	+	+	+	.	2	+	.
Gomphonema minuta	1	+	+	+	2	+	+	+	.
Navicula veneta	1	+	+	+	+	+	1	+	+
Fragillaria ulna	+	1	+	+	+	.	+	2	.
Navicula atomus	.	.	+	.	.	2	.	.	2
Achnantes minutissima	.	+	.	.	1	+	.	+	1
Melosira varians	+	+	.	+	+	+	+	2	+
Cyclotella meneghiniana	.	.	.	+	+	.	2	+	.

More evident than differences between the sites were similarities between the samples from the same seasons (Tab. 3). Certain diatoms occurred with relatively higher abundance in the summer like: *Navicula capitatoradiata, Cymbella affinis, Navicula menisculus, Cocconeis placentula.* On the other hand, diatom species like *Nitzschia dissipata* and *Navicula reichardtiana,* were more numerous in the winter samples. Two taxa, namely *Amphora pediculus* and *Gomphonema angustatum* reached the highest share in spring at all three sites and were characteristic for spring samples.

The lowest number of diatom taxa was detected at the upper site (S1) where 30 diatom species were found in each sample. The lowest S-W diversity index value (2.5) was calculated for the spring sample from the upper site, which was also the most species-poor, due to the dominance of the species *Achnanthes biasolettiana* (57%). Beside the mentioned species only *Nitzschia fonticola* exceeded the share of 5% (Tab. 3).

Water quality

The average SI value for all sampling sites was 1.76 which indicates a 2nd quality class or β-mesosaprobic level with moderate organic loading. Samples from sites S2 and S3 were classified into 2nd class. Samples from the upper site S1 were classified into 1-2nd class which is the oligo- to β-mesosaprobic class (Tab. 3).

Influence of environmental parameters on diatom species composition

Two parameters statistically significantly explained the species composition of diatom communities: O_2 saturation of the water and saprobic index values calculated on the base of all algae. Saturation of water with O_2 explained 35% and saprobic index explained 33% of the total variance of the diatom community.

Diatoms presented on the left side of Fig. 3B were found in the summer samples from all three sites (Fig. 3A) and prefer water with higher O_2 saturation, while diatoms which are found on the right side, prefer water with higher content of dissolved organic matter, that also results in lower O_2 saturation.

Figure 3 A

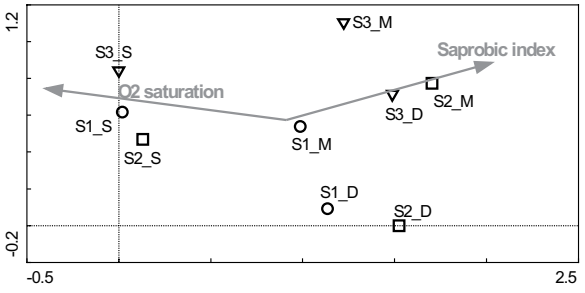

Figure 3B

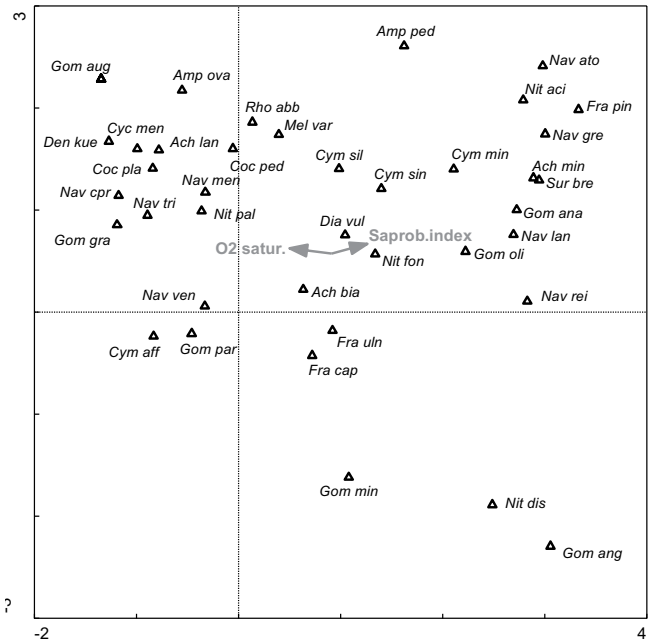

Figure 3: Detrended correspondence analysis (DCA) ordination diagram with passively projected variables of diatom assemblages from various seasons and sites of the Savinja River. Only significant ($p < 0.05$) variables are included. **A** – distribution of the samples and environmental gradients, where sites are represented with numbers (S1, S2 and S3) and seasons/months with letters (S = September, D = December, M = March; **B** – distribution of diatom species present in at least three samples are shown.

Slika 3: Ordinacijski diagram diatomejskih združb z različnih vzorčnih mest v Savinji in iz različnih sezon, narejen na osnovi korespondenčne analize z odstranjenim trendom (DCA) s pasivno projiciranimi spremenljivkami. Vključene so samo statistično značilne ($p < 0.05$) spremenljivke. **A** - razporeditev vzorcev in okoljski gradienti, kjer so vzorčna mesta predstavljena s številkami (S1, S2 in S3) in sezone/meseci s črkami (S = september, D = december, M = marec); **B** - razporeditev vrst diatomej prisotnih v vsaj treh vzorcih:

Legenda / Legend: Ach bia - *Achnanthes biasolettiana,* Ach lan - *Achnanthes lanceolata*, Ach min - *Achnanthes minutissima,* Amp ova - *Amphora ovalis*, Amp ped - *Amphora pediculus,* Coc ped - *Cocconeis pediculus,* Coc pla - *Cocconeis placentula,* Cyc men - *Cyclotella meneghiniana*, Cym aff - *Cymbella affinis,* Cym min - *Cymbella minuta,* Cym sil - *Cymbella silesiaca,* Cym sin - *Cymbella sinuata,* Den kue - *Denticula kuetzingii*, Dia vul - *Diatoma vulgaris,* Fra cap - *Fragilaria capucina,* Fra uln - *Fragilaria ulna,* Fra pin - *Fragillaria pinnata*, Gom ang - *Gomphonema angustatum,* Gom ang - *Gomphonema angustum*, Gom aug - *Gomphonema augur*, Gom gra - *Gomphonema gracile*, Gom min - *Gomphonema minuta*, Gom oli - *Gomphonema olivaceum,* Gom par - *Gomphonema parvulum,* Mel var - *Melosira varians,* Nav ato - *Navicula atomus,* Nav cpr - *Navicula capitatoradiata*, Nav rei - *Navicula reinchardtiana*, Nav ven - *Navicula veneta*, Nav gre - *Navicula gregaria,* Nav lan - *Navicula lanceolata,* Nav men - *Navicula menisculus,* Nav tri - *Navicula tripunctata,* Nit aci - *Nitzschia acicularis*, Nit dis - *Nitzschia dissipata,* Nit fon - *Nitzschia fonticola,* Nit pal - *Nitzschia palea,* Rho abb - *Rhoicosphaenia abbreviata*, Sur bre - *Surirella brebissonii*.

Correlation between environmental parameters and diversity of diatoms

Diatom species richness was positively correlated ($p < 0.01$) with saturation of water with O_2 and marginally ($p < 0.1$) with month of sampling, temperature, submerged macrophytes (Tab. 4).

Shannon-Wiener diversity index was positively correlated ($p < 0.01$) with the trophic index, with saprobic index calculated on the basis of diatoms and other algae ($p < 0.05$), and marginally significant with the saprobic index calculated on the basis of diatoms (Tab. 4).

Table 4: Summary of correlation analysis between diatom species richness (Nr. of taxa) and Shannon-Wiener diversity index (H') and some of the environmental parameters; $p < 0.01$, * $p < 0.05$, + $p < 0.1$.
Tabela 4: Povzetek analize korelacij med vrstno pestrostjo diatomej (število taksonov), Shannon-Wienerjevim indeksom (H') in izbranimi okoljskimi parametri; ** $p < 0.01$, * $p < 0.05$, + $p < 0.1$.

	S-W diversity index	Nr. of taxa
month of sampling	-0.388	-0.639+
Temperature (°C)	0.169	0.630+
O_2 saturation (%)	0.391	0.811**
Saprobic index (based on diatoms)	0.614+	-0.159
Saprobic index (based on all algae)	0.678*	-0.132
Trophic index (based on diatoms)	0.827**	0.389
submerged macrophytes	0.344	0.625+

Discussion

Distribution and diversity of diatoms

Diatoms are the most abundant primary producers in the periphyton community, especially in streams with a stony substrate, with the highest share in spring and autumn periods. In our samples diatoms represented very high share ranging from 75 to 89% of the primary producers (Tab. 3). The highest number of the diatom species was found in September in two sites downstream the town Celje.

The similarity of the species composition of the epilithic diatom communities (Fig. 2) reflected rather the season than environmental factors. The left group uniting the summer samples represents a specific group of diatom assemblages. However, this group still has three quarters of species in common with other samples. The highest similarity within the samples was found among the winter samples collected in the beginning of December. On the other hand the samples collected in the end of March showed the highest heterogeneity in species composition (Fig. 2). Diatom assemblage from the upper site was more similar to winter samples that to other two samples from the same season, which had almost identical species composition having 95% species in common. Possible reason was greater exposure to disturbance in second and third location in spring what also reflected in higher share of pioneer species *Amphora pediculus* (see Rimet and Bouchez 2012). Three quarters of species were the same in all epilithic diatom assemblages that is much higher that is reported for river Kamiška Bistrica by Toman et al. (2014), where only about a third of taxa were common to all samples. A lower rate of similarity is a consequence of stronger gradient in natural factors such as water temperature, as well as the variety of human impacts on the river ecosystem (Toman et al. 2014).

For instance, species *Achnanthes biasolettiana,* was present in all samples (Tab. 3) and was also the most abundant in almost all (except one) communities. According to Hoffman et al. (2011) *Achnanthes biasolettiana* prefers Calcium-rich, oligotrophic to mesotrophic running waters on limestone bedrock of the alpine and pre-alpine regions where they often reach high abundance. This is also the case in our research area and in accordance with our results (Tab. 3). The species *Achnanthes biasolettiana* has the ability to firmly attach to the substratum in changeable water flows (Virtanen et al. 2011) and is capable of quick

re-colonization that could be the reasons for its constant presence and dominance in the epilithic community.

Certain diatoms occurred with relatively higher abundance in the summer like: *Navicula capitatoradiata, Cymbella affinis, Navicula menisculus, Cocconeis placentula. Species Navicula capitatoradiata* even reached the highest abundance in the summer sample from the lowest site (S3), the second in the S1 and third in S2 (Tab. 3). Characteristic species for summer assemblages was also *Cymbella affinis* which was the second or third most abundant diatom.

The most abundant winter diatoms with relative abundance ≥ 5% in the winter period, beside the common *Achnanthes biasolettiana* and *Nitzschia fonticola,* were: *Nitzschia dissipata* and *Navicula reichardtiana.* Two of them - *Achnanthes biasolettiana* and *Nitzschia dissipata* were reported as most abundant in winter sample by Toman et al. (2014).

Taxa characteristic for spring samples were *Gomphonema angustatum* and pioneer species *Amphora pediculus.* The latter reached 26% share in S3 indicating the hydrological disturbance of the site due to high water levels.

The most diverse genus was *Navicula* with 13 species found, which was in accordance with findings of Soltanpour-Gargari et al. (2011) and Toman et al. (2014).

The lowest value of the diversity index (2.50) was calculated from the spring sample at the first site (S1) due to the dominance of *Achnanthes biasolettiana* (57%), which has a pioneer character.

The greatest diversity index value (4.1) and the highest periphyton biomass were in the S3 winter sample, where nitrate concentration was high (6.6 mg/L). Moreover, the highest trophic index was calculated for this sample. Measurements showed the highest concentration of dissolved oxygen and pH, as well as coverage with filamentous algae at the site S3. We can assume from this that diversity of epilithic diatom community increases with increasing amount of nutrients and dissolved organic matter (DOM), meaning that the studied part of the Savinja River is in considerably good ecological status. This sample contained 33 diatom species and had the highest number of taxa (8) with relative abundance of at least 5%, i.e., *Achnanthes biasolettiana, Nitzschia fonticola, Amphora pediculus, Nitzschia dissipata, Diatoma vulgaris, Navicula reichardtiana, Navicula lanceolata, Navicula gregaria.* Significant correlations between Shannon-Wiener diversity index and trophic as well as saprobic index (Tab. 4) were calculated, meaning that the higher the concentration of nutrients and dissolved organic matter, the higher the diverstity of diatoms.

Water quality

Water quality was evaluated using the saprobic index. Upper site belongs to oligo-β-mesosaprobic status, characteristic of moderate organic loading.

Samples from the sites downstream of the town Celje were classified into 2nd class which is the β-mesosaprobic class (Tab. 3) and indicates low organic loading. The exception was the summer sample from the second location (S2), which was classified to 1st-2nd class and displayed slightly better condition. The most abundant taxon (except in S3_P) was *Achnanthes biasolettiana* which is characteristic for β-mesosaprobic state (Hoffman et al. 2011). Our results are similar to those obtained by Koren (2009), but on the base of trophic index we found out that there was less nutrient loading on the site S2 (downstream the town) than seven years ago. However, the trophic index reached the highest values in the lowest site (S3), which can be explained with suboptimal efficiency of tertiary purification processes and nutrient removal in the Celje wastewater treatment plant (WWTP).

Influence of environmental parameters on diatom species composition

Influences of environmental factors on the diatom community were tested using RDA. The significant variables explained almost 36% of diatom species composition (Tab. 5), which is lower than results published by Passy (2007), where the share reached 60%. A possible reason for mentioned differences could be much smaller size of research area. Soininen (2007) reports that the relative importance of environmental and spatial factors varies with study scale and distance effects are negligible over small scales.

The highest share of variability of the studied epilithic diatom community was explained by O_2

saturation and saprobic index calculated on the base of all algae.

Diatoms presented on the left side of Fig. 3B seemed to prefer water with higher O_2 saturation and were found in the summer samples from all three sites (Fig. 3A). At the time of summer sampling the water temperature was relatively high (21-23 °C), which contributed to the high saturation values. Diatoms distributed on the right side of the diagram (Fig. 3B) are found in the downstream sites S2 and S3 (Fig. 3A) and prefer water with high content of dissolved organic matter that also results in lower O_2 saturation.

Parameters that influence the structure of epilithic communities often have synergistic effects making the influence of a single parameter on the species composition, diversity and other community characteristics hard to define. Furthermore, there is also the influence of biotic interactions (grazing, competition) which are very difficult to quantify. The mentioned facts could be possible reasons for the differing conclusions on the importance of various factors in structuring epilithic diatom communities (Soininen 2007, Lange et al. 2011, Beltrami et al. 2012).

Correlation between environmental parameters and diversity of diatoms

Species diversity of certain community is determined by the diversity of habitats, amount and diversity of nutrients, water temperature, flow regime and stability of the ecosystem, which depends mainly on hydrological disturbances and pollution (Moss 2010, Zelnik 2015). Velghe et al. (2012) calculated negative correlation between diatom species richness and amount of phosphorous. In our case S-W diversity index (diatoms) was unexpectedly positively correlated ($p < 0.05$) with the saprobic index calculated on the basis of all algae, moreover highly significant ($p < 0.01$) correlation was calculated with trophic index (Tab. 4). These findings indicate low content of organic matter and nutrients, too low even to enable the thriving of species-rich epilithic diatom community, which needs higher amounts of nutrients and/or organic matter to support high number of species. Diatom species richness was positively correlated with O_2 saturation, which is expected to decrease with increasing content of DOM. Due to turbulent flow and fully insolated channel, water contains sufficient amount of oxygen even in reaches with higher content of organic matter.

Conclusion

Our results revealed considerable changes in diatom species composition during the year which exceeded the changes between the sites despite all of the human influences in urban landscape, which is not in accordance with the findings of (e.g. Passy 2007), who observed minor changes in diatom community composition between seasons. However, saprobic index as well as trophic index calculated for single samples showed the differences between the sampling sites. Saprobic index was lower in the site S1 which was classified into oligo- to β-mesosaprobic class, whereas S2 and S3 were classified into β-mesosaprobic class. Trophic index reached the highest values in the site S3, which can be explained with suboptimal efficiency of nutrient removal in the WWTP.

The highest share of variability of the epilithic diatom community was explained by oxygen saturation (35%) and saprobic index (33% of TVE), which are greatly influenced by human impacts. Both above mentioned parameters were also positively correlated with diversity of diatom communities, meaning that the increasing amount of organic matter and nutrients, respectively, increase the diversity of diatom community. Since the Water Framework Directive has been accepted by the European Commission and member states, the official monitoring system is more focused on the evaluation of ecosystem status than water quality status. Community of benthic diatoms is an essential element of the mentioned monitoring, as the diatoms respond to the amount of nutrients and dissolved organic matter. These characteristics define them as good indicators of an ecological status and should be used further in monitoring. We also confirm the importance of sampling date for the monitoring.

Povzetek

Spremljanje ekološkega stanja vodnih ekosistemov je bistvenega pomena za oceno človeškega vpliva na vodno okolje in vrednotenje učinkovitosti upravljanja z vodami. Slabšanje kakovosti vode je posledica industrijskih, komunalnih in kmetijskih virov, ki proizvajajo širok spekter polutantov. Ker so alge zelo dober pokazatelj sprememb v kakovosti vode, smo jih uporabili za določanje stanja izbranega odseka reke Savinje na območju Celja in njegove okolice.

Na približno 10 km dolgem odseku smo izbrali tri vzorčna mesta. Med mesecem septembrom 2011 in marcem 2012 smo izvedli tri vzorčenja, in sicer poletno, zimsko in spomladansko. Perifiton smo vzorčili po metodi pobiranja in strganja kamnov s skalpelom in ščetko. Ob vsakem vzorčenju smo spremljali tudi hidrološke, kemijske in fizikalne parametre. S Shannon-Wienerjevim indeksom smo ocenili diverziteto perifitonske združbe, s saprobnim indeksom organsko obremenjenost vodnega okolja, s trofičnim indeksom po Rottu (1999) pa smo spremljali obremenjenost s hranili. Glede na vrednosti saprobnega indeksa smo vzorčna mesta uvrstili v kakovostne razrede.

Naši rezultati so pokazali, da se je perifitonska združba spreminjala sezonsko ter med vzorčnimi mesti, hkrati pa so se spreminjali tudi abiotski dejavniki. V vseh vzorcih so prevladovale kremenaste alge, po deležu pa so jim sledile zelene alge in cianobakterije. Ugotovili smo, da je izbran odsek reke Savinje malo ali zmerno organsko obremenjen. Na podlagi vrednosti trofičnega indeksa smo ugotovili, da je s hranili najbolj obremenjeno vzorčno mesto S3, kar pripisujemo vplivu iztoka iz centralne čistilne naprave Celje in suboptimalni učinkovitosti terciarnega čiščenja. S klastrsko analizo narejeno na podlagi Sørensenovega indeksa podobnosti, kjer upoštevamo samo prisotnost vrst, ne pa številčnosti smo potrdili našo hipotezo, da bodo razlike v vrstni sestavi in diverziteti perifitonske združbe večje med sezonami kot pa med posameznimi vzorčnimi mesti. Rezultati naše raziskave so bili zelo podobni rezultatom, ki jih je dobil Koren (2009), le na podlagi trofičnega indeksa smo ugotovili, da se je stanje obremenjenosti s hranili na mestu S2 izboljšalo. Z nizom redundančnih analiz (RDA) smo ugotovili, da ima na taksonomsko sestavo združbe diatomej statistično značilen vpliv nasičenost s kisikom in saprobni indeks izračunan s pomočjo vseh alg. Pri analizi korelacij med diverziteto in ostalimi parametri, smo izračunali pozitivno korelacijo med Shanon-Wienerjevim indeksom ter trofičnim in saprobnim indeksom, medtem ko je bilo število taksonov v statistično značilni povezavi z nasičenostjo s kisikom. Iz dobljenih rezultatov sklepamo, da na perifitonsko združbo v reki Savinji na preučevanem odseku mesto Celje kljub vsemu nima velikega vpliva in da odsek ni bil tako onesnažen kot smo pričakovali. Potrdimo lahko tudi pomembnost datuma vzorčenja v monitoringu, ki ga moramo upoštevati za ustrezno vrednotenje ekološkega stanja in primerjavo z drugimi območji in leti.

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Architectural adaptation in *Myriophyllum spicatum* L. in a lotic environment: is it caused by current velocity?

Ali so spremembe rastne oblike pri vrsti *Myriophyllum spicatum* L. v tekoči vodi posledica hitrosti vodnega toka?

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Abstract: Little information is available for aquatic plants regarding their architectural response to strong environmental drivers like water flow. We examined architectural variability in *Myriophyllum spicatum* L. in the short terminal section of a small canal earlier used for inland navigation. This stretch is characterised by decreasing water depth towards a final spill-over construction, which causes increasing current velocity. Visibly different plant beds had developed at three sampling sites, located between the upstream end of the study reach and the end at the spill-over. This situation bears some resemblance to an experimental flume due to regulated water flow and constant discharge, yet with aquatic plant beds still located in their permanent environment during the whole year. Following this precondition our hypothesis envisaged a close relationship between current velocity and realised plant architecture. Current velocity was measured with an electronic vane device, and representative architectural features of plants were recorded from plant samples at the sites of different flow. Characteristic and significant variation in the architecture of *M. spicatum* was demonstrated at the sites of different current impact. Regarding other environmental parameters like sediment composition, water chemistry or the effect of shading no influence seems likely expected, as samples were collected across the canal width at each site. The mean values of all architectural parameters of *M. spicatum* follow the same trend with high significance, regarding the increase in plant length, branching, and the overall dimension of the plant beds, which is in close relationship to the current velocity at the sampling sites. The few other records available in literature cited in this paper point into the same direction, but these studies were also carried out in the field. In our opinion the clear results may not comply with a final and experimentally generalised relationship between aquatic plant architecture and water flow. But our contribution offers some statistical proof that our hypothesis is not too far from explaining the effects of current velocity, which is one of the main environmental parameters defining aquatic plant growth.

Keywords: aquatic macrophytes, water flow velocity, architectural adaptation, *Myriophyllum spicatum*

Izvleček: O spremembah rastne oblike vodnih rastlin zaradi vodnega toka je malo znanega. Proučili smo variabilnost rastne oblike klasastega rmanca (*Myriophyllum spicatum* L). v končnem delu kanala, ki se je včasih uporabljal za plovbo. Na 70 m dolgem odseku se globina vode nižja, kar vpliva na povečanje hitrosti vodnega toka. Na treh izbranih vzorčnih mestih vzdolž raziskovanega odseka se sestoji klasastega rmanca razlikujejo že po izgledu. Zato smo predvidevali, da so spremembe v rastni obliki posledica sprememb v hitrosti vodnega toka. Na različnih vzorčnih mestih smo z elektronskim merilcem izmerili hitrost vodnega toka ter analizirali značilnosti rastne oblike vzorcev rastlin. Dokazali smo pomembne razlike v razrasti in velikosti vrste *M. spicatum* na različnih lokacijah. Drugi okoljski dejavniki, kot so sestava sedimenta, kemizem vode in učinek senčenja, niso imeli vpliva na rast rastlin, saj so bili vzorci na vseh lokacijah odvzeti po vsej širini kanala. Povprečne vrednosti vseh merjenih parametrov kot so povečanje dolžine rastlin, stopnja razvejanosti in skupna velikost sestoja, sledijo istemu vzorcu, ki je v tesni povezavi s hitrostjo vodnega toka na območjih vzorčenja. Menimo, da kljub jasnim razlikam v razraslosti rastlin ne moremo v celoti potrditi povezave med rastno obliko in hitrostjo vodnega toka.

Ključne besede: vodni makrofiti, hitrost vodnega toka, spremembe rastne oblike, *Myriophyllum spicatum*

Introduction

The architecture of a plant describes the spatial organisation of its structures in three dimensions, which is under genetic control (Reinhardt and Kuhlemeier 2002), often producing a characteristic branching pattern most easily observed even by non-experts. But exogenous constraints (Barthélémy and Caraglio 2007) determine the ontogenetic result of plant development leading to architectural variation (temperature stress: Bridge et al. 2013, planting density: Costes et al. 2012, shading: Winona 2015, Ford 2014, McKenzie-Gopsill et al. 2016)

All the examples cited above deal with terrestrial plants. Much less information is available for aquatic plant species. This is especially true when looking at the effects of water flow on aquatic plant architecture.

One of the earliest sources is information provided in Arber's (1920) study on aquatic plants. The central focus is given to leaf types regarding heterophylly, land and water forms, entire, dissected, fenestrated, cylindrical or ribbon-shaped leaves, as well as several other aspects of leaf composition. Some relation to architectural aspects is found in a comment on submerged leaves and side branches, where the development of 'juvenile-like' leaves was detected. Butcher (1933) covered the influence of water flow regarding some aspects of water chemistry and substrate (ibid. p.63), and described seasonal and inter-annual changes in species composition and cover (ibid. p.80, R. Itchen, and p.81, R. Lark), but no mention is made of phenotypic plasticity related to the water current.

In Sculthorpe's (1967) book on the biology of vascular aquatic plants the influence of water current is mentioned, with a focus on anatomical features of macrophyte leaves. But a short note refers to leaf segments of *Myriophyllum* (no species cited) being '*shorter and firmer in flowing water*' (ibid., p.109). Phenotypic variation was detected in aquatic *Ranunculus* species and in the genus *Potamogeton*, where current speed was related to variable leaf form and anatomy (ibid., p. 221/222). A perpetuated juvenile status of leaves was observed in some species of *Alisma*, *Potamogeton*, *Sagittaria* and *Sparganium* (ibid., p.232) under the impact of swift current.

Abundant information on aquatic plants and their response to a range of environmental conditions is provided in the books of Haslam (1987, 2006, 2013). The relation of aquatic macrophytes and flow conditions is covered in many diagrams, tables and drawings showing typical groups and

situations, especially those of weed beds in rivers of different character (e.g. geological background, slope, discharge, etc.). Specifically regarding *Myriophyllum spicatum* L., this species was found to be most abundant in moderate flow, but showed a marked ability to withstand fast flow as well. Among several species recorded to tolerate spates *M. spicatum* was cited as not being uprooted as easily as others. Unfortunately no numerical information on current speed is provided, and no reference to details of plant architecture is made. However, in her recent book on river plants Haslam (2013, p.71 f.) adds some architectural information regarding greater length of internodes and more elongated plants in older parts of rheophilic (i.e. current-affine) *Ranunculus* beds, and growing in greater water depth.

Gessner (1955) remains a useful source of information on the relationship between current velocity and architectural parameters for *Nuphar lutea* (L.) Sm. and *Berula erecta* (Huds.) Cov. (name used by Gessner, ibid. p.303: *B. angustifolia*).

Regarding specific information on *M. spicatum,* Whitton (1975, p.111) mentions that this species may produce smaller plants in faster flow. Miler et al. (2014) compared the biomechanical properties and morphological characteristics of lake and river plants, but *M. spicatum* samples were taken from still waters (lakes, ponds) as opposed to *M. alterniflorum* DC., which was sampled from rivers. These results relate to the architectural data reported by Wegleiter (1990), who studied *M. spicatum* in lakes of South-Tyrol (Italy), and do not correspond to our samples collected from sites in running water.

The information cited above shows that there is little quantitative data on the architecture of aquatic plants in relation to water flow in the literature. Our study aims at testing the hypothesis that variation in architectural features of *Myriophyllum spicatum* L., may be influenced by current velocity.

Sampling Site

The 'Wiener Neustädter Kanal' (Wiener Neustädter Canal, WNC), was selected as the study location, where easily visible differences in form and size of *Myriophyllum spicatum* L. beds attracted our interest.

Opened in 1803, this canal, originally 61 km long, connected Wiener Neustadt (Federal Province of Lower Austria) and the Centre of Vienna as the only part of a commercial transport canal system that was originally planned to reach Trieste/Italy (Lange 2003).

The canal receives its water from a stream that originates in alpine catchments, and the discharge is controlled by a main weir at Wiener Neustadt, where the canal is diverted from the source stream. The regulated flow and minor changes in discharge bear resemblance to an experimental flume, regarding other environmental features (nutrients, sediment type etc.), too.

Due to its rather constant flow the WNC provides favourable conditions for macrophyte development, which causes maintenance activities including some mechanical management of submersed aquatic species, usually located in the upper and middle reaches.

By 1878 the navigation was shut down due to competition from railway freight transport (Bruckmüller et al. 2004). In the early 1970s the canal was terminated at the town of Biedermannsdorf, truncating the water course to a length of 36km. Environmental purposes and leisure dominate the present use, but eight small hydropower plants replace former lock sites. The water of the canal spills into a small regulated river, the Mödlingbach (Fig. 1).

The study site was situated in the terminal reach of the WNC (Fig. 1). Three sampling sites showing easily visible differences in plant bed structure, and characterised by different current velocities, were selected in a stretch of less than 70 m.

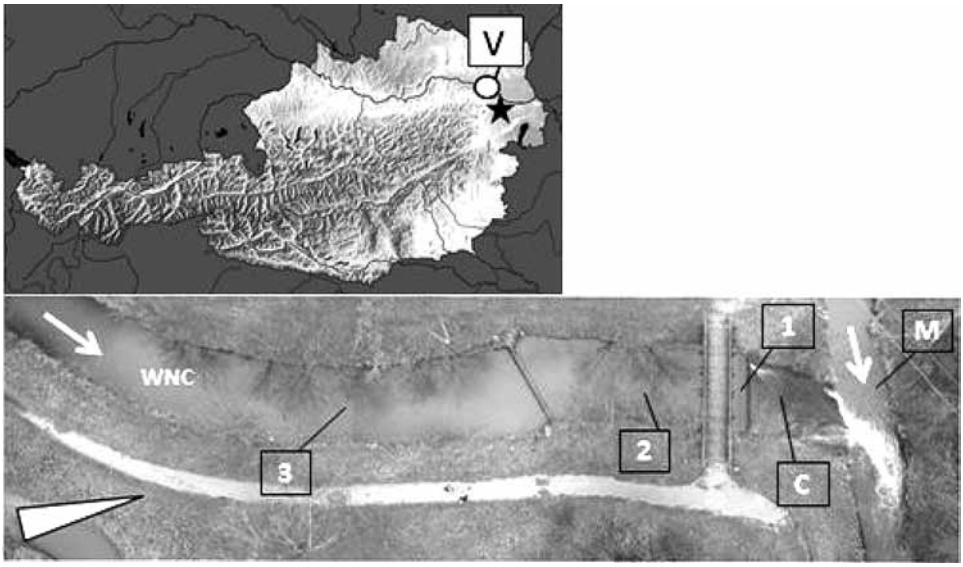

Figure 1: Sampling site.
Upper Panel: V – Location of Vienna; Five-pointed star: location of the study site. Map source: Creative Commons Attribution 3.0 Licence; http://www.ginkgomaps.com).
Lower panel: Sampling Sites 1, 2, 3. M: Mödlingbach, receiving the WNC. C: steep chute connecting the WRC with Mödlingbach. Prior to the termination at Biedermannsdorf an aqueduct had led the canal across this stream bed. The upper rim of the chute is a level concrete beam of c. 0.4 m width, crossing the whole course of the WNC, which protects the upper end of the chute against erosion. Arrows indicate flow direction. Triangle: north arrow. All sites are open to the South, ensuring little effect of shading. Image: FDG Austria, DI Norbert Exler, PhD © 2016. All plant samples were collected from the upstream end of the plant beds, where water flow exerts its full force.

Slika 1: Mesto raziskav.
Zgornja slika: V - Mesto Dunaj; zvezica: mesto vzorčenja. Vir Map: Creative Commons 3.0 Dovoljenje; http://www.ginkgomaps.com).
Spodnja slika: Mesta vzorčenja 1, 2, 3 M: Mödlingbach, ki prejemajo WNC. C: strm žleb, ki povezuje WRC s vodotokom Mödlingbach. V kraju Biedermannsdorf je v preteklosti akvadukt vodil kanal preko struge. Zgornji rob žleba je 0,4 m širok. Puščice kažejo smer toka. Trikotnik: Severna usmerjenost. Vsa mesta vzorčenja so orientirana južno, kar zagotavlja odsotnost senčenja. Vir: FDG Avstrija, DI Norbert Exler, PhD © 2016. Vsi vzorci rastlin so bili nabrani gorvodno, kjer vodni tok doseže svoj polno hitrost.

Site 1 is situated at the pedestrian bridge at the upper end of the chute. The concrete beam across the whole canal width provides a level base for current measurement (Fig. 1). At the vertical upstream face of the beam the bottom of the canal rises to a depth of just 0.11 m, due to sediment accumulation at this barrier, forcing the water across its top. This results in an increase in current velocity over the top of the beam (mean current velocity: 1.44 m s^{-1}, S.D. 0.20; n = 15 across the canal; specification of the measuring device: see below) before the water starts rushing down the chute. At this site 6 complete plants of *M. spicatum* were sampled for architectural measurement.

Site 2 is located in a short reach of quite abundant plant growth in a section between 8 to 15 m upstream of Site 1. This part of the canal is characterised by 0.30 m mean water depth (S.D. 0.08) and a mean current velocity of 0.92 m s^{-1} (S.D. 0.26). At this site 4 samples were collected.

Site 3 is located in a reach 45 to 65 m upstream of Site 1 where very large plant stands had developed. The characteristic features of this site are 0.32 m mean water depth (S.D. 0.14) and a

mean current velocity of 0.39 ms^{-1} (S.D. 0.05) in the peripherals of the plant stands. At this site 5 samples of *M. spicatum* were collected, growing closer to the right bank.

Methods

Measurement of current velocity

Current velocities were measured at the sampling sites with an electronic flow meter (Type µP – ASDI; vane diameter: 16 mm; Höntzsch GmbH, Waiblingen, Germany). The small diameter of the vane allowed for setting the probe close to either the canal surface or bottom or very close to the plant stands, respectively.

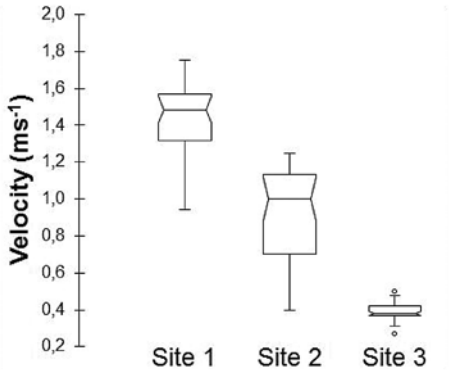

Figure 2: Current velocities across the canal at the three sampling sites.
Box: Median; 25 and 75% Quartile; Whiskers: Min/Max (Significance: Kruskal-Wallis-Test: $p < 0.0001$ for all combinations of the three Sites); Circles: outliers.

Slika 2: Hitrosti vodnega toka na treh mestih vzorčenja.
Okvir: Mediana; 25 in 75 % četrtine; Brki: Min / Max (Značilnost razlik: Kruskal-Wallis-test: p <0,0001 za vse kombinacije); krožci: izstopajoči podatki.

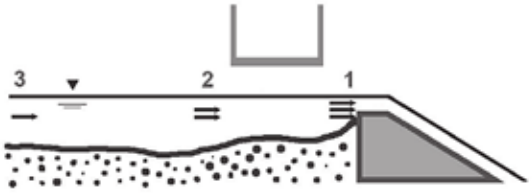

Figure 3: Location of sampling sites, concrete bar and chute (schematic).
The water level is indicated by the triangle. Arrows indicate increasing current velocity. The concrete bar and the chute are located to the right. Actual water depth on top of the bar: 0.17 m. Position of water level, width of the concrete bar top and canal bottom (depth related to the middle of the water body) are approximated to the real situation, including the pedestrian bridge. The average gradient of the chute is 18.1%.

Slika 3: Lokacija mest vzorčevanja (shema).
Vodostaj je označen s trikotnikom. Puščice kažejo povečanje hitrosti toka. Betonski preliv in žleb se nahajata na desni. Globina vode na vrh: 0,17 m. Položaj vodostaja, širina betonskega preliva ter zgornji in spodnji del kanala (globina je je sorazmerna s sredino vodnega telesa) je prikaz dejanskega stanja, vključno z mostom za pešce. Povprečni naklon žleba je 18,1 %.

Selection of the tested species

Due to the regulated conditions in the WNC neither water chemistry nor discharge show high variability, except for periods of closure of canal parts for repair and maintenance, or during aquatic plant removal, which is not conducted very frequently and never over the complete canal length at a time. Such conditions favour macrophyte growth, but only a few species have established in this artificial water body on a rather permanent basis.

The first macrophyte surveys covering the full length of the WNC were carried out in 1992 and 1994 (Janauer, unpublished). In 1992 the occurrence of *Elodea canadensis* Michx., *M. spicatum, Potamogeton crispus* L., *P. pectinatus* L. [syn. *Stuckenia pectinata* (L.) Börner], *P. perfoliatus* L., and *Ranunculus trichophyllus* Chaix was recorded. In 1994 *E. canadensis*, *M. spicatum*, *P. pectinatus*, *P. perfoliatus*, *R. trichophyllus*, and *Zannichellia palustris* L. were detected. In Gasteiner's survey of 1999 (Diploma Thesis, 2001), *M. spicatum* was the macrophyte species with highest frequency regarding the number of survey units over many years (Frequency: 1992 – 35.1; 1994 – 39.2; 1999 – 48.9).

The samples taken at the three sites were the complete plants at Site 1, but since Site 2 and 3 were subject to slower water flow, larger beds of plants were present. From these beds, individual fully grown plants were collected, which consisted of a central axis, rooted at some length, and the respective branches of different order.

The samples were transported in pails of water to a nearby location, where the material was spread out on a large table covered with plastic film. Location and hierarchy of branching (Bornkamm et al. 1991) was recorded with a metal tape measure, to assess the architectural structure of each plant sampled. The axes and branches of terrestrial plants display a stable structure which can be described mathematically (Godin et al. 1999). Submersed aquatic plant stands – the '*waving plants of the river*' (Haslam 2013) – move constantly following the flow and turbulence of the water, and a fixed three dimensional geometry cannot be defined *in situ*. We focused on several parameters of the branching pattern as an indicator of variation in plant architecture related to water flow velocity.

Statistics

Based on measuring and categorising individual features of the plant architecture the data were analysed with SPSS 15.0 for WINDOWS. Normal distribution was tested by applying Kolmogorov-Smirnov, or Shapiro-Wilks tests, respectively. ANOVA was applied to normally-distributed data together with Duncan Post-Hoc mean separation test, testing for significance between means. For testing the significance of non-normal data Kruskal-Wallis test was used, followed by the Mann-Whitney-U-Test to discriminate between significantly different categories. Methods followed Untersteiner (2007, pp. 133-182). We used the following references as a nomenclature sources: Casper and Krausch (1980), Casper and Krausch (1981) and The Plant List, 2010. Version 1. http://www.theplantlist.org/.

Results

Typical examples of *Myriophyllum spicatum* architecture are shown below, which are characteristic for the three sampling sites and the effect of current velocity (Fig. 4).

Characteristic examples of growth form at Sites 1-3

The following passage shows the relationship between different architectural characteristics assessed for *Myriophyllum spicatum* at the three sampling sites. Significance of differences is shown in Table 2.

Length of the main stem and Total length of all axes

Length of the main stem regards BO 1 axes (BO = branching order), which covers all sampled individuals at a Sampling Site. Total length of all axes combines main stem length plus all branches (BO 1-5)

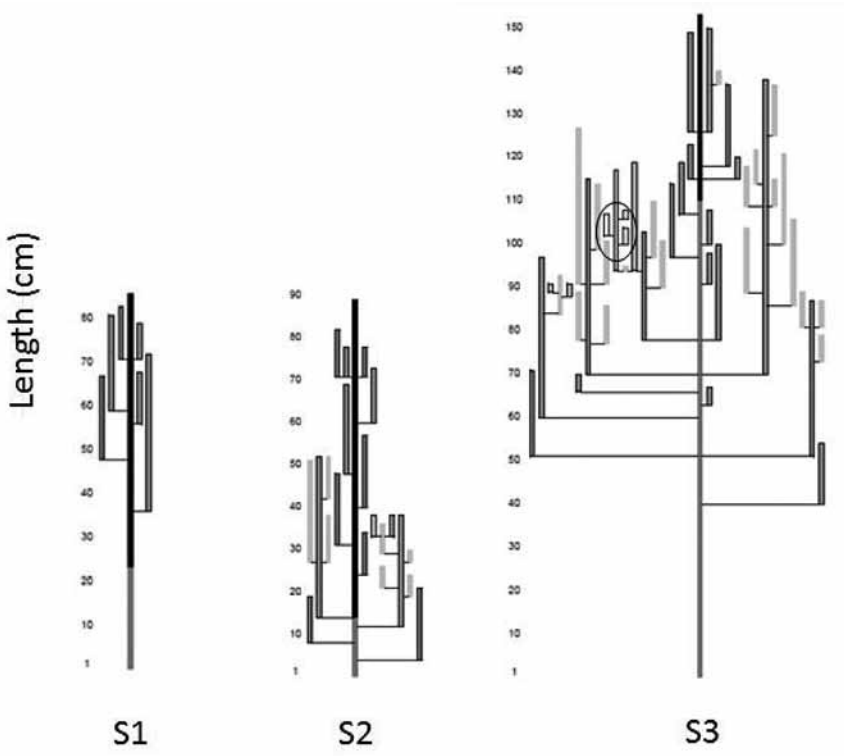

Figure 4: *Myriophyllum spicatum* - Examples of branching at Sites 1 – 3
BO = branching order. Grey: BO 1 – main stem, rooted; Black: BO 1 – main stem, floating; Dark Grey, black outline: BO 2; Light Grey, no outline: BO 3; White, black outline: BO 4; Additional branches on BO 4 axis: BO 5 (marked by oval shape).
S1: High current velocity impact. Few branches developed. S2: Intermediate current velocity impact. The central bulk of the bed is composed of BO2 and BO3. S3: Lower current velocity impact. Note the extremely short BO 3 axis between the two BO 4 axes. The left one developed three short BO 5 branches.

Slika 4: *Myriophyllum spicatum* - Primeri razvejanosti na lokacijah 1 - 3
BO = stopnja razvejanosti, temno sivo: BO 1 - glavno steblo - ukoreninjeno; črno: BO 1 - glavno steblo, plavajoče; temno sivo, črno obrobljeno: BO 2; svetlo sivo, neobkroženo: BO 3; belo, črno obrobljeno: BO 4; dodatne veje na BO 4. osi: BO 5 (obkroženo). S1: velik vpliv hitrosti toka. Razvejanost majhna. S2: srednji vpliv hitrosti toka. Osrednji del sestoja je sestavljen iz BO 2 in BO 3. S3: majhen vpliv hitrosti toka. Izjemno kratka BO 3 os med dvema BO 4 osema. Na levi so razvite tri kratke BO 5 veje.

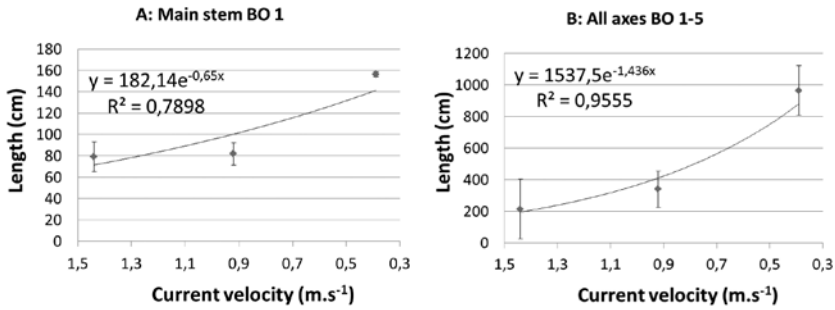

Figure 5: **(A):** Main stem (BO 1) – Length **(B)**: All axes (BO 1 – 5) - Length
Regarding all Figures: $n_{Site\ 1}$: 6, $n_{Site\ 2}$: 4, $n_{Site\ 3}$: 3. Whiskers: S.D., $p = 0.05$; Significance: see Table 1. Regression line: exponential.

Slika 5: **(A):** Glavno steblo (BO 1) – dolžina **(B)**: vse osi (BO 1 – 5) - dolžina
($n_{Site\ 1} = 6$, $n_{Site\ 2} = 4$, $n_{Site\ 3} = 3$). Brki: S.D., p = 0,05; Značilne razlike: glej Tabelo 1. Regresijska premica: eksponentna.

Figure 5A shows that the main stem (BO 1) of sampled individuals increases in length with increasing distance from the downstream end of the canal, which is equivalent to decreasing current velocity. The main stems of Site 1 and 2 are very similar regarding the mean, but S.D. is smaller at Site 2. The length of main stems (BO 1) at Site 3 is significantly longer than that of Site 2, which is not significantly different from Site 1.

In Figure 5B the means of Site 1 and Site 2 are still closely related, but Site 3 is clearly different from the other sites.

Total number of branches (BO 2 – 5)

The development of branches is a conspicuously different feature of the plants sampled at the three sampling sites, related to the change from fast to slower water flow.

The distances between the means of Sites 1 and 2 are larger for branches than for main stems but the S.D. values are overlapping. The number of branches at Site 3 is significantly higher than that of Site 1 and 2.

During the examination of the sampled material it became obvious that some of the branches (BO 2 – 5) showed no furcation, i.e. no further branching, representing terminal axes, often of considerable length. In contrast other branches of lower, as well as higher BO level had developed additional branches. These two types of branches were analysed separately.

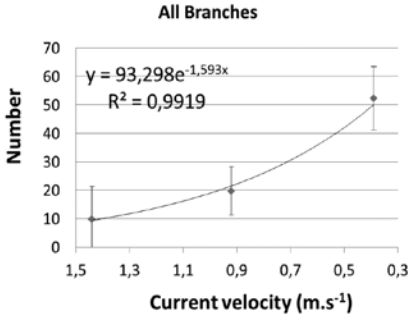

Figure 6: Total number of branches (BO 2-5).
Slika 6: Skupno število stranskih poganjkov (BO 5/2).

Branches without furcation – terminal axes

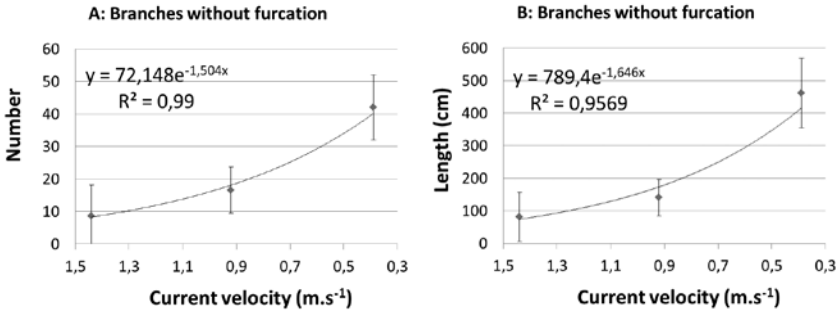

Figure 7: Branches (BO 2 – 5) without furcation – (**A**) number, (**B**) length.
Slika 7: Stranski poganjki (BO 2 – 5) brez razvejanja – (**A**) število, (**B**) dolžina.

The number of BO 2 – 5 terminal axes (Fig. 7A and B) increased from the location with fastest flow towards the one with the slowest. Sites 1 and 2 did not show significant difference, but the samples at Site 3 were significantly separated regarding both the number and the length of these branches (Table 2).

Branches (BO 2 – 4 -type) with furcation

When viewing individual beds of *M. spicatum* in the WNC from the bank the intensive development of bed size was instantly recognised for locations with decreasing flow velocity. This feature is caused by additional branching of BO 2 – 4 -type branches. The examples of characteristic individual plants given in Figure 8 clearly show that effect.

Branches of BO 2 – 4 -type producing branches of higher order (BO 3 – 5) define, in part, the overall size of the beds of *M. spicatum*. The means in Figure 8 are clearly separated and indicate the intensive development of additional axes, which increase the visual bulk of beds in locations with lower impact of current velocity. Despite the relatively high variation in branching of individual plants sampled from the sites statistical analysis (Table 2) reveals some significant differences.

Significance of the results shown in Figures 5 - 8 is presented in Table 2.

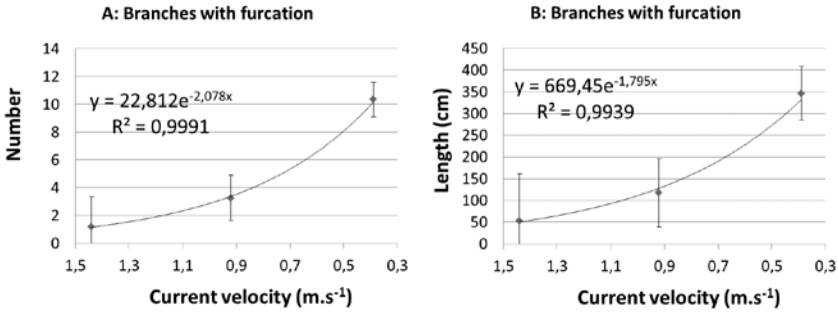

Figure 8: Branches (BO 2 – 4) with furcation – (**A**) number, (**B**) length. Consider the different scale of the ordinate, as compared to Fig. 7.
Slika 8: Stranski poganjki (BO 2-5) z razvejanjem – (**A**) število, (**B**) dolžina. Ordinate se razlikujejo v primerjavi s sliko 7.

Table 2: Relationship of architectural parameters of *Myriophyllum spicatum*
Sampling Sites 1, 2, 3 (means); Square brackets: Figure number. t. a.*: terminal axes without furcation. Different letters indicate significant difference (p = 0.05).

Tabela 2: Razlike v razraslosti rastlin vrste *Myriophyllum spicatum* na različnih mestih vzorčenja. Mesta vzorčenja: 1, 2, 3 (srednje vrednosti); Oglati oklepaji: Številka slike. t. a. *. os brez razvejanja. Različne črke kažejo pomembne razlike (p = 0,05).

Architectural parameters	Differences among sampling sites		
Main stem (BO 1) – length [5A]	1[a]	2[a]	3[b]
All axes (BO 1 – 5) – cumulated length [5B]	1[a]	2[a]	3[b]
Branches (BO 2 – 5) – total number [6]	1[a]	2[a]	3[b]
Branches (BO 2 – 5) / t. a.* – number [7A]	1[a]	2[a]	3[b]
Branches (BO 2 – 5) / t. a.*– length [7B]	1[a]	2[a]	3[b]
Branches (BO 2 – 4) / furcate – number [8A]	1[a]	2[a]	3[b]
Branches (BO 2 – 4) / furcate – length [8B]	1[a]	2[a]	3[b]

Discussion

Myriophyllum spicatum L. is a widespread aquatic plant, with a potential to inhabit still, as well as running water bodies. When comparing the architectural features of *M. spicatum* in lakes (Wegleiter 1990) and that recorded in our survey of the Wiener Neustädter Canal (WNC), the phenotypic differences in *M. spicatum* had become evident. This triggered our interest in the final reach of the WNC, where flow conditions change markedly over a rather short distance and phenotypic plasticity was apparent in the size of the plant beds, as well as in the architectural variation we had studied then.

In general all the parameters tested in this study reacted quite similar regarding the architectural features, especially when comparing the mean values at each sampling site. This fact is obviously expressed by the regression lines in Figures 5 – 8. But tests revealed that plant features were not significantly different for Site 1 and Site 2 (Tab. 2). Site 3, in contrast, was clearly different from the other two sampling sites. As the difference is highly significant in current velocity at all three sites one can reflect on the different results recorded for the architectural parameters at Sites 1 and 2. Since different individuals were sampled across the width of the canal this caused a greater standard deviation and no significance ($p = 0.05$) when compared to that of the current velocity. On the other hand this stronger scatter in plant architecture may provide different micro-scale habitats for organisms associated with plant beds.

A comparison of our data with results from a still water can illustrate possible differences in two architectural features of *M. spicatum*: Length of the main stem (BO 1-type) and the Length of Internodes (Table 3; both parameters recommended by Lehmann et al., 1997; results reported below: Wegleiter, 1990, pp. 77-79, Montiggler See, South Tyrol, Italy).

Table 3: *Myriophyllum spicatum* - Comparison of architectural parameters in still (Montiggler Lake) and running water (WNC)
Range of main stem length and mean internode length
Tabela 3: *Myriophyllum spicatum* - primerjava arhitekturnih parametrov med stoječo (Montiggler jezera) in tekočo vodo (WNC)
Obseg dolžine glavnega stebla in povprečna dolžina internodija

	Montiggler Lake		Wiener Neustädter Canal (this study)		
Date	Main stem[1] (m)	Internodes[2] (cm)	Sites	Main stem[2] (m)	Internodes[2] (cm)
15.7.1986	0.75 - 0.9	2.17	1	0.79	2.36
12.8.1986	1.10 - 1.35	2.24	2	0.81	2.42
12.9.1986	1.75 - 1.90	2.78	3	1.56	6.17
3.10.1986	2.30 - 2.40	3.38			

[1] range, [2]mean

The mean length of the main stem of the WNC samples at Site 1 and 2 is about equivalent to that of early summer samples from the lake. Main stems of Site 3, the longest in the WNC, are considerably shorter than those in the lake during the final summer period. On the other hand, under flow stress in the WNC the length of internodes is greater than that in the lake. These examples indicate substantial differences in architectural adaptation to still as opposed to running water conditions, in *M. spicatum*.

One of the most conspicuous features of the reaction of *M. spicatum* beds to decreasing flow velocity in the WNC is the increase in general bulk, which shows the relationship between 'biomass density and plant architecture', according to Duarte and Roff (1991), who also studied *M. spicatum*. When analysing the causes for that phenomenon the types of branches provide answers for the samples collected at the WNC. Figure 4 provides graphic examples of this phenomenon and Figure 6 shows the total number of branches recorded (Branching Order, BO 2 – 5). Two groups showing 'bulk production' were easily differentiated as part of the BO 2 – 5 sample. The first group is characterised by branches directly emerging from the main axis (BO 2), as well as from branches of higher order (BO 3 – 5) , which failed in producing additional axes and ended up as axes without furcation or 'terminal axes' (Fig. 7A, 7B). The second group comprises axes of BO 2 to BO 4-type, which produced additional branches (Fig. 8A, 8B). The effect of slower water flow effectively results in additional bulk production. This is in full accord with the findings of Chambers et al. (1991), who showed that even a modest increase in water flow decreased the biomass and abundance of aquatic weed beds, providing proof with experimental transplants. Hrivnák et al. (2013) also listed flow regime prior to fine substrate as the most important driver for species and environmental conditions relationship.

Searching for much earlier information on flow impact affecting aquatic plants Gessner's (1955) examples are in full support with our results, as shown in the following citation on two macrophytes of different growth form. Though not listing *M. spicatum* among rheopbiont [*associated with faster flow*] aquatic species (70 – 120 cm.sec^{-1}), nor those occurring in more moderate flow (13 – 70 cm.sec^{-1}) either, this author reports a general restraint on stem, internode and leaf length by faster flow (ibid. p.302), indicating '*Myriophyllum'* without defining the species (Tab. 4, after Ruess, probably by personal communication to Gessner, as Ruess is not listed in Gessner's references).

Table 4: Attenuation of plant parameters in *Myriophyllum sp.* related to water flow
Catagories and numbering format according to the source. * no record.
Tabela 4: Spremembe parametrov rastlin pri vrsti *Myriophyllum sp.* v povezavi s pretokom vode. Kategorije in vrednosti glede na vir. * ni podatka.

Water flow (cm s^{-1})	Length of stem (cm)	Internode length (cm)	Leaf length (cm)
0	-*	3 - 6	2.5
20	100	2 - 3	2
70	50	1.5 - 2.0	1.2-1.5

Another example, quite identically referred to in several textbooks, is found in the same source (Gessner 1955, p.305) for petiole length and leave size of *Nuphar lutea* (L.) Sm., regarding the high flow impact at the upstream end of the plant stand (described as 'luv', no numerical values provided), and sites with lower flow within the same plant stand drawn by Gessner (after Ruess, personal communication). Despite missing a differentiation between submersed and floating leaves of this species the essence of the contribution is clear (Tab.5).

In her most recent book Haslam (2013, p.24) referred to the reduction of hydraulic resistance by shorter shoot length and a lack of branching in *Ranunculus peltatus* Schrank. Haslam (2013, p.188) also reports about *R. fluitans* Lam. showing shoots 'barely branched' while being affected by 'torrential' flow, but adapting to a 'well branched' status with 'much longer shoots' after installation of a sluice upstream which reduced flow. This example compares well with our experience in the WNC, where fast flow resulted in barely branched plants at Site 1 and much longer and well-branched plants at Site 3. But, following the same source, when torrential flow was restored, the beds ('clumps' *sensu* Haslam) returned to their original 'barely branched' form over some time, indicating the phenotypic plasticity of aquatic plants.

Our own data on the impact of different flow velocities, as well as Haslam's example of development, and successive reduction in plant bulk, indicate the importance, and capacity, of aquatic macrophytes to falling back on architectural variation for sustaining survival under variable environmental conditions. According to Grosfeld et al. (1999) such effects may help to distinguish ontogenetic variation from environmental plasticity, as shown by their studies on *Araucaria araucana* (Molina) K.Koch. This aspect was also discussed by Dingkuhn et al. (2005), seeking solutions with simulation models. Stein and Boyer (2006) also proposed ontogenetic drivers like environmental boundary conditions determining the final architecture realised by an individual plant. This should, with respect to the phylogenetic potential in general, enable a plant to adapt to variable conditions of habitat, which are the drivers behind the effects of flow on *M. spicatum* shown in our study.

Table 5: Impact of flow on architectural features of *Nuphar lutea* (L.) Sm.
Numbering format according to the source.
Tabela 5: Vpliv toka na arhitekturne značilnosti vrste *Nuphar lutea* (L.) Sm.
Vrednosti so podane kot v viru.

Flow impact	Petiole length*	Leaf length*	Leaf width*
'luv'= upstream end	5	4.5	5
within stand	25 - 30	9.5	14

* in cm

On the same basis Wolfe and Mazer (2005) argued that the responses to environmental heterogeneity are related to fitness against variable habitat conditions. Following the same line of arguments, but referring to examples of terrestrial plants, Barthelemy and Caraglio (2007) state that genetic determination of architectural features of a species is affected by the environment 'only under extreme ecological conditions' (e.g. determining the crown physiognomy of trees, ibid. p.387 and p. 390), and the final expression of structure and architectural position of a particular plant element 'may be modulated by environmental or technical factors' (ibid. p. 396). According to this consideration substantial changes in environmental conditions and subsequent architectural adaptation are linked to enable the survival of plant species under pressure of mechanical forces, as described by Puijalon et al. (2011) for wind in terrestrial habitats. Barthelemy and Caraglio (2007) cited 'extreme ecological conditions' as causing variation in plant architecture: when comparing the different density of air and water it does not seem unlikely to expect strong effects of water flow at higher current velocities on aquatic plant architecture. At least currents recorded at the Sites 1 and 2 in this study will exclude the occurrence of many macrophyte species common in the majority of running waters, and phenotypic plasticity (Dingkuhn et al. 2005) may support *M. spicatum* in sustaining current impact in our test sites.

Summary

Information focusing on architectural features of aquatic macrophytes in running water has been rather scarce, which triggered the authors' interest on *Myriophyllum spicatum* L. growing in a part of the Wiener Neustädter Canal (WNC), which provides 'quasi-experimental' conditions due to the almost constant discharge, very uniform other environmental conditions, and significantly different flow conditions caused by technical adaptations in its very final stretch.

Results show that architectural variation of *M. spicatum* is closely related to water flow, yielding the most reduced growth form at the site with the highest current velocity. Comparison with older and more recent literature points into the same direction in most cases, but the present results comprise the first detailed record on architectural adaptation of *M. spicatum* in running water. In accord with the conception of other authors, this study shows that intensive environmental impact of increasing current velocity at the test sites occurs together with a marked variation in the basic architectural concept in this aquatic plant species. It seems worth trying to confirm this insight with other aquatic macrophytes, too.

Povzetek

Raziskave o arhitekturnih značilnostih vodnih makrofitov v tekočih vodah so redke, zato smo raziskovali zgradbo poganjkov vrste *Myriophyllum spicatum* L., ki raste v delu kanala Wiener Neustädter (WNC). V kanalu so bile prisotne ugodne razmere primerne za izvedbo poskusa, zaradi skoraj konstantnega odtoka, podobnih okoljskih razmer, in zelo različne hitrosti vodnega toka.

Rezultati kažejo, da so spremembe v razrasti klasastega rmanca tesno povezane s hitrostjo vodnega toka. Najmajše in najmanj razrasle rastline so uspevale na mestu z najvišjo hitrostjo vodnega toka. Primerjava rezultatov s starejšimi in novejšimi viri kaže podobne odzive v prilagajanju rastnih oblik, vendar rezultati naše študije podajajo prve natančne podatke o prilagoditvah rastne oblike potopljene rste *M. spicatum* na tekočo vodo. V skladu z drugimi avtorji, je študija pokazala jasen vpliv hitrosti vodnega toka na testnih lokacijah, skupaj s spremembo spremembo osnovnega koncepta razrasti vodnih rastlinskih vrst. Te rezultate bi bilo potrebno preveriti tudi na drugih vrstah vodnih makrofitov,

Acknowledgements

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16th International Symposium on Microbial Ecology

16. mednarodni simpozij mikrobne ekologije - ISME 16

21. - 26. avgust 2016, Montréal, Kanada

Srečanja ISME potekajo redno že vse od 1977, leta 1986 pa je srečanje gostila tudi Ljubljana. 16. mednarodni simpozij ISME, ki je letos potekal v Montrealu v Kanadi, je privabil kar 2106 udeležencev iz 53 držav. V okviru simpozija se je zvrstilo šest plenarnih predavanj, čemur je sledilo kar 29 različnih sekcij s predstavitvami posterjev tekom treh dni. Udeleženec simpozija si je lahko dnevno izbral eno izmed sedem ali osem simultano potekajočih sekcij, ki so pokrivale izjemno širok nabor mikrobne ekologije od evolucije, modeliranja mikrobnih interakcij, pregleda novih in obstoječih metabolnih poti pri mikroorganizmov in habitatov, ki jih le-ti naseljujejo, (meta) genomike, interakcij med mikrobi in gostitelji, biogeokemijskih ciklov, napredka v programskih orodjih za obdelavo ekoloških podatkov, novih metod ter inovativnih postopkov bioremediacije.

Največji mikrobiološki inkubator na svetu je postal Mehiški zaliv po nesreči leta 2010, ko je prišlo do iztekanja nafte v ocean. Mikrobe v oceanu/okolju lahko analogno primerjamo s stanjem v ozračju. Klima predstavlja neko povprečno stanje, npr. povprečno mikrobno abundanco ali diverziteto, medtem ko vreme predstavlja trenutno stanje v ozračju; podobna dnevna nihanja opažajo tudi pri mikrobih. Zanimivo, ko v morju umre celica diatomeje, le-ta sprosti prosto v okolje kar 75% raztopljene organske snovi (DOM), če je v njeni okolici 10^6 bakterij na ml, če pa je gostota bakterijskih celic njeni v okolici 10^7 pa znaša delež sproščenega DOM v morje le 5%. V morju živi tudi cianobakterija *Prochlorococcus*, najbolj abundanten fotosintetski organizem na zemlji (10^{27} celic). Za mikrobno ekologijo predstavlja izjemen izziv proučevanje metaviroma v tem in ostalih naravnih okoljih.

Globalne klimatske spremembe se bodo vedno bolj izrazito kazale tudi v globalnem mikrobiomu, še zlasti v hladnih okoljih. Spremembe v mikrobiomu kanadskega javorja, ki ga uporabljajo za pridobivanje sirupa, predstavlja za lokalno kanadsko gospodarstvo precejšen problem. Za kriomikrobiologijo in astrobiologijo predstavlja velik izziv razvoj sekvencijskega detekcijskega sistema (angleško, biosignature detection sequencing device), ki bi v realnem času odkril prisotnost življenja. Izziv je tudi aseptično vzorčevanje v takšnih okoljih, npr. vrtanje skozi ledeni pokrov na Antarktiki do enega izmed 400 spodaj ležečih jezer. Vsekakor se je treba še veliko naučiti od mikrobov v ekstremnih habitatih, kot je npr. *Planococcus halocryophillus*, ki raste v širokem temperaturnem razponu od minus 25 do plus 35°C. Čeprav je v puščavah nizka mikrobna diverziteta, z izjemo arhej, obstajajo hipolitični kremenasti habitati (prepuščajo le 0,05 do 5% svetlobe) s primarnimi producenti, ki so pomembno gonilo mikrobne dinamike v tem okolju.

Prst niti ni tako gostoljuben mikrobni habitat kot se zdi na prvi pogled, saj je le 0,1 do 1% organskega ogljika dostopnega za mikrobni metabolizem. Proučevanje ekologije prsti je pomembno tudi pri kmetijski proizvodnji, npr. prisotnost nekaterih praživali izrazito pospeši rast določenih kultivarjev. Z intenzivno rabo zemlje so povezane tudi emisije N_2O, ki ima kar 300-krat večji toplogredni učinek kot CO_2. Njegove emisije iz prsti se lahko enostavno zniža s povišanjem pH tal na 7.

Vedno večji pomen se v mikrobni ekologiji pripisuje številnim kometaboličnih in medvrstnim interakcijam. Precej raziskovalnih skupin po svetu se zato ukvarja z odkrivanjem novih in sklopljenih metabolne poti, npr. sklopljenja fototrofna fiksacije ogljika z istočasno redukcijo železa ali oksidacija Fe(II) z istočasno redukcijo nitrata. Zanimivo interakcijo predstavlja bakterijski endosimbionti (*Wolbachia*), ki usmerja razvoj spola pri vinski mušici (*Drosophila*).

Primer evolucije v laboratoriju opazujejo v poskusu, ki neprekinjeno poteka že vse od leta 1988, ko dnevno v izbrano gojišče precepljajo 12 populacij *Escherichia coli*. Do sedaj imajo že 65000 generacij. V poskusu so ugotovili, da se postopoma izboljšuje fitnes organizma, prihaja pa tudi do veliko nesinonimnih mutacij zlasti v genih za metabolizem in regulatorne funkcije. V tem času je prišlo tudi do izgube 1,4% začetnega genoma. Po 30000 generacijah se je pojavila mutacija, ki omogoča izrabo citrata; ta subpopulacija postaja nova vrsta.

Farmacevtski pripravki v okolju predstavljajo prav poseben izziv. Npr. bioremediacija odpadnih voda, ki vsebujejo farmacetvske pripravke, bi morala obvezno vključevati filtracijo in ločeno obdelavo te vode, da se izognemo širjenju anbitiobičnih rezistenc. Vendar pa, kot poročajo, je mikrobni rezistom v okolju splošno prisoten in zelo star. To so podkrepili s primerom jame Lechugilla (ZDA), kjer so v jamskem mikrobiomu odkrili rezistenčne gene brez zunanjega (človekovega) vnosa antibiotikov.

Številna bolezenska stanja pri ljudeh danes že pripisujejo človeškemu mikrobiomu. Danes je že znano, da človeški intestinalni mikrobiom z nizko diverziteto pomeni slabo za naše zdravje. Mikrobi v prebavilih pomembno sodelujejo pri regulaciji imunskega sistema, odstranjevanju toksinov ter preko metabolitov komunicirajo tudi z ostalimi organi, npr. z živčnim sistemom in možgani. V posebnem kemostatu lahko kultivirajo že 90% črevesne mikrobiote. To brozgo so poimenovali tekoče zlato (angleško, liquid gold), saj na podlagi predhodnih spodbudnih rezultatov že izvajajo transplantacijo mikrobioma zdravih ljudi (angleško, feacal transplant) v ljudi, ki jih pestijo težave, vezane na prebavo, npr. pseudomembranozni kolitis, ki ga povzroča bakterija *Clostridium difficile*.

Novi predsednik združenja mikrobnih ekologov z dvoletnim mandatom je postal Colin Murrell iz Velike Britanije. Naslednji simpozij ISME 17 bo v Leipgizu v Nemčiji, za tem pa se leta 2020 srečanje seli v Južnoafriško republiko.

Janez Mulec

INSTRUCTIONS FOR AUTHORS

1. Types of Articles

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.

9. Illustrations and Tables
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.

10. The quality of graphic material
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).

11. Conclusions
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.

13. Literature
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 published in the same year are cited, the individual works are indicated with the added letters a, b, c, etc.:

(Ward 1994a,b). If direct quotations are used, the page numbers should be included: Toman (1992: 5) or (Toman 1992: 5–6).The bibliography shall be arranged in alphabetical order beginning with the surname of the first author, comma, the initials of the name(s) and continued in the same way with the rest of the authors, separated by commas. The names are followed by the year of publication, the title of the article, the international abbreviation for the journal (periodical), the volume, the number in parenthesis (optional), and the pages. Example:

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.

Pullin, A.S., McLean, I.F.G., Webb, M.R., 1995. Ecology and Conservation of *Lycaena dispar*: British and European Perspectives. In: Pullin A. S. (ed.): Ecology and Conservation of Butterflies, 1st ed. Chapman & Hall, London, pp. 150-164.

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

14. Format and Form of Articles

The manuscripts should be sent exclusively in electronic form. The format should be Microsoft Word (*.doc) or Rich text format (*.rtf) using Times New Roman 12 font with double spacing, align left only and margins of 3 cm on all sides on A4 pages. Paragraphs should be separated by an empty line. The title and chapters should be written bold in font size 14, also Times New Roman. Possible sub-chapter titles should be written in italic. All scientific names must be properly italicized. Used nomenclature source should be cited in the Methods section. The text and graphic material should be sent to the editor-in-chief as an e-mail attachment. For the purpose of review the main *.doc or *.rtf file should contain figures and tables included (each on its own page). However, when submitting the manuscript the figures also have to be sent as separate attached files in the form described under paragraph 10. All the pages (including tables and figures) have to be numbered. All articles must be proofread for professional and language errors before submission.

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)
Table legends; each in English and in Slovene – (Times New Roman 12, title – Times New Roman 14 bold, table designation and table title – Times New Roman 12 bold)
Acknowledgements – (Times New Roman 12, title – Times New Roman 14 bold)
Literature – (Times New Roman 12, title – Times New Roman 14 bold)
Figures, one per page; figure designation indicated top left – (Times New Roman 12 bold)
Tables, one per page; table designation indicated top left – (Times New Roman 12 bold)
Page numbering – bottom right – (Times New Roman 12)

15. Peer Review

All Scientific Articles shall be subject to peer review by two experts in the field (one Slovene and one foreign) and Brief Note articles by one Slovene expert in the field. With articles written in Slovene and dealing with a very local topic, both reviewers will be Slovene. In the compulsory accompanying letter to the editor the authors must nominate one foreign and one Slovene reviewer. However, the final choice of referees is at the discretion of the Editorial Board. The referees will remain anonymous to the author. The possible outcomes of the review are: 1. Fully acceptable in its present form, 2. Basically acceptable, but requires minor revision, 3. Basically acceptable, but requires important revision, 4. May be acceptable, but only after major revision, 5. Unacceptable in anything like its present form. In the case of marks 3 and 4 the reviewers that have requested revisions have to accept the suitability of the corrections made. In case of rejection the corresponding author will receive a written negative decision of the editor-in-chief. The original material will be erased from the ABS archives and can be returned to the submitting author on special request. After publication the corresponding author will receive the *.pdf version of the paper.

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