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The effect of thyme essential oil on germination and early growth of wheat

Vpliv timijanovega eteričnega olja na kalitev in zgodnjo rast pšenice

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Abstract: Essential oils (EOs) are becoming an important alternative as seed decontaminating agents opposite to synthetic seed preservatives due to their antimicrobial activities. They also inhibit seed germination which may be problematic for seeds of cultural plants. In the present study, different treatments of wheat (*Triticum aestivum*) grain with thyme (*Thymus serpyllum*) EO were tested. Wheat grains were treated with thyme EO for 6, 12 and 24 hours to determine the optimal time of EO treatment in order to reduce fungal infection of the grain surface and at the same time preserve high germination rate of seeds and good physiological status of seedlings during early stages of their growth. Germination rate, fresh mass and length of shoots and roots, and physiological status of the seedlings were compared according to the duration of the treatment. The 6 h-EO treatment was the most optimal procedure that did not affect seed germination, was not harmful for the seedlings and prevented fungal infections. The thyme EO could potentially be used as a protective agent for wheat grain intended for sowing and food production.

Keywords: essential oil, germination, seedling growth, thyme, wheat

Izvleček: Eterična olja (EO) postajajo pomembna alternativa sintetičnim pripravkom za zaščito semen pred mikrobi. Ker EO zavirajo tudi kalitev semen, je njihova uporaba pri kultiviranih rastlinah lahko problematična. V raziskavi smo preučili različne načine tretiranja semen pšenice (*Triticum aestivum*) z EO timijana (*Thymus serpyllum*). Pšenična semena smo tretirali z EO timijana 6, 12 in 24 ur ter določili optimalni čas tretiranja, ki zavre glivne okužbe na površini semen in obenem ohrani visoko stopnjo kalivosti semen ter dobro fiziološko stanje kalic v začetnem obdobju rasti. Določili smo delež kalivosti, svežo maso, dolžino poganjka in korenin ter fiziološko stanje kalic in jih primerjali glede na čas tretiranja. Šest-urno tretiranje z EO je bilo najbolj optimalno, ker se glivne okužbe niso razvile in hkrati ni vplivalo na kalitev semen in rast kalic. Timijanovo EO bi lahko bilo uporabno kot zaščitni pripravek za pšenična semena, ki se jih uporablja za setev in kot živilo.

Ključne besede: eterično olje, kalitev, rast kalic, timijan, pšenica

Introduction

Wheat (*Triticum aestivum)* is a cultural plant and an important baking cereal. After rice, it is the second most important source of calories in human nutrition. In terms of production worldwide, it is the second most important cereal after maize, but latter is mostly used as animal forage (Awika 2011). With the production of 161 023 tons in 2016, wheat is also the most important cereal in baking industry in Slovenia (Statistical Office of the Republic of Slovenia). On the field or during the storage, wheat grains can be infected with different fungi that cause economic losses and health problems for humans and animals. Especially fungi of the genera *Fusarium*, *Aspergillus* and *Penicillium* pose a big threat for human health due to the production of harmful secondary metabolites, called mycotoxins (D'Mello et al. 1998, Hussein and Brasel 2001). High yield losses are caused by the fungi of the genus *Fusarium* which are transferred from infected grains to the seedlings and affect their growth and development (Knudsen et al. 1995), while fungi of the genera *Alternaria* and *Aspergillus* reduce seed germination (Ruza et al. 2004).

Post harvesting treatment of stored grains with synthetic fungicides affects the quality of cereals and may pose serious hazard for consumers' health (Osman 2011). Essential oils (EOs) are becoming an important alternative as seed decontaminating agents opposite to synthetic seed preservatives. In addition to strong fungicidal effects they are biodegradable and show low toxicity to human and animal health (Sivakumar and Bautista-Baños 2014).

EOs are synthesized as secondary metabolites in aromatic plants. They are very complex natural mixtures that contain volatile terpenes, terpenoids and aromatic and aliphatic compounds. Because of high number of components, EOs seem to have no specific cellular targets. It has been proposed that the antimicrobial activity of EOs may be attributed to their ability to pass through the cytoplasmic membrane, disrupt its structure and permeabilize it. Once inside the cell, they can inhibit cellular functions (Calo et al. 2015). Thyme (*Thymus*) is one of the most widely used plant genera in folk medicine. The EOs of *Thymus vulgaris* are known to have antiseptic, antiviral and antimicrobial activities (Anžlovar et al. 2014, Dorman and Deans 2000, Reichling et al. 2009). EOs from other aromatic plants from genera *Origanum* and *Lavandula* also possess antifungal properties. Lebanese Za'atar (*Origanum syriacum*) EO acts against *Aspergillus niger*, which is a common food spoilage fungus, *Penicillum* spp. and *Fusarium oxysporum* which infect variety of fruits (Daouk et al. 1995). *Lavandula stoechas* ssp. *stoechas* EO also acts against *F. oxysporum* and is highly active against *Rhizoctonia solani,* known for causing various plant diseases such as collar rot, root rot and wire stem (Angioni et al. 2006).

Previously, we isolated five fungal endophytes from wheat seeds: *Alternaria alternata*, *Alternaria infectoria, Aspergillus flavus, Epiccocum nigrum* and *Fusarium poae,* and tested the effectiveness of thyme EO on fungal radial growth *in vitro* (Anžlovar et al. 2017). The thyme EO showed remarkable *in vivo* efficacy in protecting the EO fumigated wheat seeds from fungal infection. Čepin et al. (2016) also studied the effectiveness of thyme EO in order to reduce fungal infection of wheat grain. They found that EO treatment significantly decreased fungal infection but also inhibited seed germination regardless of the duration of the EO treatment. Germination inhibition and high phytotoxic activity of thyme EO has been reported in the past (Angelini et al. 2003, De Almeida et al. 2010).

The aim of this research was to determine the optimal time of seed treatment with thyme EO in order to reduce the infection of seeds with fungi and at the same time preserve high germination rate and good physiological status of wheat seedlings during early stages of their growth.

Material and methods

Materials

We used 100% natural, pure thyme essential oil extracted from *Thymus serpyllum* (Primavera Life GmbH, Germany). Wheat seeds (*Triticum aestivum* L. cv. 'Savinja') were obtained from the Agricultural Institute of Slovenia.

Essential oil treatment

Wheat seeds were soaked in 0.2% thyme essential oil in 10% dimethylsulphoxide (DMSO). 10% DMSO was prepared by diluting 5 ml of concentrated DMSO (Sigma, USA) in 45 ml of distilled water. Approximately 160 wheat seeds (two replicates, each with 80 seeds) were placed in a plastic Petri dish (2r = 5.5 cm) and poured over with 4 ml of 10% DMSO and 8 µl of thyme EO. Petri dishes were sealed with parafilm to prevent EO evaporation and placed on a shaker (Gerhardt, Germany) for 6, 12 and 24 hours at room temperature and 90 rpm. Control treatment was done with 10% DMSO and 6 hours shaking. After the EO treatment, the seeds were placed into automatic sprouters (EasyGreen® MicroFarm System, Easy-Green Factory Inc., Nevada, USA) with programmed water spraying in the intervals of 5-4-4-3-3-5 hours over a 24 h period. The seeds germinated and seedlings grew in the chambers for 7 days at room temperature and ambient light.

Growth of wheat seedlings

Every day, shoot height was measured for 21 randomly assigned seedlings for each replicate and treatment (control and 6, 12, 24 h of EO). At the end (day 7), germination rate, fresh shoot and root mass, shoot height and root length were measured. 100 mg of wheat shoots and roots per each treatment were separated and frozen in liquid nitrogen for further biochemical analysis.

Lipid peroxidation

Lipid peroxidation was estimated indirectly via malondialdehyde (MDA) content. Previously frozen roots and shoots (100 mg) were homogenized in 2 ml of acidic reagent, consisting of 0.3% (w/v) 2-thiobarbituric acid (Fluka, Spain) and 10% (w/v) trichloroacetic acid (Acros Organics, Germany). After homogenization, samples were incubated at 95 °C for 30 min and then chilled on ice and centrifuged (Eppendorf Centrifuge 5417 R) at 10 000 rpm and 4 °C for 15 min. The absorbance of the supernatant was spectrophotometrically (UV-1800 Shimadzu spectrophotometer, Japan) measured at 532 nm and 600 nm. The concentration of MDA was calculated using the extinction coefficient $\varepsilon = 155\ mM^{-1}\ cm^{-1}$ (Dolenc Koce et al. 2014).

Statistical analysis

The experiment was independently performed twice. Mean values and standard errors were calculated for all treatments. The differences among treated and control plants were tested using the One-way ANOVA. The level of significance was set at $p < 0.05$.

Results

Germination rate, mass and length of shoots and roots, and physiological status of wheat seedlings that were treated with thyme EO were compared according to the duration (6, 12, 24 h) of the treatment.

In control treatment, more than 90% of the seeds germinated on the second day, while in EO treatments a delay in germination was observed. This delay was related to the duration of the treatment, i.e. greater delay in longer EO treatments (12 and 24 h). Germination of wheat seeds was delayed and decreased for up to 93.3% and 97.4% on the second day in 12 and 24 h treatments, respectively. Shorter (6 h) treatment inhibited germination on the first three days but then germination rate increased and after 7 days it reached 85.7%. After 7 days, when the experiment ended, a difference of 11.9% in germination rate between control and the 6 h-treatment was noticed, while in the 12 h- and 24 h-treatments the percentages of germinated seeds were 52.4% and 45.2%, respectively (Tab. 1).

The delayed germination reflected on the growth dynamics during the first 7 days after EO treatment. Wheat seedlings were least affected after the 6 h-treatment with thyme EO while 12 h- and 24 h-treatments inhibited shoot growth more severely (Fig. 1).

The germination of EO-treated seeds was delayed and further growth of seedlings was reduced as indicated by the values in trend line equations (Fig. 1). The 6 h-treatment with thyme EO affected germination and growth moderately (coefficient of the second order higher than

value 2 as in control treatment) while 12 h- and 24 h-treatments inhibited shoot growth more severely (coefficient of the second order had value 1 or lower, negative coefficients of the first order).

Table 1: Germination rate of wheat seeds during 7 days of growth after the treatment with thyme EO for 6, 12 and 24 h (n=160).
Tabela 1: Delež kalivosti pšeničnih semen v 7 dneh po 6, 12 in 24-urnem tretiranju s timijanovim EO (n=160).

	Germination rate (%)			
	Control	6 h	12 h	24 h
Day 1	69.0	0 .0	0.0	0.0
Day 2	92.9	50.0	7.1	2.4
Day 3	95.2	61.9	28.6	9.5
Day 4	97.6	78.6	35.7	28.6
Day 5	97.6	83.3	42.9	31.0
Day 6	97.6	85.7	50.0	40.5
Day 7	97.6	85.7	52.4	45.2

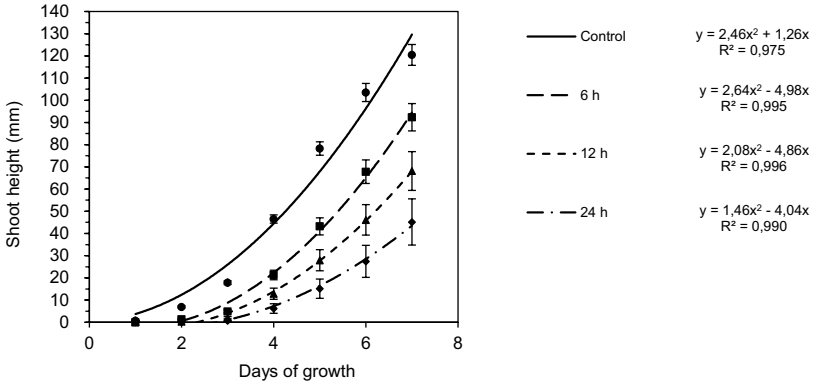

Figure 1: Shoot growth of wheat seedlings during 7 days of growth after the treatment with thyme EO for 6, 12 and 24 h. Mean values ± standard errors are shown (n=42). Trendline equation describes the polynome of the 2nd order. R^2 – coefficient of determination.
Slika 1: Rast poganjka pšeničnih kalic v 7 dneh po 6, 12 in 24-urnem tretiranju s timijanovim EO. Prikazane so povprečne vrednosti ± standardne napake (n=42). Enačba trendne črte opisuje polinom 2. stopnje. R^2 – koeficient determinacije.

After 7 days, the length of shoots was reduced for 23.3% ($p < 0.001$) in 6 h-treatment when compared to control. The reduction of root length was smaller and non-significant ($p = 0.610$). Other two EO treatments, 12 and 24 h, significantly inhibited growth of both shoots and roots. Shoot length was reduced for 43.4% in 12 h-treatment and for 64.7% in 24 h-treatment, while root length was not affected as much: it was reduced for 19.6% in 12 h and 52.8% in 24 h-treatment (Fig. 2).

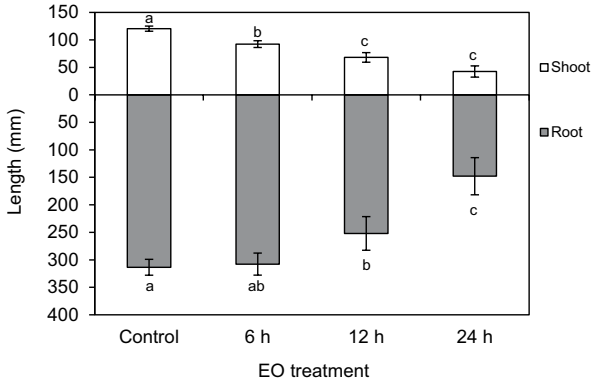

Figure 2: Shoot and root length of wheat seedlings after 7 days of growth and treatment with thyme EO for 6, 12 and 24 h. Mean value ± standard error is shown (n=42). Different letters indicate statistically significant differences (ANOVA, $p < 0.05$).

Slika 2: Dolžina poganjka in korenine pšeničnih kalic po 7 dneh rasti po 6, 12 in 24-urnem tretiranju s timijanovim EO. Prikazane so povprečne vrednosti ± standardne napake (n=42). Različne črke označujejo statistično značilne razlike (ANOVA, $p < 0,05$).

The same reduction was observed in fresh mass of shoots and roots of wheat seedlings (Fig. 3). After 7 days, the inhibitory effect of thyme EO was most evident in 24 h-treatment which significantly ($p < 0.001$) reduced shoot mass for 55.5% and root mass for 50.3% ($p = 0.067$). The 12 h-treatment showed similar effects: shoot mass was significantly smaller for 44.1% ($p < 0.001$), while root mass non-significantly decreased for 14.2% ($p = 0.497$). The 6 h-treatment significantly decreased shoot mass for 23.5% ($p < 0.001$) in comparison to the control treatment while root mass was at the control level.

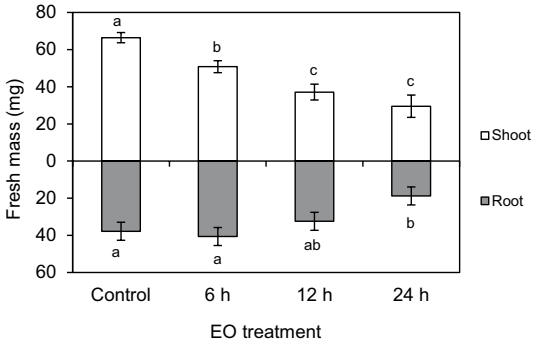

Figure 3: Fresh mass of wheat seedlings after 7 days of growth and treatment with thyme EO for 6, 12 and 24 h. Mean value ± standard error is shown (n=42). Different letters indicate statistically significant differences (ANOVA, $p < 0.05$).

Slika 3: Sveža masa pšeničnih kalic po 7 dneh rasti po 6, 12 in 24-urnem tretiranju s timijanovim EO. Prikazane so povprečne vrednosti ± standardne napake (n=42). Različne črke označujejo statistično značilne razlike (ANOVA, $p < 0,05$).

The physiological status of wheat seedlings was estimated by lipid peroxidation which is a marker of oxidative stress. None of the treatments with thyme EO had any effect on lipid peroxidation in shoots of wheat seedlings (Fig. 4A). There was also no effect on the roots except in seedlings, treated with thyme EO for 24 hours, where higher MDA content was measured (Fig. 4B).

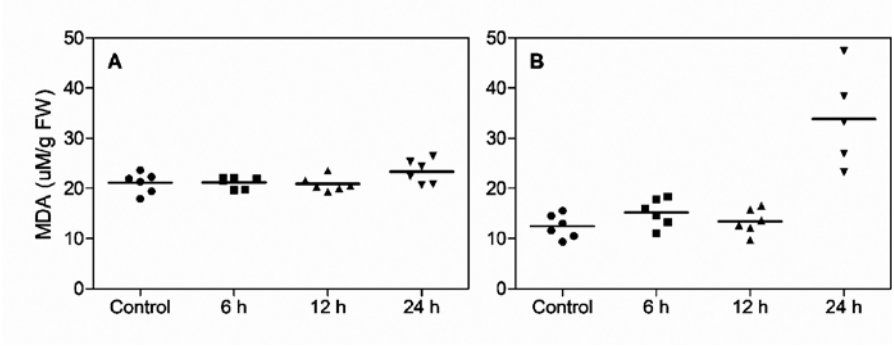

Figure 4: Malondialdehyde (MDA) content in shoots (A) and roots (B) of wheat seedlings after 7 days of growth and treatment with thyme EO for 6, 12 and 24 h. Mean value is indicated with the line (n=6).

Slika 4: Vsebnost malondialdehida (MDA) v poganjkih (A) in koreninah (B) pšeničnih kalic po 7 dneh rasti po 6, 12 in 24-urnem tretiranju s timijanovim EO. Povprečna vrednost je označena s črto (n=6).

Discussion

Wheat is susceptible to fungal infection, which can affect crop yield and have negative effect on human health (Anžlovar et al. 2017). The negative effects of synthetic chemicals on human and animal health and on the environment have caused an increasing demand for natural additive options. Essential oils exhibit antimicrobial properties which can make them suitable alternatives to synthetic chemicals (Calo et al. 2015). In the present study wheat seeds were exposed to thyme essential oil for 6, 12 and 24 h. During the 7-day experiment the seeds and later, the seedlings were monitored and a few growth related (fresh mass and length of shoots and roots) and physiological parameters (lipid peroxidation) were measured. In comparison to the study of Čepin et al. (2016), this experiment was carried out in automatic sprouters instead of Petri dishes. Germination tests in Petri dishes allow the germination rate to be monitored only for the first three days when seedlings are very small, while sprouters are larger and allow for longer growth. Also, humidity conditions can be better controlled in sprouters and field conditions can be more adequately imitated. In sealed Petri dishes, the effect of essential oil can be enhanced due to limited aeration.

Kotan et al. (2013) found the direct application of the essential oil suspension of *Satureja hortensis* to have a toxic effect on the tomato seeds, but the concentrations of essential oil were 4 and 8 times higher than in our experiment. In our case, even with lower concentration of EO, the 24 h-treatment showed toxic effect on the wheat seedlings. Most favourable thyme EO treatment that reduced fungal infection and at the same time preserved germination rate and normal physiological parameters of wheat seedlings was the 6 h-treatment. The growth of wheat seedlings was very similar to the control and the germination rate reached 85.7%. The 6 h-treatment was also selected as the most suitable by Čepin et al. (2016). In their experiment more seeds germinated in 6 h-treatment than in 12 h- and 24 h- while fungal infection decreased by 48.0% in comparison to the control.

The germination of seeds treated with thyme EO for 12 and 24 hours was delayed and further growth of these seedlings was reduced as well. However, 6 h-treatment caused only a delay of seed germination while seedlings growth was not affected. Poonpaiboonpipat et al. (2013) also noticed a delay in germination when barnyardgrass seeds were exposed to lemongrass essential oil. Our experiment did not focus on fungal infection of the seeds, but we noticed more fungi on the seeds in control treatments and after 6 h- and 12 h-treatment compared to 24 h-treatment. Based on the results of lipid peroxidation, which is a marker of oxidative stress, these fungal infections had no effect on the physiological status of wheat seedlings. On the contrary, increased levels of MDA were measured in older rotten tissues of *Phaseolus vulgaris* infected with *Botrytis cinerea* (Muckenschnabel et al. 2001).

The fact that after 7 days there were no differences in MDA content in the shoots for any of the EO treatments when compared to the control, proves that thyme EO did not induce stress in shoots of wheat seedlings. MDA content was at the control level also in roots, except in the case of 24 h-treatment when significantly higher MDA content was measured (high coefficient of variation of 28.18%). This finding indicates that this EO treatment represented stress for the roots of wheat seedlings. Longer monitoring of seedlings growth would reveal if this change reflects on the structure and function of treated plants.

Based on our results we can conclude that the optimal protective treatment with thyme EO that reduces fungal infection, but still does not affect seed germination and is not toxic for the seedlings, is the 6 h-treatment. This treatment has a potential for use as a protection agent for grain intended for sowing and food production, but further analysis to determine the cost and safety for the cultural plants should be performed.

Povzetek

Eterična olja (EO) postajajo pomembna alternativa sintetičnim pripravkom za zaščito semen pred mikrobi. Ker EO zavirajo tudi kalitev semen, je njihova uporaba pri kultiviranih rastlinah lahko problematična. Za timijanovo EO je dokazano, da ima dobro protimikrobno aktivnost, tako proti bakterijam kot proti glivam. Poleg tega je timijan znano zelišče in zdravilna rastlina z blagodejnimi učinki za človeka.

V raziskavi smo želeli določili optimalni čas tretiranja semen pšenice (*Triticum aestivum*) z EO timijana (*Thymus serpyllum*), ki zmanjša glivne okužbe semen ter hkrati ohrani visoko raven njihove kalivosti in dobro fiziološko stanje kalic v zgodnjih fazah rasti. Pšenična semena smo namočili v 0,2 % EO timijana v 10 % DMSO za 6, 12 in 24 ur. Po tretmaju smo jih prenesli v kalilnike s programiranim načinom pršenja, kjer so kalila 7 dni pri sobni temperaturi in osvetlitvi. Vsak dan smo pri 21 naključno izbarnih kalicah izmerili dolžino poganjka. Po 7 dneh smo za vsak tretma določili delež kalivosti ter dolžino in svežo maso poganjkov in korenin. Po 100 mg poganjkov in korenin smo zamrznili za meritve lipidne peroskidacije, ki je pokazatelj oksidativnega stresa.

Ugotovili smo, da je 6-urno tretiranje pšeničnih semen s timijanovim EO najbolj optimalno, saj je bilo glivnih okužb malo, kalitev semen in rast kalic pa sta ostali na ravni kontrolnega tretmaja. Daljše tretiranje z EO je tudi zmanjšalo glivne okužbe, vendar je upočasnilo kalitev semen in rast kalic. Pri 24-urnem tretmaju z EO se je tudi povišala lipidna peroksidacija v koreninah kalic. Rezultati raziskave kažejo, da bi bil kratkotrajen (6-urni) tretma s timijanovim EO uporaben kot zaščitni protiglivni postopek za pšenična semena, ki se jih uporablja za setev in kot živilo.

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References

Angelini, L. G., Carpanese, G., Cioni, P. L., Morelli, I., Macchia, M., Flamini, G., 2003. Essential oils from mediterranean lamiaceae as weed germination inhibitors. Journal of Agricultural and Food Chemistry 51, 6158-6164.

Angioni, A., Barra, A., Coroneo, V., Dessi, S., Cabras, P., 2006. Chemical composition, seasonal variability, and antifungal activity of *Lavandula stoechas* L. ssp. *stoechas* essential oils from stem/leaves and flowers. Journal od Agricultural and Food Chemistry 54, 4364-4370.

Anžlovar, S., Baričevič, D., Ambrožič Avguštin, J., Dolenc Koce, J., 2014. Essential oil of common thyme as a natural antimicrobial food additive. Food Technology and Biotechnology 52, 263-268.

Anžlovar, S., Likar, M., Dolenc Koce, J., 2017. Antifungal potential of thyme essential oil as a perservative for storage of wheat seeds. Acta Botanica Croatica 76, 64-71.

Awika, J. M., 2011. Major cereal grains production and use in the world. In: Awika, J. M., Piironen, V., Bean S. (eds.), Advances in cereal science: Implications to food processing and health promotion, 1-13. American Chemical Society, Washington, DC.

Calo, J. R., Crandall, P. G., O'Bryan, C. A., Ricke, S. C., 2015. Essential oils as antimicrobials in food systems – A review. Food Control 54, 111-119.

Čepin, T., Kogej, Z., Sever, T., Skubic, L., Šoln, K., 2016. Thyme essential oil as natural fungicide in wheat germination (Timijanovo eterično olje kot naravni fungicid pri kalitvi pšenice). Collectanea Studentium Physiologiae Plantarum 7, 28-30.

Daouk, R. K., Dagher, S. M., Sattout, E. J., 1995. Antifungal activity of essential oil of *Origanum syriacum* L. Journal of Food Protection 58, 1147-1149.

De Almeida, L. F. R., Frei, F., Mancini, E., De Martino, L., De Feo, V., 2010. Phytotoxic activities of mediterranean essemtial oils. Molecules 15, 4309-4323.

D'Mello, J. P. F., Macdonald, A. M. C., Postel, D., Dijksma, W. T. P., Dujardin, A., Placinta, C. M., 1998. Pesticide use and mycotoxin production in *Fusarium* and *Aspergillus* phytopathogens – Mini review. European Journal of Plant Pathology 104, 741-751.

Dolenc Koce, J., Drobne, D., Klančnik, K., Makovec, D., Novak, S., Hočevar M., 2014. Oxidative potential of ultraviolet-A irradiated or nonirradiated suspensions of titanium dioxide or silicon dioxide nanoparticles on *Allium cepa* roots. Environmental Toxicology and Chemistry 33, 858-867.

Dorman, H. J. D., Deans, S. G., 2000. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. Journal of Applied Microbiology 88, 308-316.

Hussein, S. H., Brasel, J. M., 2001. Toxicity, metabolism, and impact of mycotoxins on humans and animals – A review. Toxicology 167, 101-134.

Knudsen, I. M. B., Hockenhull, J., Jensen, D. F., 1995. Biocontrol of seedling diseases of barley and wheat caused by *Fusarium culmorum* and *Bipolaris sorokiniana*: effects of selected fungal antagonists on growth and yield components. Plant Pathology 44, 467-477.

Kotan, R., Dadasoğlu, F., Karagoz, K., Cakir, A., Ozer, H., Kordali, S., Cakmakci, R., Dikbas, N., 2013. Antibacterial activity of the essential oil and extracts of *Satureja hortensis* against plant pathogenic bacteria and their potential use as seed disinfectants. Scientia Horticulturae 153, 34-41.

Muckenschnabel, I., Williamson, B., Goodman, B. A., Lyon, G. D., Stewart, D., Deighton, N., 2001. Markers for oxidative stress associated with soft rots in French beans (*Phaseolus vulgaris*) infected by *Botrytis cinerea*. Planta 212, 376-381.

Osman, K. A., 2011. Pesticides and human health. In: Stoytcheva, M. (ed.), Pesticides in the modern world – Effects of pesticides exposure, 205-230. InTech, Rijeka.

Poonpaiboonpipat, T., Pangnakorna, U., Suvunnameka, U., Teerarakb, M., Charoenyingc, P., Laosinwattanab, C., 2013. Phytotoxic effects of essential oil from *Cymbopogon citratus* and its physiological mechanisms on barnyardgrass (*Echinochloa crus-galli*). Industrial Crops and Products 41, 403-407.

Reichling, J., Schnitzler, P., Suschke, U., Saller, R., 2009. Essential oils of aromatic plants with antibacterial, antifungal, antiviral, and cytotoxic properties – an overwiev. Forschende Komplementärmedizin 16, 79-90.

Ruza, A., Linina, A., Gaile, Z., Bankina, B., 2004. Possibilities of long-term storage of cereal seeds. Vagos 64, 72-76.

Sivakumar, D., Bautista-Baños, S., 2014. A review on the use of essential oils for postharvest decay control and maintenance of fruit quality during storage. Crop Protection 64, 27-37.

Statistical office of the Republic of Slovenia. Crop production (ha, t, t/ha), Slovenia, per year. Retrieved February 2, 2018 from http://pxweb.stat.si/pxweb/Dialog/Saveshow.asp (in Slovene).

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Seasonal dynamics of fungal colonisation of *Plantago altissima* roots in a water-fluctuating wetland

Sezonska dinamika glivne kolonizacije korenin visokega trpotca v presihajočem mokrišču

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Abstract: Fungal colonisation in an amphibious plant species from an intermittent lake may differ significantly among plant specimens growing under contrasting water regimes. We examined the effect of the presence of surface water on the level of fungal colonisation in tall plantain (*Plantago altissima* L.). We investigated the presence and abundance of arbuscular-mycorrhizal (AM) structures, dark-septate-endophyte (DSE) fungal structures, and plant growth parameters. The observed overall frequency of fungal structures during the vegetative season was relatively high, ranging from 50% to 90%. Mycorrhizal frequency was little affected by water level fluctuations. However, it dropped significantly at the end of the season in the senescence phase. The densities of arbuscules, vesicles, and hyphal coils reached the highest levels in peak season in June and July. The density of DSE structures, namely microsclerotia, showed similar dynamics over the season as for AM structures. Mycorrhizal frequency was positively related to leaf water content.

Keywords: intermittent lake, *Plantago altissima*, roots, fungal colonisation, arbuscular mycorrhiza, dark septate endophytes

Izvleček: Glivna kolonizacija amfibijskih rastlinskih vrst na presihajočem jezeru se lahko znatno razlikuje med rastlinami, ki rastejo v razmerah različnega vodostaja. Preučili smo učinek prisotnosti površinske vode na raven glivne kolonizacije pri visokem trpotcu (*Plantago altissima* L.). Raziskali smo prisotnost in številčnost struktur arbuskularne mikorize (AM), struktur temnih septiranih endofitov (DSE) in parametre rasti rastlin. Pogostnost glivnih struktur med je bila med rastno sezono rarmeroma visoka, saj je segala od 50 % do 90 %. Pogostnost mikorize je ostala na isti ravni ne glede na spremembe vodostaja, vendar se je ob koncu sezone v fazi senesence znatno znižala. Gostota arbuskulov, veziklov in svitkov je dosegla najvišjo raven v juniju in juliju. DSE strukture, mikrosklerociji, so imele podobno dinamiko kot AM strukture. Pogostnost mikorize je bila pozitivno povezana z vsebnostjo vode v listih.

Ključne besede: presihajoče jezero, *Plantago altissima*, korenine, glivna kolonizacija, arbuskularna mikoriza, temni septirani endofiti

Introduction

Water regime shapes physical, chemical, and biological processes in wetlands (Cronk and Fennesy 2001). Intermittent wetlands are characterised by extreme water level fluctuations that affect habitat conditions in time and space (Dolinar et al. 2016). Alternations of flooded and dry periods result in a unique flora with specific adaptations that enable plants to cope with these outstanding conditions (Braendle and Crawford 1999, Gaberščik et al. 2003, Urbanc-Berčič et al. 2005, Šraj et al. 2006). Roots of the majority of wetland species are colonised by fungi (Escudero and Mendoza 2005, Stevens et al., 2010). Variations in mycorrhizal colonisation among different species in wetlands are crucial for structuring fen plant communities (Cornwell et al. 2001). Surveys of plant root systems of these species revealed the presence of dark septate endophytes (DSE) and arbuscular mycorrhizal fungi (AM) (Šraj et al. 2006, Gaberščik et al. 2017). DSE are common especially in habitats with frequent stress conditions (Mandyam and Jumpponen 2008). DSE are a diverse group of Ascomycetes that colonises root tissues inter- and intracellularly and form clusters of inflated, rounded, and thick-walled cells within cortical cells called microsclerotia (Jumpponen and Trappe 1998). Mycorrhizal fungi positively affect cycling of macronutrients because they mobilise phosphorus and nitrogen, and are an important carbon sink in the soil (Bonfante and Genre 2010, Johnson et al. 2015). AM fungi are the most widespread fungal symbionts of plants, belonging to the phylum Glomeromycota (Smith and Read 2008). AM fungi form symbiotic relations with plants that have multiple benefits for both partners. In exchange for oxygen and organic carbon, AM fungi enhance plant tolerance to variable water conditions, protect plants against toxic metals and pathogens, and improve their nutrient acquisition (Jacott et al. 2017, Idoia et al. 2004). Therefore, mycorrhizal plants often exhibit improved water status in comparison with non-mycorrhizal plants, which is thought to be a consequence of their greater plant growth (Augé 2004). In the case of carbon limitation, it has been shown that the symbiotic associations become stronger rather than weaker, which suggests that the support of mycorrhizal fungi is of high priority for stressed plants (Gange and West 1994, Ayres et al. 2006, Hartley and Gange 2009, Fontana et al. 2009, Zhang et al. 2015).

Many plant species colonising wetlands exhibit an amphibious character, therefore they can thrive both in water and on dry land, where they develop structurally and/or functionally different growth forms (Gaberščik 1993, Germ and Gaberščik 2003, Boeger and Poulson 2003, Klančnik et al. 2014). Studies of fungal colonisation carried out in amphibious species from an intermittent lake have revealed that the frequency of fungal colonisation may differ significantly among plant specimens of the same species when growing under contrasting water regimes (Šraj et al. 2006, Dolinar et al. 2010, 2016, Gaberščik et al. 2017). However, very little is known about the seasonal dynamics and functionality of these root fungal structures in amphibious plant species that are subjected to seasonal changes of water level in their habitats (Miller 2000, Šraj et al. 2006).

Tall plantain, *Plantago altissima* L., is an amphibious species that can sustain moderate water level fluctuations (Martinčič and Leskovar 2002). We aimed to investigate the seasonal dynamics of fungal colonisation of roots of this species growing in the area of intermittent Lake Cerknica, subjected to frequent water level fluctuations during the vegetation season. We investigated the presence and abundance of AM fungal structures and dark-septate-endophyte fungal structures. To evaluate the possible interactions between fungi and plant conditions, we also examined growth parameters. We hypothesised that the presence of surface water in the habitat of tall plantain negatively affects plant root colonisation with AM fungi and positively affects the occurrence of dark-septate-endophyte fungal structures that mitigate stress conditions, and that the level of fungal colonisation during the season is related to growth parameters and plant phenology.

Material and methods

Study site

The study was performed in the area of intermittent Lake Cerknica. Lake Cerknica is situated at the bottom of the karst polje Cerknica Polje (38 km^2).

Due to abundant precipitation in spring and autumn and occasionally also in other seasons, Cerknica Polje changes into a lake that has an average size of 20–25 km^2. Floods last on average 260 days a year and the dry period usually starts in late spring (Kranjc 2003). The vegetation pattern shows a clear zonal distribution that depends on the extent and frequency of floods (Martinčič and Leskovar 2003).

Studied species

Plantago altissima L. belongs to the family of plantains (Plantaginaceae). It is a perennial herbaceous plant that blooms from May to July (Martinčič et al. 2007). It is distributed in the south-eastern Europe ranging from Pannonian lowland to northern Italy, where it reaches its western limit. Plant material was sampled at wet meadow at Rešeto region, which is located at the edge of the lake in the vicinity of the village Dolenje Jezero (N 45°46'17" (45.7715°) E 14°21'24" (14.3568°)), at a site where soil was water-saturated and even flooded most of the vegetation season except in September. Wet meadow is habitat of a community *Deschampsio - Plantaginetum altissimae*. Beside *Plantago altissima* and *Dechampsia caespitose,* species like *Sanguisorba officinalis*, *Allium ungulatum*, *Genista tinctoria Gratiola officinalis* and some sedges could be found in the area. The area is flooded part of the year, usually in spring and autumn (Martinčič and Leskovar 2002). The extent and duration of floods affect different plant species in different way. In some species environmental extremes accelerate the senescence processes. Water level fluctuations during the experimental season are presented in Figure 1. The water level measurements took place close to the sampling site in the river Stržen (cca. 500 m far) that meanders through the Cerknica Polje. High water level in the peak season (June) resulted in a die-off of the aboveground organs of *P. altissima* that developed once again after a water level drop in July. The presence of surface water at the sampling location is indicated in Figure 3 by grey symbols. From 10 to 20 plants were sampled randomly four times during the vegetation period.

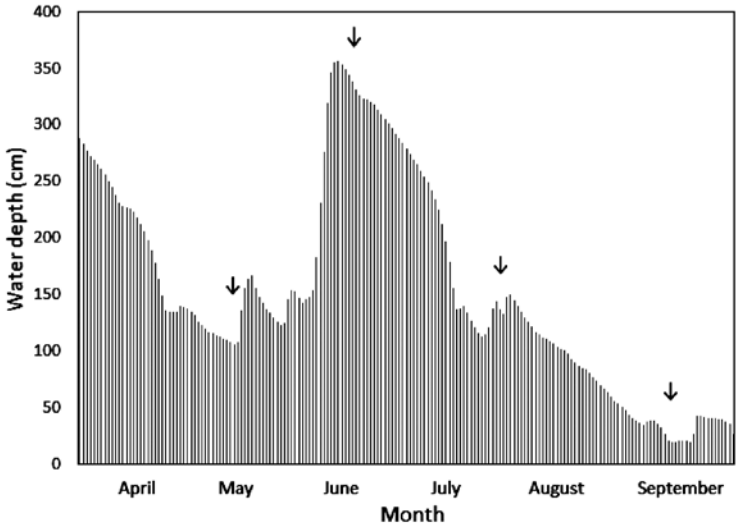

Figure 1: Water level fluctuations measured in the riverbed of Stržen at the weather station Stržen – Dolenje jezero during sampling period (http://vode.arso.gov.si/hidarhiv/pov_arhiv_tab.php). Arrows indicate sampling dates.

Slika 1: Spremembe vodostaja, merjene v koritu Stržen pri meteorološki postaji Stržen - Dolenje jezero v času vzorčenja (http://vode.arso.gov.si/hidarhiv/pov_arhiv_tab.php). Puščice označujejo datume vzorčenja.

Root colonisation with fungi

A block of soil (20 cm × 20 cm × 25 cm) was dug out around the roots of each plant, with the soil and the roots taken from the ground together, and then being transferred to the laboratory. Fine roots were carefully separated from the soil and fixed in ethanol, for the determination of fungal colonisation. These roots fixed in ethanol were later thoroughly washed with distilled water, cleared with 10% KOH, and stained with 0.05% Trypan blue, according to the procedure described by Philips and Hayman (1970). Fungal colonisation of each plant sample was determined on 30 1-cm-long fine-root segments, according to Trouvelot et al. (1986). The root segments were examined under a microscope (Olympus CX41; Olympus, Hamburg, Germany) and photographed using a CCD camera (Colourview II; Soft Imaging System, Münster, Germany) using the Cell Olympus Imaging software. Root segments were ranked into six classes of mycorrhizal colonisation [%] (0, <1, 1−10, 11−50, 51−90, >90) (Trouvelot et al. 1986). The factors determined were: mycorrhizal frequency (proportion of infected root segments by any fungal structure); mycorrhizal intensity (abundance of mycorrhizal colonisation for each root segment); and abundance of different arbuscular-mycorrhizal and dark-septate-endophyte fungal structures, namely arbuscules, vesicles, hyphal coils, and microsclerotia per sample. In addition to the above-mentioned structures, mycorrhizal frequency also comprised the frequency of fungal hyphae.

Growth analysis

We performed growth analysis of whole plants. For each of the 10 to 20 plants per sampling site, we determined fresh and dry mass of below- and aboveground organs, number of flowers, length, width, dry mass of leaves, number of leaves per plant, and plant and leaf water contents.

Statistical analyses

Differences in plant traits including colonisation of roots with fungi were tested using ANOVA or non-parametric Kruskal–Wallis tests with Bonferroni's correction. Normal distribution was tested using the Shapiro-Wilk test. SPSS Statistics 19.0 was used for calculations. To find possible relationships between measured parameters, Spearman's rank correlation analysis was performed (IBM SPSS Statistics 19). Redundancy analysis (RDA) was used to determine whether variations in response variables were related to explanatory variables using CANOCO for Windows 4.0. Monte Carlo permutation tests were carried out to test the significance of the relationships between fungal and plant parameters. The level of significance was accepted at $p \leq 0.05$.

Results

Plant traits

In May, when the majority of plants were still in the vegetative phase, leaf width and length differed considerably from the other three samplings, while they were more or less the same in June, July and September (Table 1). Inflorescences have become very numerous in June, while only a few buds had already been present in May. Belowground biomass was at the same level until September when leaf biomass along with belowground biomass both reached peak values. The lowest values of leaf and plant water content were recorded in September. Mycorrhizal intensity was highest in July and dropped significantly in September.

The overall frequency of fungal structures (including hyphae) was relatively high, ranging from 50% to 90% (Figure 2, upper graph). In plants from the vegetative phase, we observed similar levels of mycorrhizal frequency as during the flowering phase, whereas it significantly decreased during the senescence phase in September. The number of vesicles as storage organs and arbuscules, which indicate active mycorrhiza, was significantly higher during the flowering phase and declined towards the end of the season (Figure 2, lower graph). Similar trend was observed for microsclerotia density, which was significantly reduced in September.

Table 1: Plant growth parameters, plant and leaf water content, and mycorrhizal intensity in *Plantago altissima* in different phases of the vegetative season. Different letters indicate differences in measured parameters among different months ($p<0.05$).

Tabela 1: Parametri rasti rastlin, vsebnost vode v rastlinah in listih ter obseg mikorize pri visokem trpotcu v različnem času vegetacijskega obdobja. Različne črke označujejo razlike med merjenimi parametri v različnih mesecih ($p<0,05$).

	Month of sampling			
Parameter	May	June	July	September
Number of buds/plant	0.25±0.42 [b]	1.2±0.49 [c]	0 [a]	0 [a]
Number of inflorescences/plant	0 [a]	7.5±7.6 [b]	0 [a]	0 [a]
Number of leaves/plant	6.1±2.0 [ab]	4.4±1.7 [a]	6.8± 2.6 [b]	4.9±1.0 [ab]
Average leaf length (cm)	12.6±1.7 [c]	27.1±3.2 [b]	21.4± 2.3 [a]	28.4±1.8 [ab]
Average leaf width (cm)	0.79±0.23 [b]	1.39±0.21 [a]	1.20±0.22 [a]	1.27±0.2 [a]
Leaf dry mass /plant (g)	1.16±0.66[b]	1.91±0.65 [a]	1.84±1.09 [a]	3.53±0.86[a]
Belowground dry mass/plant (g)	1.04±0.76 [a]	0.82±0.33 [a]	1.05±0.81 [a]	1.74±0.76 [b]
Water content (whole plant) (%)	0.60±0.10 [ab]	0.64±0.06 [a]	0.63±0.04 [ab]	0.58±0.06 [b]
Leaf water content (%)	0.67±0.06 [ab]	0.73±0.04 [a]	0.66±0.03 [b]	0.47±0.06 [c]
Mycorrhizal intensity (M%)	6.63±2.67 [ab]	7.95±1.06 [a]	10.01±4.13 [b]	3.73±1.69 [c]

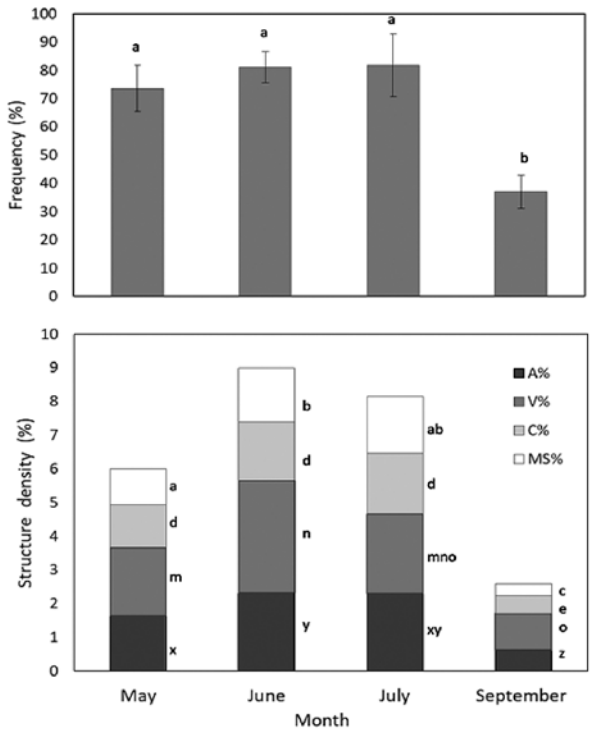

Figure 2: The frequency (upper graph) and density (lower graph) of different fungal structures, i.e., arbuscules (A%), vesicles (V%), hyphal coils (C%), and microsclerotia (MS%) in *Plantago altissima* roots in different phases of the vegetative season. Different letters indicate differences in measured parameters among different months ($p<0.05$).

Slika 2: Pogostost (zgornji graf) in gostota (spodnji graf) različnih glivnih struktur, tj. arbuskulov (A%), veziklov (V%), svitkov (C%), in mikrosklerocijev (MS%) v koreninah visokega trpotca v različnem času vegetacijskega obdobja. Različne črke označujejo razlike med merjenimi parametri v različnih mesecih ($p<0,05$).

Relations between fungal and leaf parameters

Plant water content was positively related to the density of fungal structures ($p<0.05$),. The correlation coefficients were 0.35 for arbuscules, 0.46 for vesicles, 0.39 for coils and 0.46 for microsclerotia, respectively. Belowground dry mass wasnegatively related to mycorrhizal frequency ($R^2= -0.33$; $p<0.05$). The linear regression between leaf water content and overall mycorrhizal frequency was shows positive relation to ($r^2 = 0.56$; $p<0.05$) (Figure 3).

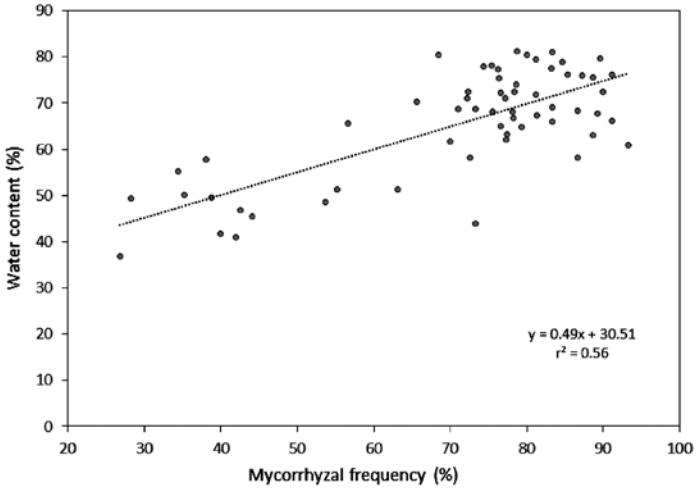

Figure 3: The linear regression of leaf water content and mycorrhizal frequency in *Plantago altissima.* (n=60; p<0.05)
Slika 3: Regresija vsebnosti vode v listih in pogostosti mikorize pri visokem trpotcu (n=60; p<0,05).

Redundancy analysis that was run to explain the variability of growth parameters with fungal structures revealed that out of all fungal parameters, only mycorrhizal frequency revealed to be significant. This parameter explained only 15% of the variability of growth parameters. However, when redundancy analysis was run to explain the variability of fungal structures abundance with growth parameters, it showed that altogether these parameters explained 33% of the variability of fungal structures frequency. Mycorrhizal frequency was positively related to leaf water content and negatively to morphometric leaf parameters, namely leaf length and width, leaf water content, number of leaves, and leaf dry mass (Fig. 3). The density of specific structures per infected segments was negatively related to growth parameters as well as to leaf water content.

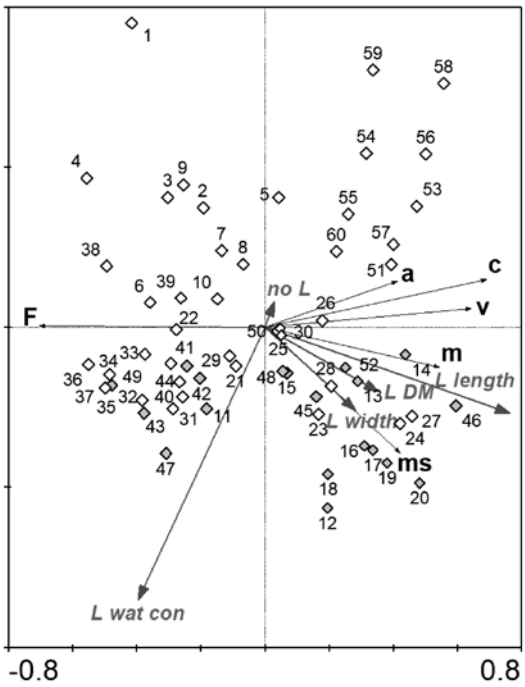

Figure 4: Redundancy analysis plot showing the relationship between leaf properties and mycorrhizal frequency (F), and density of arbuscules (a), vesicles (v), microsclerotia (ms), and coils (c) per colonised root segment in *Plantago altissima* roots; May (1-20), June (21-40), July (41-50), and September (51-60); open symbols indicate samples growing at locations without surface water during the sampling, while grey symbols indicate flooded locations at the time of sampling. Growth parameters: no L – number of leaves, L length – leaf length, L DM – leaf dry mass, L width – leaf width, L wat con – leaf water content.

Slika 4: Graf analize redundance, ki prikazuje razmerje med lastnostmi listov in mikorizno frekvenco (F) ter gostoto arbuskulov (a), veziklov (v), mikrosklerocijev (ms) in svitkov (c) na koloniziranem segmentu korenin visokega trpotca; maj (1-20), junij (21-40), julij (41-50) in september (51-60); odprti simboli označujejo vzorce, ki rastejo na lokacijah brez površinske vode v času vzorčenja, medtem ko sivi simboli nakazujejo poplavljene lokacije v času vzorčenja. Parametri rasti: no L - število listov, L length - dolžina listov, L DM - listna suha masa, L width - širina listov, L wat - vsebnost vode v listih.

Discussion

Plant habitats in Lake Cerknica are permanently exposed to disturbances due to water level fluctuations that can damage the present vegetation (Gaberščik et al. 2003). New colonisation of disturbed area with plants needs efficient strategies of local vegetation. Asmelash et al. (2016) showed that AM fungi play important role in plant community succession, since they increase above- and belowground biodiversity, even though their effect on primary productivity may be negligible. In *Plantago altissima*, the overall frequency of fungal structures was relatively high, ranging from 50% to 90%, while specific AM structures were somewhat less abundant. According to the stress gradient hypothesis (Brooker et al. 2008), the strength of mutualism between symbiotically associated organisms increases as habitat stress conditions worsen (Zhang et al. 2015).

Water level fluctuations affect mineralisation rate and nutrient release as shown by different studies (Boulton and Brock 1999). This is also the case in Lake Cerknica, where the availability of nutrients is low during dry period, while it increases during wetting (Urbanc-Berčič and Gaberščik 2001). The interaction between water and nutrient availability could therefore be crucial in determining the extent of mycorrhizal symbiosis between some wetland plant species (Cornwell et al. 2001). Seasonal dynamics of the frequency of fungal structures in *Plantago altissima* showed high level with increasing trend throughout the season with the exception of September. In September, the frequency significantly decreased, possibly due to the senescence processes and abundant storage in plant rhizomes. A stable level of fungal colonisation throughout the season, irrespective of the hydrological conditions, is probably the result of the preservation of fungal structures that developed in a favourable environment even in adverse conditions (Miller and Sharitz 2000).

The frequency of the majority of fungal structures became more abundant in June and July. In June, plants were in the flowering phase that increased the need for nutrients, as also shown in other studies (Naveed et al. 2012, Dolinar et al. 2016). Regvar et al. (2006) report that in some plant species active AM can be limited to specific periods of plant ontogenesis, especially to the fruiting and seeding period. In *Plantago altissima*, new assimilation areas developed in July, since deep water from the previous month caused the decay of aboveground structures, including of the assimilation areas.

AM colonization affects a number of plant and soil characteristics, among which the effect on plant water relations is very important (Augé 2004). In our study, plant water content was positively related to the density of the majority of fungal structures, with the exception of arbuscules and coils.

DSE are common plant root colonizers in extreme environments. The hyphae of DSE fungi produce melanin in their cell walls, which increases the toughness of cell walls, increases plant tolerance to pathogens, and protects against dehydration (Jumpponen 2001). This is of great importance in water-fluctuating habitats, however in the case of *Plantago altissima* in Lake Cerknica, microsclerotia density showed similar dynamics over the vegetative season as AM structures, which revealed the potential to increase resources availability in studied plant species..

Summary

Intermittent wetlands are characterised by extreme water level fluctuations that affect habitat conditions in time and space. Studies of fungal colonisation carried out in amphibious plant species in an intermittent lake revealed that the frequency of fungal colonisation differs significantly among plant specimens growing under contrasting water regimes. We hypothesized that the presence of surface water in the habitat of tall plantain (*Plantago altissima* L.) negatively affects plant root colonisation with AM fungi, and positively affects the occurrence of dark-septate-endophyte fungal structures that mitigate stress conditions, and that the level of fungal colonisation during the season is related to growth parameters and plant phenology. We investigated the presence and abundance of AM fungal structures and dark-septate-endophyte fungal structures. To evaluate the possible interactions between fungi and plant conditions, we also examined growth parameters. In May, when the majority of plants were still in the vegetative phase, leaf width and length differed the most from the other three samplings (Table 1). Inflorescences have become numerous in June, while only a few buds had already been present already in May. The decay of aboveground organs due to high floods in peak season was followed by new growth of leaves and in the following month the level of fungal colonisation remained the same. Belowground biomass was also at the same level until September when leaf biomass along with belowground biomass both reached peak values. Leaf and plant water content were lowest in September. Mycorrhizal intensity was highest in July and dropped significantly in September. The total frequency of fungal structures (including hyphae) was relatively high, ranging from 50 % to 90 %. In plants from the vegetative phase, we observed similar levels of mycorrhizal frequency as during flowering, whereas it significantly decreased during the senescence phase in September. The number of vesicles increased significantly during

the flowering phase and declined towards the end of the season. Similar trend was observed for DSE structures, namely microsclerotia, as their number was significantly reduced in September. However, the frequency of DSE structures microsclerotia was significantly positively related to AM structures that was not in accordance with our hypothesis. Plant water content was positively related to the density of different fungal structures. Leaf water content was positively related to the mycorrhizal frequency and to the density of specific fungal structures. Belowground dry mass was negatively related to mycorrhizal frequency. All these data revealed the importance of fungal colonisation for plant water status.

Povzetek

Presihajoča mokrišča zaznamujejo skrajna nihanja ravni vodostaja, ki vplivajo na habitate v času in prostoru. Raziskave glivne kolonizacije amfibijskih rastlinskih vrst v presihajočih ekosistemih so pokazale, da se pogostnost glivne kolonizacije znatno razlikuje med rastlinami, ki rastejo različnih vodnih razmerah. Predpostavili smo, da prisotnost površinske vode v habitatu visokega trpotca (*Plantago altissima* L.) negativno vpliva na kolonizacijo rastlinskih korenin z AM glivami in pozitivno na pojavljanje struktur temnih septiranih endofitov, ki blažijo stresne razmere, ter da je obseg glivne kolonizacije med sezono povezan s parametri rasti in fenologijo rastlin. Raziskovali smo prisotnost in obseg AM glivnih struktur ter struktur temnih septatnih endofitov. Za oceno morebitnih interakcij med glivami in rastlinskimi parametri smo preučili tudi parametre rasti. Maja, ko je bila večina rastlin še vedno v vegetativni fazi, so se širine in dolžine listov najbolj razlikovale od drugih vzorcev. Cvetovi so se pojavili junija. Zaradi obsežnih poplav na višku sezone, je prišlo do propada nadzemnih organov in ob upadu vode rasti novih listov, kljub temu pa je raven glivne kolonizacije korenin ostala podobna. Tudi podzemna biomasa je ostala na isti ravni do septembra, ko sta biomasa listov in podzemna biomasa dosegli svoje najvišje vrednosti. Vsebnost vode v listih je bila najnižja v septembru. Intenzivnost mikorize je bila najvišja v juliju in je občutno upadla v septembru. Pogostnost vseh glivnih struktur (vključno s hifami) je bila relativno visoka, saj je segala od 50 % do 90 %. Pri rastlinah v vegetativni fazi smo opazili podobno raven pogostnosti mikorize kot med cvetenjem, medtem ko se je v septembrskem obdobju znatno zmanjšala. V času cvetenja se je število veziklov znatno povečalo in se zmanjšalo proti koncu sezone. Podoben trend smo opazili tudi pri mikrosklerocijih, strukturah DSE, katerih pogostnost se je septembra bistveno zmanjšala. Frekvenca struktur DSE je bila v pozitivni povezavi z AM strukturami, kar ni bilo v skladu z našo hipotezo. Vsebnost vode v rastlinskem tkivu je bila pozitivno povezana z gostoto večine glivnih struktur, razen z arbuskuli in svitki. Suha masa podzemnih organov je bila negativno povezana s pogostnostjo mikorize. Rezultati potrjujejo pomen glivne kolonizacije za rastline v presihajočih mokriščih.

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References

Asmelash, F., Bekele, T., Birhane, E., 2016. The potential role of arbuscular mycorrhizal fungi in the restoration of degraded lands. Front. Microbiol., 7, 1095.

Ayres, R.L., Gange, A.C., Aplin, D.M., 2006. Interactions between arbuscular mycorrhizal fungi and intraspecific competition affect size, and size inequality, of *Plantago lanceolata* L. J. Ecol., 94, 85–294.

Augé, R.M., 2004. Arbuscular mycorrhizae and soil/plant water relations. Can. J. Soil Sci., 84, 373–381.

Boeger, M.R.T., Poulson, M.E., 2003. Morphological adaptations and photosynthetic rates of amphibious *Veronica anagallis-aquatica* L. (Schropulariaceae) underdifferent flow regimes. Aquatic Botany, 75, 123–135.

Bonfante, P., Genre, A., 2010. Mechanisms underlying beneficial plant–fungus interactions in mycorrhizal symbiosis. Nat. Commun, 1, 48.

Boulton, A.J., Brock, M.A., 1999. Australian Freshwater Ecology: Processes and Management. Gleneagles Publishing, Glen Osmond, Australia.

Braendle, R., Crawford, R.M.M., 1999. Plants as amphibians. Perspectives in Plant Ecology, Evolution and Systematics, 2, 56–78.

Brooker, R.W., Maestre, F.T., Callaway, R.M., Michalet, R., 2008. Facilitation in plant communities: the past, the present, and the future. J. Ecol, 96, 18–34.

Cornejo, P., Meier, S., García, S., Ferrol, N., Durán, P., Borie, F., Seguel, A., 2017. Contribution of inoculation with arbuscular mycorrhizal fungi to the bioremediation of a copper contaminated soil using *Oenothera picensis*. J. Soil Sci. Plant Nutr., 17, 1, on-line version ISSN 0718-9516.

Cornwell, W.K., Bedford, B.L., Chapin, C.T., 2001. Occurrence of arbuscular mycorrhizal fungi in a phosphorus-poor wetland and mycorrhizal response to phosphorus fertilization. American Journal of Botany, 88, 1824–1829.

Cronk, J.K., Fennessy, M.S., 2001. Wetland plants: biology and ecology. Lewis Publishers, Boca Raton.

Dolinar, N., Gaberščik, A., 2010. Mycorrhizal colonization and growth of *Phragmites australis* in an intermittent wetland. Aquatic Botany, 93, 93-98.

Dolinar, N., Regvar, M., Abram, D., Gaberščik, A., 2016. Water-level fluctuations as a driver of *Phragmites australis* primary productivity, litter decomposition, and fungal root colonisation in an intermittent wetland. Hydrobiologia, 774, 69-80.

Fontana, A., Reichelt, M., Hempel, S., Gershenzon J., Unsicker S.B., 2009. The effects of arbuscular mycorrhizal fungi on direct and indirect defense metabolites of *Plantago lanceolata* L. J Chem Ecol., 35, 833.

Gaberščik, A., 1993. Measurements of apparent CO_2 flux inamphibious plant *Polygonum amphibium* L. growing over environmental gradient. Photosynthetica, 29, 473–476.

Gaberščik, A., Urbanc-Berčič, O., Kržič, N., Kosi, G., Brancelj, A., 2003. The intermittent lake Cerknica: various faces of the same ecosystem. Lakes and Reservoirs: Research and Management, 8, 159–168.

Gaberščik, A., Dolinar, N., Šraj, N., Regvar, M., 2017. What have we learnt from studying mycorrhizal colonisation of wetland plant species? In: Varma et al. (eds.): Mycorrhiza - function, diversity, state of the art. 4th ed. Cham: Springer, pp. 291-304.

Gange, A.C., West, H.M., 1994. Interactions between arbuscular mycorrhizal fungi and foliar-feeding insects in *Plantago lanceolata* L. New Phytol., 128, 79–87.

Germ, M., Gaberščik, A., 2003. Comparison of aerial and submerged leaves in two amphibious species, *Myosotis scorpioides* and *Ranunculus trichophyllus*. Photosynthetica, 41, 91–96.

Hartley, S.E, Gange, A.C., 2009. Impacts of plant symbiotic fungi on insect herbivores: Mutualism in a multitrophic context. Annu. Rev. Entomol, 54, 323–342.

Idoia, G., Nieves, G., Jone, A., 2004. Plant phenology influences the effect of mycorrhizal fungi on the development of Verticillium-induced wilt in pepper. European Journal of Plant Pathology, 110, 227.

Jacott, C.N., Murray, J.D., Ridout, C.J., 2017. Trade-offs in arbuscular mycorrhizal symbiosis: Disease resistance, growth responses and perspectives for crop breeding. Agronomy, 7, 75.

Johnson, N.C., Wilson, G.W.T., Wilson, J.A., Miller, R.M., Bowker, M.A., 2015. Mycorrhizal phenotypes and the law of the minimum. New Phytologist, 205, 1473-84.

Jumpponen A., 2001. Dark septate endophytes – are they mycorrhizal? Mycorrhiza, 11, 207–211.

Jumpponen A., Trappe J.M., 1998. Dark septateendophytes: a reiew of facultative biotrophic root-colonizing fungi. New Phytologist, 140, 295-310.

Klančnik, K., Pančić, M., Gaberščik, A., 2014. Leaf optical properties in amphibious plant species are affected by multiple leaf traits. Hydrobiologia, 737, 121.

Kranjc, A., 2003. Geologija in geomorfologija. In: Gaberščik, A. (ed), Jezero, ki izginja: Monografija o Cerkniškem jezeru. Društvo ekologov Slovenije, Ljubljana, 18–37.

Mandyam, K., Jumpponen, A., 2008. Seasonal and temporal dynamics of arbuscular mycorrhizal and dark septate endophytic fungi in a tallgrass prairie ecosystem are minimally affected by nitrogen enrichment. Mycorrhiza, 18, 145-155.

Martinčič, A., Leskovar, I., 2002. Vegetacija. In: Jezero, ki izginja - Monografija o Cerkniškem jezeru. Gaberščik A. (ed.). Društvo ekologov Slovenije, Ljubljana, 81-94.

Martinčič, A., Wraber, T., Jogan, N., Ravnik, V., Podobnik, A., Turk, B., Vreš, B., Frajman, B., Strgulc-Krajšek, S., Trčak, B., Bačič, T., Fischer, M.A., Eler, K., Surina, B., 2007. Mala flora Slovenije. Tehniška založba Slovenije, Ljubljana, 967 p.

Miller, S.P., 2000. Arbuscular mycorrhizal colonization of semi-aquatic grasses along a wide hydrological gradient. New Phytologist, 145, 145-155.

Miller, S.P., Sharitz, R.R., 2000. Manipulation of flooding and arbuscular mycorrhiza formation influences growth and nutrition of two semiaquatic grass species. Functional Ecology, 14, 738–748.

Naveed, D., Jason, B., Nishanta, R., 2012. Mycorrhizal Colonization of *Hypericum perforatum* L. (Hypericaceae) from Serpentine and Granite Outcrops on the Deer Isles, Maine. Northeastern Naturalist 19, 517-526.

Regvar, M., Vogel-Mikuš, K., Kugonič, N., Turk, B., Batič F., 2006. Vegetational and mycorrhizal successions at a metal polluted site-indications for the direction of photostabilisation. Environmental Pollution, 144, 976-984.

Salehi, A., Kouchaksaraei, M.T., Goltapeh, E.M., Shirvany, A., Mirzaei, J., 2016. Effect of mycorrhizal inoculation on black and white poplar in a lead-polluted soil. Journal of Forest Science, 62, (5): 223–228.

Smith, S. E., Read, D.J., 2008. Mycorrhizal Symbiosis. Academic Press.

Šraj, N., Pongrac, P., Klemenc, M., Kladnik, A., Regvar, M., Gaberščik, A., 2006. Mycorrhizal colonisation in plants from intermittent aquatic habitats. Aquatic Botany, 85, 331-336.

Šraj, N., Pongrac, P., Regvar, M., Gaberščik, A., 2009. Photon-harvesting efficiency and arbuscular mycorrhiza in amphibious plants. Photosynthetica, 47, 61-67.

Trouvelot, A., Kough, J.L., Gianinazzi-Pearson, V., 1986. Estimation of VA mycorrhizal infection levels. Research for methods having a functional significance. In: Gianinazzi, S., (ed.): Physiological and Genetical Aspects of Mycorrhizae, Service des Publications INRA, Paris. pp. 217–221.

Urbanc-Berčič, O., Gaberščik, A., 2001. The influence of water table fluctuations on nutrient dynamics in the rhizosphere of common reed (*Phragmites australis*). Water Science and Technology, 44, 245-250.

Urbanc-Berčič, O., Kržič, N., Rudolf, M., Gaberščik, A., Germ, M., 2005. The effect of water level fluctuation on macrophyte occurrence and abundance in the intermittent Lake Cerknica. In: Vymazal, J. (ed.): Natural and Constructed Wetlands: Nutrients, Metals and Management. Backhuys Publishers, Leiden, pp. 312–320.

Zhang, H., Ziegler, W., Han, X., Trumbore, S., Hartmann. H., 2015. Plant carbon limitation does not reduce nitrogen transfer from arbuscular mycorrhizal fungi to *Plantago lanceolata.* Plant Soil, 396, 369–380.

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Heavy metal accumulation and changes in phenylpropanoid metabolism induced by mining activities in leaves of *Pinus nigra* and *Pinus eldarica*

Akumulacija težkih kovin in spremembe fenilpropanoidne presnove v listih vrst *Pinus nigra* in *Pinus eldarica*

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Abstract: Plants can absorb pollutants produced through industrial activities. In this research, biochemistry and lignin biosynthesis processes are studied in *Pinus nigra* and *P. eldarica* trees, that were exposed to pollutants from Sarcheshmeh copper complex and from the Kentuiyeh as control area (both Iran). Needles were collected in areas adjacent to mining factory, where heavy metals (Cu, Zn, Cd and Pb) are accumulated in higher concentrations than in plants from control areas. Comparison of the two studied pine species reveals that *P. eldarica* needles amass heavy metals 27% more than *P. nigra*. There was a higher content of total phenolic compounds and flavonoids in needles growing in close vicinities of the mining activity. Lignin content was similar in both species of pine and remained unchanged regardless of the pollution level. Higher activities of enzymes in phenylpropanoid pathway in needles from polluted areas were measured for both pine species. Findings of this study suggest involvement of general phenylpropanoid pathway in heavy metal resistance of pine trees. It seems that *P. eldarica* can accumulate more heavy metals in its needles and it has greater resistance to pollutants.

Keywords: flavonoids, heavy metals, lignin, phenolic compounds, pollutants

Izvleček: Rastline lahko absorbirajo onesnažila, ki nastanejo pri različnih industrijskih aktivnostih. V raziskavi smo preučili različne biokemijske in s sintezo lignina povezane procese pri borih *Pinus nigra* in *P. eldarica*, ki so rastli na onesnaženem področju ob industrijskem kompleksu Sarcheshmeh in na kontrolnem področju Kentuiyeh (Iran). V iglicah borov, ki so rastli blizu obrata, se je kopičilo več tečkih kovin (Cu, Zn, Cd in Pb) kot v kontrolnih rastlinah. Iglice vrste *P. eldarica* so kopičile za 27% več kovin kot *P. nigra*. Iglice onesnaženih dreves so vsebovale več fenolnih spojin. Količina lignina je bila podobna pri obeh vrstah bora in ni bila povezana z onesnaženostjo. Pri obeh vrstah bora so bile izmerjene tudi višje aktivnosti encimov fenilpropanoidne presnove. Izsledki raziskave nakazujejo, da so temeljne fenilprpanoidne poti vključene v odpornost borovih dreves proti težkim kovinam. Vrsta *P. eldarica* akumulira več težkih kovin in je bolj odporna proti onesnažilom.

Ključne besede: flavonoidi, težke kovine, lignin, fenolne spojine, onesnažila

Introduction

Mining activities and smelting produce toxic gases and other pollutants, such as hydrochloric acid, sulphuric acid, fluoride, magnesium, and heavy metals on a continuous basis (Salomons 1995). Near the non-ferrous metal smelters, high concentrations of toxic compounds have been detected in soils and vegetation. Recent reports indicate that regardless of the form of anthropogenic trace metals, their availability to plants is significantly higher than those of natural origin (Kabata-Pendias 2010).

Plants growing in industrial areas absorb a variety of pollutants. Toxic particles cause reduced growth and yield, as well as leaf injury in sensitive plants. Before exhibiting visible damage to leaves, plants encounter physiological changes when exposed to pollutants (Seyyednejad et al. 2011). Activation of phenylpropanoid metabolism has been reported for responses to biotic and abiotic stress, such as heavy metals, UV irradiation, and drought stress (Dixon and Paiva 1995). Phenylpropanoids are a diverse group of plant secondary metabolites, including anthocyanin, flavonols, proanthocyanidins (PAs), and lignin that accumulate in wide variety of plant tissues (Deluc et al. 2006).

Phenolic compounds such as phenolic amides are part of the plant cell walls. Overexpression of the enzymes responsible for their biosynthesis and the increase in their incorporation into the cell wall has been proved in biotic and abiotic stress conditions (Moura et al. 2010). Functions of phenylpropanoid compounds in defending the plant range from preformed or inducible physical and chemical barriers against infections to signal molecules involved in local and systemic signaling for defense genes induction (Dixon et al. 2002).

Both deficiency and high concentration of nutrients can cause abnormalities in accumulation of phenolic compounds and the lignin content (Grabber 2005). Lignin renders rigidity and strength to the cell wall and supports standing and plants resistance to pests and microorganisms (Jones et al. 2001). Phenolic precursors are polymerized to lignin through an oxidative mechanism. It is reported that peroxidases (PODs, a H_20_2-dependent hemoprotein) and laccases (an oxygen-dependent oxidase) perform the polymerization of monolignols into lignin (ten Have and Teunissen 2001).

Open pit mining at Sarcheshmeh Copper factory, the largest porphyry copper deposit in Iran, has been active for more than 30 years; and the concentration, melting, and molding plants are currently operating at full capacity. Reverb and converter stacks of the smelting plant respectively release 136 000 and 163 000 m^3/h of gaseous emissions, 24 hours a day, every day. Emissions include NOX, SO_2, CO_2, as well as metal particles (Ardejani et al. 2008). Different plant species have been planted in areas surrounding the factory for ornamental purposes, amongst them two pine species of *Pinus eldarica* and *P. nigra.* These trees grow in vicinity of both reverb and converter stacks and in areas at longer distances from the mining zone.

To determine the probable physiological response of pine trees to anthropogenic mining activities, some of the biochemical compounds and enzymes found in lignin biosynthesis pathways were studied in needles of two pine species (*P. nigra* and *P. eldarica*) collected in the vicinity of the copper mining factory and compared to needles of trees that grow in control areas distant from the factory.

Material and methods

Sarcheshmeh Copper mining factory, the largest porphyry copper deposit in the central region of the Zagros Mountain Range is located 160 km southwest of Kerman and 50 km south of Rafsanjan in Kerman Province, Iran. Kantuiyeh area, 9 km away from the Sarcheshmeh mining factory, was used for comparison. This area was selected because it is located upwind from the factory and the surrounding hills give it some protection from the factory`s pollution. Some plant species such as *P. eldarica* and *P. nigra* were ornamentally planted at different distances from the factory. In this study, three 8 m^2 plots dominated by *P. eldarica* and *P. nigra* were selected as replicates in area adjacent to the factory (as heavily polluted area) and Kentuiyeh (as control area). Needles were collected from the lower branches of the 15 years old tress and compared for their lignin content, total phenolic, flavonoids, and enzymes

involved in lignin biosynthesis. The content of some heavy metals such as Cu, Pb, Cd and Zn were also measured in needle samples.

Total phenolic content

Phenolic compounds were determined using the modified method of Singleton and Rossi (1965). 0.5 gr of fresh needles were extracted in 95% ethanol. The extract was mixed with 0.5 ml of Folin–Ciocalteu's reagent (diluted 1:1 with water) and 1 ml of a 5% sodium carbonate solution added. The absorption at 725 nm was measured after 1 h (Singleton and Rossi 1965). Gallic acid was used as a standard and results were expressed as mg g^{-1}dw.

The total phenolic content was expressed as gallic acid equivalents (GAE) in microgram per gram of extract (µg GAE g^{-1} extract).

Flavonoids content

The flavonoids content was analyzed as described by Chang et al. (2002). Based on this method, 0.1 g of each sample was pulverized in 10 ml methanol and centrifuged at 12 000 g. 0.5 ml of supernatant was added to 1.5 mL of methanol, 0.1 mL of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate, and 2.8 ml of distilled water, and left at room temperature for 30 min. Absorbance of the reaction mixture was measured at 415 nm with a double beam UV/Visible spectrophotometer (Perkin Elmer, USA). Quercetin was used for calibration curve (Chang et al. 2002).

Lignin content

Lignin content was assayed based on acetyl bromide protocol (Fukushima and Hatfield 2001). For that, 100 mg of air-dried samples were pulverized in 12.0 ml distilled water. The reagent then centrifuged at 10,000 g for 10 min and the resulting pellet was washed twice, using pure ethanol, each time for 30 min after filtration and then incubated in mixture of choloroform and ethanol (2:1 V/V) overnight. The filtered solution was centrifuged at 10 000 g for 20 min. The supernatant was removed and the obtained pellet was air dried. The dried sample was weighed, grinded and stored in desiccator.

6 ml of stored sample was digested in 2.5 mL of a 25% acetyl bromide in acetic acid reagent containing 0.1 ml 70% perchloric acid at 71 ºC for 30 min, with occasional mixing. After cooling in cold water, 6.0 ml glacial acetic acid and 5 ml 0.3 M NaOH was added to the volume. After bleaching, the volume was brought up to 25 ml using glacial acetic acid and centrifuged (3 000 g, 15 min). Optical density was measured at 280 nm and concentration was determined using the following equation:

$$\text{Lignin (\%)} = \frac{100\,(\text{As} - \text{Ab}) \times \text{V}}{a \times w}$$

where, As is the sample absorbance at 280 nm, Ab is blank absorbance at 280 nm, V is final volume, w is dry weigh of pellet and a is extinction coefficient (20.09 nm^{-1} cm^{-1}). A blank was included to correct for reagent background absorbance (Lacerda et al. 2006).

Preparation of extracts for enzyme activity

0.5 g of frozen leaves were homogenized in 50 mM phosphate buffer (pH 7.2) containing 1 mM EDTA, 1 mM PMSF and 1% PVP. The homogenate was centrifuged at 14 000 g for 15 min at 4 ˚C. The supernatant was used directly for the assay of enzyme activity and Bradford protein content assay using bovine serum albumin as the standard.

Coniferyl alcohol dehydrogenase (CAD, EC 1.1.1.195)

CAD activity was measured following the oxidation of appropriate hydroxycinnamyl alcohol at 30 ºC. The assay mixture (1 mL) contained 50 µl of 2M coniferyl alcohol, 100 mM Tris-HCl buffer (pH 9.3), 100 µl NADPH, and 10 µl of enzyme extract. The formation of coniferyl aldehyde was assayed at 390 nm (Halpin et al. 1998).

Phenylalanine ammonia lyase (PAL, EC 4.3.1.5)

Enzyme activity was assayed radio-chemically using L-Phe as the substrate at 35 ºC, based on the method of Thorpe and Hall (1984). The

reaction mixture consisted of 0.5 ml 10 mM L-phenylalanine, 0.4 ml deionized distilled water and 100 µl of supernatant. The reaction mixture was incubated at 37 ºC for 1 h. The reaction was terminated by the addition of 0.5 ml 6M chloridric acid (Thorpe and Hall 1984). PAL activity in the buffer was determined from production of cinnamate during 1 h as measured from absorbance change at 260 nm. A unit of enzyme activity was defined as the amount of enzyme required for the formation of 1 mol of product in 1 min under assay conditions. Cinnamic acid standard curve was used for determination of product content.

Polyphenol oxidase (PPO, EC 1.14.18.1)

The reaction mixture for polyphenoloxidase activity consisted of 2800 µl 0.2 M tris buffer, pH 6.8 in 0.02 M pyrogallol, and 100 µl enzyme extract. The absorbency of the purpurogallin formed was taken at 420 nm. The content of the rest of pyrogallol in reaction mixture was calculated using extinction coefficient of 6.2 $mM^{-1}cm^{-1}$ (Kar and Mishra 1976). PPO activity was expressed as units per mg of protein. A unit of enzyme activity was defined as the enzyme required for formation of 1 M pyrogallol to purpurogallin**.**

Guiacol peroxidase (GPX, EC 1.11.1.7)

Peroxidase activity was measured using the method of Plewa et al. (1991). Guaiacol oxidation (tetraguaiacol formation) was monitored by reading the absorbance at 470 nm. The increase in absorbance was recorded until constant which was estimated about 3 min in this study. Activity was calculated using extinction coefficient 25.5 $mM^{-1}cm^{-1}$ at 470 nm for tetraguaiacol and expressed in units per gram of protein. The enzyme unit in the extract was calculated for the formation of 1 mM tetraguaiacol for 1 minute.

Atomic absorption spectroscopy

To determine the accumulation of Cu, Zn, Pb and Cd in plants' needles, atomic absorption spectroscopy analysis was conducted. 5 ml of 65% nitric acid and 1 ml 75% hydrochloric acid were added to 1 g of dried needle sample. The mixture was heated for 1 hour at 80 ºC in water bath. After digestion, the final total volume of 50 ml was reached using ion-free water. The obtained solution was analyzed using GFA spectra-220 atomic adsorption spectrometer. GTA-110 graphite tube atomizer was used for determination of copper, zinc, lead and cadmium.

Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) followed by Duncan's test. Mean values of three replicates were calculated and the differences were considered significant for $p<0.05$.

Results and discussion

Heavy metals accumulation in pine needles

Cd, Pb, Cu and Zn contents were significantly higher in needles collected from trees in polluted areas adjacent to the mining factory relative to amounts found in needles collected from control trees in Kentuiyeh (Tab. 1). *P. eldarica* accumulated higher amounts of Cu, Zn and Cd, while *P. nigra* trees had higher amounts of Pb in their needles (Tab. 1). Both species collected high amounts of Cd in needles. *P. eldarica* accumulated much more contents of Cd collecting 310.10 µg g^{-1} DW compared to Cu with 240.06 µg g^{-1} DW.

It's been confirmed that copper smelting and toxic emissions at Sarcheshmeh Copper Complex have polluted the surrounding soil. Sarcheshmeh Copper Complex surface soil was shown an area polluted with potentially toxic metals such as As, Cu, Pb, Zn, Mo, and Cd (Rastmanesh et al. 2011). Our results indicate higher contents of heavy metals, such as copper, zinc, cadmium and lead accumulated in plants grown in vicinity of Sarcheshmeh copper mining factory, as compared to plants at Kentuiyeh area, located 9 km upwind from the factory surrounded by hills. Results also indicate that *P. eldarica* trees have greater capacity for amassing heavy metals from polluted areas than *P. nigra* trees. The result of other researchers show that majority of contaminated areas are in the prevailing wind directions coming from North and North-East (Rastmanesh et al. 2011).

Table 1: Content of Cu, Zn, Cd and Pb in needles of *P. eldarica* and *P. nigra* near Sarcheshmeh copper mining factory (polluted area) and in Kantuiyehas (control area). Mean value ± SE is shown (n = 3). Different letters in superscript indicate statistical significance between both areas and pine species (ANOVA and Duncan test, $p< 0.05$).

Tabela 1: Vsebnost Cu, Zn, Cd in Pb v iglicah *P. eldarica* in *P. nigra* na onesnaženem področju blizu bakrovega rudnika Sarcheshmeh in na kontrolnem področju Kantuiyehas. Prikazane so povprečne vrednosti ± SN (n = 3). Različne črke v nadpisu prikazujejo statistično značilne razlike med področjema in borovima vrstama (ANOVA in Duncan-ov test, $p< 0,05$).

		Heavy metal content (μg g^{-1} DW)			
		Cu	Zn	Cd	Pb
P. eldarica	Polluted	240.06±8.21^{a}	34.61±2.08^{a}	310.10±12.16^{a}	14.47±2.90^{b}
	Control	48.32±3.65^{c}	18.35±0.57^{b}	11.72±1.82^{c}	3.39±0.68^{c}
P. nigra	Polluted	175.41±6.40^{b}	38.66±2.01^{a}	176.39±4.68^{b}	49.64±7.61^{a}
	Control	46.18±3.10^{c}	19.44±.06^{b}	8.12±2.90^{c}	3.81±1.14^{c}

DW – dry weight

Phenylpropanoids compounds content

The contents of the total phenolic compounds ($p=0.22$) and total flavonoids ($p=0.01$) (Fig. 1) in needles collected from the polluted site were significantly higher than amounts found in Kentuiyeh area; for both pine species studied here. On the other hand, there was no significant difference ($p=0.165$) between lignin contents in needles collected from either of the two areas for both species (Fig. 1).

Based on the results from current project, mining activities influenced the phenylpropanoid pathway, suggesting a possible role of phenolic in response to environmental pollutants. Enhanced accumulation of phenolic is considered to be one of the most common reactions of plants to stressful conditions (Wild and Schmitt 1995). It has been demonstrated that long-term exposure to air pollutants leads to enhanced accumulation of phenolic compounds (Gostin 2009a, Gostin 2009b). Based on our results that show higher phenolic and flavonoid contents in pine trees in area close to mining activity, it seems that chronic exposure to toxic emissions of Sarcheshmeh Copper Complex stimulates carbon flux in the secondary metabolic pathways; hence, inducing a shift of the available resources towards the synthesis of secondary products. Researchers proposed specification of the total and simple phenolic compounds and also flavonoid contents in the needles as biological indicators of pollution and air quality in Aleppo pine (*Pinus halepensis*) (Pasqualini et al. 2003, Robles et al. 2003). In vitro studies have shown that flavonoids can directly scavenge molecular species of active oxygen: superoxide, hydrogen peroxide, hydroxyl radical, singlet oxygen or peroxyl radical. Their antioxidant activity is due to their ability in donating electrons or hydrogen atoms (Michalak 2006).

It has been proposed that heavy metals such as Al or Pb may suppress metabolic pathways for lignin biosynthesis in plants. In this study, no significant difference in lignin content was found in either of the pine species. However, higher lignin biosynthesis in presence of excess Cu concentrations has been reported (Michalak 2006). It has been demonstrated that cellulose and lignin biosynthesis activities (enzymes and transcripts) are reduced by the ozone in hybrid poplar wood; as a consequence of decrease in cambial activity and cell wall production (Richet et al. 2011). From the results of current study, it seems that pine trees and with more specification, *P. elderica*, raise phenolic and flavonoids production throughout activation of phenylpropanoid pathway to tolerate stressful condition in area close to mining activities. While little amounts of phenylpropanoid compounds go through lignin biosynthesis in pine needles.

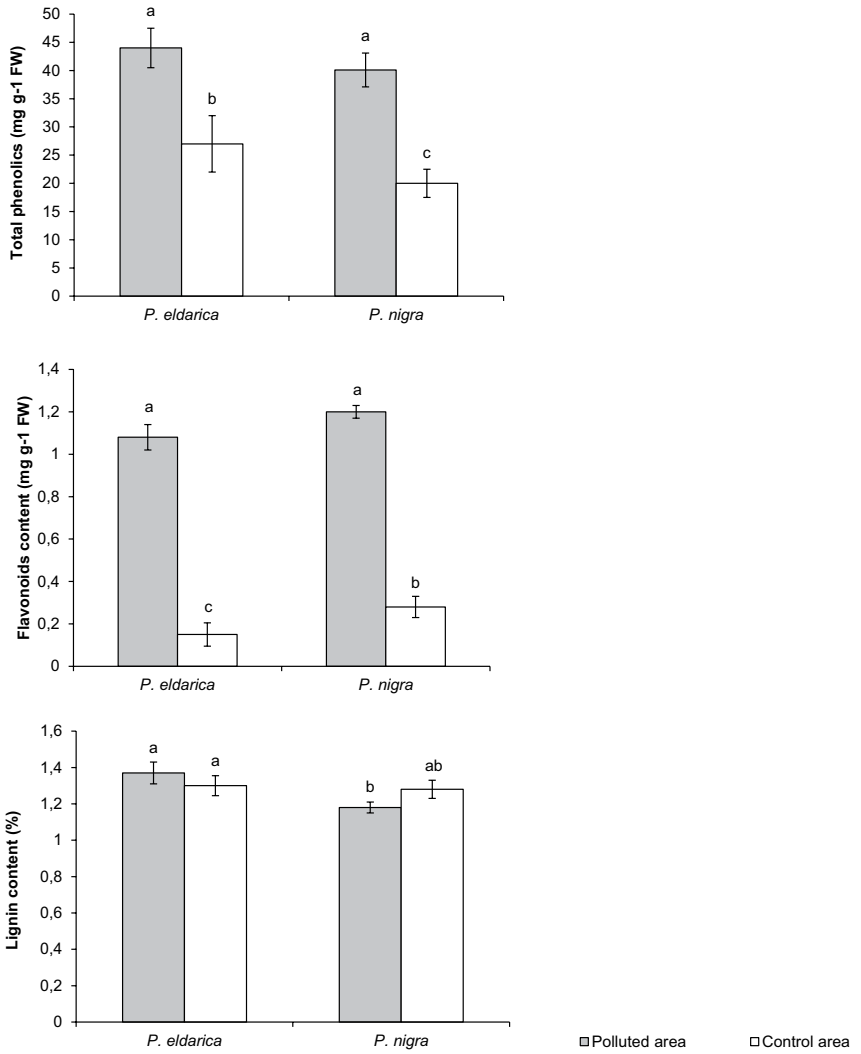

Figure 1: The content of total phenolic compounds, total flavonoids and lignin in needles of *P. eldarica* and *P. nigra* near Sarcheshmeh copper mining factory (polluted area) and in Kantuiyehas (control area). Mean value ± SE is shown (n = 3). Different letters in superscript indicate statistical significance (ANOVA and Duncan test, $p < 0.05$).

Slika 1: Vsebnost fenolnih spojin, flavonoidov in lignina v iglicah *P. eldarica* in *P. nigra* na onesnaženem področju blizu bakrovega rudnika Sarcheshmeh in na kontrolnem področju Kantuiyehas. Prikazane so povprečne vrednosti ± SN (n = 3). Različne črke v nadpisu prikazujejo statistično značilne razlike (ANOVA in Duncan-ov test, $p < 0,05$).

Activity of enzymes involved in phenylpropanoid pathway

In this study, activities of CAD and PAL were higher in *P. eldarica* needles in areas adjacent to the factory. In *P. nigra,* CAD activity was similar in both sites and PAL was higher in the control site in *P. nigra* needles (Fig. 2).

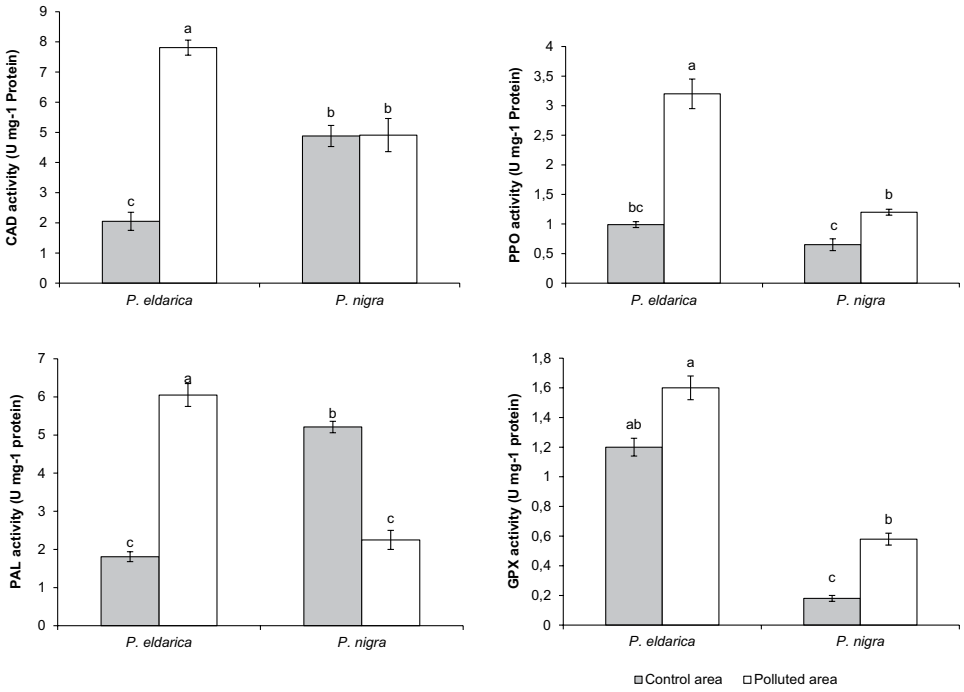

Figure 2: The enzyme activity of coniferyl alcohol dehydrogenase (CAD), phenylalanine ammonia lyase (PAL), polyphenol oxidase (PPO) and guiacol peroxidase (GPX) in needles of *P. eldarica* and *P. nigra* near Sarcheshmeh copper mining factory (polluted area) and in Kantuiyehas (control area). Mean value ± SE is shown (n = 3). Different letters in superscript indicate statistical significance (ANOVA and Duncan test, $p< 0.05$).

Slika 2: Vsebnost koniferil alkohol dehidrogeneze (CAD), fenilalanin amonij liaze (PAL), polifenol oksidaze (PPO) in gvajakol peroksidaze (GPX) v iglicah *P. eldarica* in *P. nigra* na onesnaženem področju blizu bakrovega rudnika Sarcheshmeh in na kontrolnem področju Kantuiyehas. Prikazane so povprečne vrednosti ± SN (n = 3). Različne črke v nadpisu prikazujejo statistično značilne razlike (ANOVA in Duncan-ov test, $p< 0,05$).

CAD is an indicator of lignin biosynthesis due to its specific role at the monolignol biosynthesis (Boerjan et al. 2003) which catalyzes the reduction of hydroxycinnamaldehyde to hydroxycinnamyl alcohols. CAD is expressed under stressful conditions (Galliano et al. 1993). In poplar, it has been proposed that stimulation of CAD was correlated to the increase in the first enzyme of the general phenylpropanoid pathway. PAL which catalyzes deamination of phenylalanine to cinnamate is one of the enzymes that has a key role in phenylpropanoid metabolic pathway (Iriti and Faoro 2009). It was reported that high concentrations of Cu causes accumulation of phenolic compounds and lignin along with an enhancement of polyphenol oxidase, glucose-6-phosphate dehydrogenase, shikimate dehydrogenase, PAL and CAD activities in *Panax ginseng* root suspension culture (Ali et al. 2006). Cu has indirect impact on oxidation and polymerization of monolignols (Moura et al. 2010). These finding supports our results on *P. eldarica* having higher CAD and PAL activities on areas polluted by heavy metals. However, CAD activity was the same in P. *nigra* in both areas. There was a contradiction in our findings where there were no significant changes in lignin content of

pine species in each area, while CAD and PAL activities increased in polluted areas. However, from the results achieved by phenolic and flavonoids measurements, it seems that activation of phenylpropanoids pathway caused increment of phenolic compounds not involved in lignin biosynthesis. Since gravimetric and spectrophotometric methods may suffer from interference caused by non-lignin components, there is no ideal method for measuring the lignin content (Cabané et al. 2004); hence, it is proposed that lignin structure, content and composition in different parts of the tree should be studied using other more relevant techniques.

PPO and GPX activities were higher in *P. eldarica* needles in areas adjacent to the factory. In *P. nigra* the same changes were seen for PPO and GPX activities (Fig. 2). Peroxidases are oxidoreductases that catalyze the oxidation of a diverse group of organic compounds using hydrogen peroxide as the ultimate electron acceptor (Dawson 1988). The last step in the synthesis of lignin and suberin has been proposed to be catalyzed by peroxidases (Quiroga et al. 2000). There are some evidence suggesting the role of specific peroxidases in lignin polymerization (Marjamaa et al. 2009). In soybean plants, cadmium has raised GPX and laccase activity as well as the lignin content (Finger-Teixeira et al. 2010). Peroxidases are effective quenchers of reactive oxygen species (ROS) and play an important role in the adaptation and ultimate survival of plants during periods of stress (Verma and Chandra 2014). Quiroga et al. (2000) have reported that TPX1 overexpression in tomato significantly increased the lignin content in transgenic tomato plants. However, determining the activity of peroxidases exclusively as involved in biosynthesis of lignin peroxidase is a more appropriate approach to identifying their role in phenylpropanoids pathway metabolism.

In this project, PPO showed higher activity in trees grown on polluted area. Increase in enzymes involved in phenolic compound biosynthesis has also been reported under other stress conditions such as ozone. In poplar (*Populus maximorwizzii×Populus trichocarpa*), higher levels of PAL activity were found to be associated with a greater ozone tolerance. Researchers also illustrated cadmium-induced increases in phenols, peroxidase and polyphenol oxidase activities from plant extracts (Lavid et al. 2001b). It is suggested that polymerization of polyphenols by peroxidases, enhanced after heavy-metal uptake and detoxification, is responsible for the binding of heavy metals in *Nymphaea* epidermal glands (Lavid et al. 2001a). Increase in PPO and PAL activities could be considered as the main reason for production of phenolic compounds, and flavonoids content in pine trees grown close to Sarcheshmeh Copper Complex. These changes convey the plants the ability to withstand stressful condition resulted from mining activities.

Conclusions

In summary, *P. eldarica* needles accumulated significant amounts of Cu, Zn and Cd while the accumulated contents in *P. nigra* were lower. However, Pb content was higher in *P. nigra* needles.

Our findings indicate that phenolic compounds and activities of enzymes, involved in phenylpropanid metabolism, increased in both pine species because of exposure to pollutants from the Sarcheshmeh Copper Complex. Higher content of phenolic compounds, flavonoids and higher activity of enzymes PPO, PAL and CAD in needles of both pine species suggest potential tolerance to pollution induced by stressful conditions in areas adjacent to mining activities. However, no difference in the lignin content was recorded.

Although concentrations of total phenolic, flavonoids and lignin were similar in both species, the activity of CAD, PPO and PAL was higher in *Pinus eldarica*. These findings suggest higher potential for *P. eldarica* plants to tolerate stressful conditions as created in polluted areas of Sarcheshmeh copper mining factory as compared to *P. nigra* species.

Povzetek

Iglice vrste *P. eldarica* so akumulirale značilno več Cu, Zn in CD kot iglice *P. nigra*, medtem, ko je bila vsebnost Pb višja v iglicah *P. nigra*. Rezultati so pokazali, da so se pri obeh vrstah bora povišale vsebnost fenolnih spojin in aktivnosti encimov fenilpropanoidne presnove, če so bile rastline izpostavljene onesnaženju iz

bakrovega industrijskega obrata Sarcheshmeh. Višja vsebnost fenolnih spojin, flavonoidov in višja encimska aktivnost polifenol oksidaze (PPO), fenilalanin amonij liaze (PAL) in koniferil alkohol dehidrogeneze (CAD) v borovih iglicah kaže na potencialno tolerance za stresne dejavnike, ki jih na bližnjih območjih povzroča onesnaženje. Vsebnost lignina se ni spremenila.

Kljub temu, da je bila celokupna koncentracija fenolov, flavonoidov in lignina podobna, so bile encimske aktivnosti višje pri *P. eldarica*, kar kaže, da vrsta lažje prenaša stresne razmere, ki nastanejo zaradi onesnaženja z bakrovim rudniškim obratom kot *P. nigra*.

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References

Ali, M.B., Singh, N., Shohael, A.M., Hahn, E.J., Paek, K.-Y., 2006. Phenolics metabolism and lignin synthesis in root suspension cultures of *Panax ginseng* in response to copper stress. Plant Science, 171, 147-154.

Ardejani, F.D., Karami, G., Assadi, A., Dehghan, R.A., 2008. Hydrogeochemical investigations of the Shour River and groundwater affected by acid mine drainage in Sarcheshmeh porphyry copper mine. In: 10th International Mine Water Association Congress. pp 235-238.

Boerjan, W., Ralph, J., Baucher, M. 2003, Lignin biosynthesis. Annual Review of Plant Biology, 54, 519-546.

Cabané, M., Pireaux, J.-C., Léger, E., Weber, E., Dizengremel, P., Pollet, B., Lapierre, C., 2004. Condensed lignins are synthesized in poplar leaves exposed to ozone. Plant Physiology, 134, 586-594.

Chang, C.-C., Yang, M.-H., Wen, H.-M., Chern, J.-C., 2002. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. Journal of Food and Drug Analysis, 10, 178-182.

Dawson, J.H., 1988. Probing structure-function relations in heme-containing oxygenases and peroxidases. Science, 240, 433-439.

Deluc, L., Barrieu, F., Marchive, C., Lauvergeat, V., Decendit, A., Richard, T., Carde, J.P., Merillon, J.M., Hamdi, S., 2006. Characterization of a grapevine R2R3-MYB transcription factor that regulates the phenylpropanoid pathway. Plant Physiology, 140, 499-511.

Dixon, R.A., Achnine, L., Kota, P., Liu, C.J., Reddy, M., Wang, L., 2002. The phenylpropanoid pathway and plant defence—a genomics perspective. Molecular Plant Pathology, 3, 371-390.

Dixon, R.A., Paiva, N.L., 1995. Stress-induced phenylpropanoid metabolism. The Plant Cell, 7, 1085-1097.

Finger-Teixeira, A., Ferrarese, M.d.L.L., Soares, A.R., da Silva, D., Ferrarese-Filho, O., 2010. Cadmium-induced lignification restricts soybean root growth. Ecotoxicology and Environmental Safety, 73, 1959-1964.

Fukushima, R.S., Hatfield, R.D., 2001. Extraction and isolation of lignin for utilization as a standard to determine lignin concentration using the acetyl bromide spectrophotometric method. Journal of Agricultural and Food Chemistry, 49, 3133-3139.

Galliano, H., Cabané, M., Eckerskorn, C., Lottspeich, F., Sandermann H.Jr., Ernst, D., 1993. Molecular cloning, sequence analysis and elicitor-/ozone-induced accumulation of cinnamyl alcohol dehydrogenase from Norway spruce (*Picea abies* L.). Plant Molecular Biology, 23, 145-156.

Gostin, I.N., 2009a. Air pollution effects on the leaf structure of some Fabaceae species. Notulae Botanicae Horti Agrobotanici Cluj-Napoca, 37, 57-63.

Gostin, I.N., 2009b. Structural modification induced by air pollutants in *Plantago lanceolata* leaves. Analele Universitãtii din Oradea, Fascicula Biologie, 16, 61-65.

Grabber, J.H., 2005. How do lignin composition, structure, and cross-linking affect degradability? A review of cell wall model studies. Crop Science, 45, 820-831.

Halpin, C., holt, K., Chojecki, J., Oliver, D., Chabbert, B., Monties, B., Edwards, K., Barakate, A., Foxon, G.A., 1998. Brown-midrib maize (bm1)–a mutation affecting the cinnamyl alcohol dehydrogenase gene. The Plant Journal, 14, 545-553.

Iriti, M., Faoro, F., 2009. Chemical diversity and defence metabolism: how plants cope with pathogens and ozone pollution. International Journal of Molecular Sciences, 10, 3371-3399.

Jones, L., Ennos, A.R., Turner, S.R., 2001. Cloning and characterization of irregular xylem4 (irx4): a severely lignin-deficient mutant of Arabidopsis. The Plant Journal, 26, 205-216.

Kabata-Pendias, A., 2010. Trace elements in soils and plants, 4th ed. CRC press, Taylor and Francis Group, Boca Raton, London, New York.

Kar, M., Mishra, D., 1976. Catalase, peroxidase, and polyphenoloxidase activities during rice leaf senescence. Plant Physiology, 57, 315-319.

Lacerda, R.S., Gomide, C.A., Fukushima, R.S., Schmidt, R.J., Herling, V.R.,2006 . Lignin concentration in oat (*Avena byzantina* L.) aerial part as measured by four analytical methods. Brazilian Journal of Veterinary Research and Animal Science 43, 400-407.

Lavid, N., Schwartz, A., Lewinsohn, E., Tel-Or, E., 2001a. Phenols and phenol oxidases are involved in cadmium accumulation in the water plants *Nymphoides peltata* (Menyanthaceae) and *Nymphaeae* (Nymphaeaceae). Planta, 214, 189-195.

Lavid, N., Schwartz, A., Yarden, O., Tel-Or, E., 2001b. The involvement of polyphenols and peroxidase activities in heavy-metal accumulation by epidermal glands of the waterlily (Nymphaeaceae). Planta, 212, 323-331.

Marjamaa, K., Kukkola, E.M., Fagerstedt, K.V., 2009. The role of xylem class III peroxidases in lignification. Journal of Experimental Botany, 60, ,367-376

Michalak, A., 2006. Phenolic compounds and their antioxidant activity in plants growing under heavy metal stress. Polish Journal of Environmental Studies, 15, 523-530.

Moura, J.C.M.S., Bonine, C.A.V., De Oliveira Fernandes Viana, J., Dornelas, M.C., Mazzafera, P., 2010. Abiotic and biotic stresses and changes in the lignin content and composition in plants. Journal of Integrative Plant Biology, 52, 360-376.

Pasqualini, V., Robles, C., Garzino, S., Greff, S., Bousquet-Melou, A., Bonin, G., 2003. Phenolic compounds content in *Pinus halepensis* Mill. needles: a bioindicator of air pollution. Chemosphere, 52, 239-248.

Plewa, M.J., Smith, S.R., Wagner, E.D., 1991. Diethyldithiocarbamate suppresses the plant activation of aromatic amines into mutagens by inhibiting tobacco cell peroxidase. Mutation Research, 247, 57-64.

Quiroga, M. Guerrero, C., Botella, M.A., Barcelo, A., Amaya I., Medina, M.I., Alonso, F.J., de Forchetti, S.M., Tigier, H., Valpuesta, V., 2000. A tomato peroxidase involved in the synthesis of lignin and suberin. Plant Physiology, 122, 1119-1128.

Rastmanesh, F., Moore, F., Kopaei, M.K., Keshavarzi, B., Behrouz, M., 2011. Heavy metal enrichment of soil in Sarcheshmeh copper complex, Kerman, Iran. Environmental Earth Sciences, 62, 329-336.

Richet, N. Afif, D., Huber, F., Pollet B., Banvoy, J., El Zein, R., Lapierre, C., Dizengremel, P., Perre, P., Cabane, M., 2011. Cellulose and lignin biosynthesis is altered by ozone in wood of hybrid poplar (*Populus tremula* × *alba*). Journal of Experimental Botany, 62, 3575-3586.

Robles, C., Greff, S., Pasqualini, V., Garzino, S., Bousquet-Melou, A., Fernandez, C., Korboulewsky, N., Bonin, G., 2003. Phenols and flavonoids in Aleppo pine needles as bioindicators of air pollution. Journal of Environmental Quality, 32, 2265-2271.

Salomons, W., 1995. Environmental impact of metals derived from mining activities: processes, predictions, prevention. Journal of Geochemical Exploration, 52, 5-23.

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Vital fluorescent staining for non–destructive studies of neuromast topography in urodele amphibians

Uporaba vitalnega fluorescentnega barvila kot nedestruktiven pristop za raziskave razporeditve nevromastov pri repatih dvoživkah

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Abstract: Neuromasts are mechanosensory organs found in primarily aquatic vertebrates, including many species of amphibians, and are arranged as specific patterns to form the lateral-line system on the head and along the body. We used a hair-cell-specific fluorescent dye, DiASP, to analyze the distributional pattern of neuromasts in the lateral line system of live captive-born larvae of the Italian crested newt, *Triturus carnifex* (Laurenti, 1768). We confirmed that DiASP presents a safe and accurate alternative method for non-destructive studies of neuromast ontogeny and distribution in live amphibians. All newt larvae subjected to analyses survived and no teratogenic effects of DiASP on their further development were observed. We were able to use these data to completely characterize the distribution of neuromasts in this species and to infer the functional significance of this distribution. Cross-species comparison of general topography points to neuromast arrangement as a conserved trait in urodelans.

Keywords: fluorescent staining, DiASP, neuromasts topography, salamanders

Izvleček: Nevromasti so mehanosenzorični organi primarno vodnih vretenčarjev, vključno z mnogimi vrstami dvoživk. Nameščeni so v specifičnih vzorcih in oblikujejo sistem bočne linije na glavi in vzdolž telesa. Z nedestruktivno metodo barvanja z vitalnim fluorescentnim barvilom DiASP smo analizirali vzorec razporeditve nevromastov v bočni liniji v ujetništvu rojenih ličink velikih pupkov *Triturus carnifex* (Laurenti, 1768). Potrdili smo, da DiASP predstavlja varno in natančno alternativo za nedestruktivne študije ontogenije nevromastov in njihove razporeditve pri živih dvoživkah. Vse tretirane ličinke so preživele, teratogenih učinkov DiASP na nadaljni razvoj nismo zasledili. S pridobljenimi podatki smo v celoti karakterizirali razporeditev nevromastov pri tej vrsti in sklepali na funkcionalen pomen razporeditve. Medvrstna primerjava kaže na razporeditev nevromastov kot konzervativno lastnost repatih dvoživk.

Ključne besede: flourescentno barvanje, DiASP, topografija nevromastov, repate dvoživke

Introduction

Neuromasts are mechanosensory organs constituting the functional units of the lateral-line system of aquatic vertebrates including hagfish, lampreys, fishes and amphibians (Russell 1976, Duellman and Trueb 1986, Lannoo 1988, Coombs et al. 1988, Webb 2014). In amphibians they are present in aquatic larvae and adult salamanders that retain an aquatic lifestyle (e.g. *Amphiuma, Andrias, Cryptobranchus, Necturus, Pleurodeles, Proteus, Siren*, and some species of *Ambystoma*), as well as in permanently aquatic pipid frogs (e.g. *Pipa, Xenopus*). A few species of urodeles (e.g. *Notophtalmus viridescens*) retain their lateral line system throughout life, although it partially regresses during the terrestrial subadult ("red eft") phase (Dawson 1936); the neuromasts become fully functional again when the adults return to water.

Neuromasts in aquatic amphibians enable the animals to detect water disturbances caused by currents, the movements and sounds of nearby animals, and a variety of other sources (Dijkgraaf 1962, Shelton 1970, Russell 1976, Duellman and Trueb 1986, Lannoo 1987, Hong et al. 2000). They function as a "distant touch" sensory system important to locate objects in their environment and also play an active role in localization and orientation. Unlike other vertebrate sensory systems such as eyes and nostrils, which are located in a pair of complex organs on the head, the neuromasts are distributed widely over the head and body in stereotyped patterns established during embryogenesis, and function together with electroreceptive ampullary organs to form the lateral-line component of the functionally integrated vestibular system (Dijkgraaf 1962, Lanoo 1988, Coombs and Bleckmann 2014).

The neuromasts of amphibians lie in the epidermis of the skin and consist of three types of cells. The mantle cells lie peripherally and surround the centrally positioned sensory hair cells and supporting cells that in turn surround and separate the sensory cells (Shelton 1970, Sato 1976, Russell 1976, Lannoo 1985, Coombs, et al. 1988, Webb 2014). At the apical part of every sensory hair cell are a kinocilium and many stereocilia that decrease in length with increasing distance from the kinocilium. The sensory hair cells are covered with a gelatinous cupula secreted by the supporting cells of neuromast (Dijkgraaf 1962, Webb 2014). Each hair cell is polarized with the kinocilium always located on the periphery (Dijkgraaf 1962, Flock and Wersäll 1962, Flock and Duvall 1965). The neuromasts are also arranged in lines and in different orientations that maximize the overall sensitivity of the sensory system (Lannoo 1987). Individual neuromasts are maximally sensitive to water currents in one plane only along their long axis, and different neuromasts are oriented so that their planes of maximal sensitivity are in different directions (Dijkgraaf 1962, Flock and Wersäll 1962).

Embryonically, neuromasts develop from both the neural crest cells and epidermal placodes, specifically from pre- and post-auditory placodes, ectodermal thickenings of the temporal region of head, which are situated near the inner-ear primordia (Sato 1976, Northcutt et al. 1994, Colazzo et al. 1994). The lateral line system is completely formed right before hatching of larvae (Sato 1976, Smith et al., 1988, Northcutt et al. 1994). Each neuromast is innervated by one efferent and two afferent nerve fibers of the lateral-line nerves (Dijkgraaf 1962, Flock and Jørgensen 1974, Russell 1976, Webb 2014). Most neuromasts on the head are innervated by fibers of the anterior lateral-line nerve (lateralis anterior VII), and all remaining neuromasts by the posterior lateral line nerve (lateralis posterior X) (Russell 1976, Fritzsch 1981, Duellman and Trueb 1986).

The distribution of the neuromasts of the lateral-line system of amphibians, and especially that of urodeles, has been the subject of many studies (review of Fritzsch 1981, Lannoo 1985, 1987). However, previous studies of neuromast topography in urodelans have been done in a destructive way, requiring euthanizing the animal in order to obtain specimens suitable for conventional light microscopy and/or scanning electron microscopy. An alternative, non-destructive method using fluorescent staining for neuromast topography has been widely used for the study of neuromast topography in live fish specimens (Colazzo et al. 1994, Schuster and Ghysen 2011), but has never before been used on live urodeles. One of the most commonly used hair-cell-specific fluorescent dyes for the study of the topography of neuromasts in live zebrafish is the cationic styryl pyridinium dye DiASP (Colazzo et al. 1994, Schuster and Ghysen

2011). Here we test the applicability of DiASP fluorescent dye in live urodele larvae in order to optimize the procedure and to use it to analyze the ontogeny of neuromast distribution in larvae of the Italian crested newt (*Triturus carnifex*) and to compare with available data for other urodele species described in the literature.

Material and methods

We used a sample (n = 24) of larvae of the Italian crested newt (*Triturus carnifex* Laurenti, 1768) representing a range of ages from pre-hatching to approximately 10 weeks post-hatching. The larvae were obtained from eggs laid in captivity in the laboratory of the Chair of Zoology, Department of Biology, Biotechnical Faculty, University of Ljubljana. Adult male and female newts (total body length 14-16 cm) were collected during the mating season from a pond in the University Botanical Garden in Ljubljana, Slovenia with the approval of the Slovenian Ministry of the environment and spatial planning (permit No. 35601-23/2016-4). The adults were kept in 20 liter tanks with aerated dechlorinated tap water at 20˚C with plastic strips for the attachment of eggs during laying. After spawning the adults were immediately released back into their natural habitat at the same location.

After hatching, the young larvae were kept in plastic containers (5 x 23 x 30 cm) at low density and later kept individually in smaller plastic containers (5x 10 x 18 cm) to avoid cannibalism. The larve were fed three times per week with artemia larvae and later with enchytraeid worms cultured in the laboratory. The water in the containers was replaced with decholorinated tap water three times per week after feeding.

For neuromast topography analyses we used the hair-cell-specific cationic styryl pyridinium fluorescent dye 2-Di-4-ASP (Sigma-Aldrich D3418), which has been used for studies of neuromasts in zebrafish (Colazzo et al. 1994). Live newt larvae were incubated in 5 μM Di-4-ASP in dechlorinated tap water for 10 min followed by anesthesia in 0.3 % tricaine methane-sulphonate solution (MS222, Sigma Chemical Co., St. Louis, Mo.) buffered with 0.2 % sodium bicarbonate (pH = 7) for 2 min. The lightly anaesthetized larvae were mounted in 0.5 % agar in a Petri dish for quick observation under a stereomicroscope (Leica MZ FLIII) using a GFP1 filter and photographed with a Leica DFC290 HD digital camera and Leica LAS 4 software. After observation, which usually took 10-15 minutes, the larvae were released by dissolving the agar in dechlorinated water. Larvae were then transferred back to their plastic containers in order to monitor their further development.

Results

The fluorescent dye has at a low concentration specifically labelled all neuromasts, following a short incubation period. The neuromasts could be well discerned from other, auto-fluorescing body parts (Figs. 1-4). The staining persistent sufficiently to allow the imaging of the living larvae, but faded away after 15 - 20 minutes under illumination. All larvae of *T. carnifex* used in this study survived and developed normally.

The neuromasts lie in the epidermis of the skin and are grouped together in specific clusters on the head and in lines along the rest of the body (Figs. 1A-C, 2A-B, 3). On the head they appear more closely clustered on the snout, while the rest of the other groups form rows around the eyes, in the posterior part of head, along the lower jaw and skeletal elements of the hyoid apparatus of the ventral side of the head (Figs. 1A-C, 2A-B, 3). The typical arrangement of neuromasts on the head and trunk was completely formed before the hatching of the larvae (Fig. 1A-C).

The rows of the neuromasts on the head were divided on the basis of their position and colocalization with the skull elements into eight major groups: nasal, maxillar, circumorbital (supraorbital and infraorbital), postorbital, parietal, postotic, mandibular and submandibular groups (Figs. 1A-C, 2A-B, 3). The supraorbital neuromasts are located dorsal and medial to the eyes, while infraorbital neuromasts are located posterior and ventral to the eyes (Figs. 2A-B). The orientation of neuromasts in each circumorbital row (supra and infraorbital) is tangentially to the eye (Fig. 4A). Nasal neuromasts are arranged in two rows anterior to the supraorbital and the rows are perpendicular to each other (Figs. 2A, 4A). The maxillary neuromasts form three to four rows

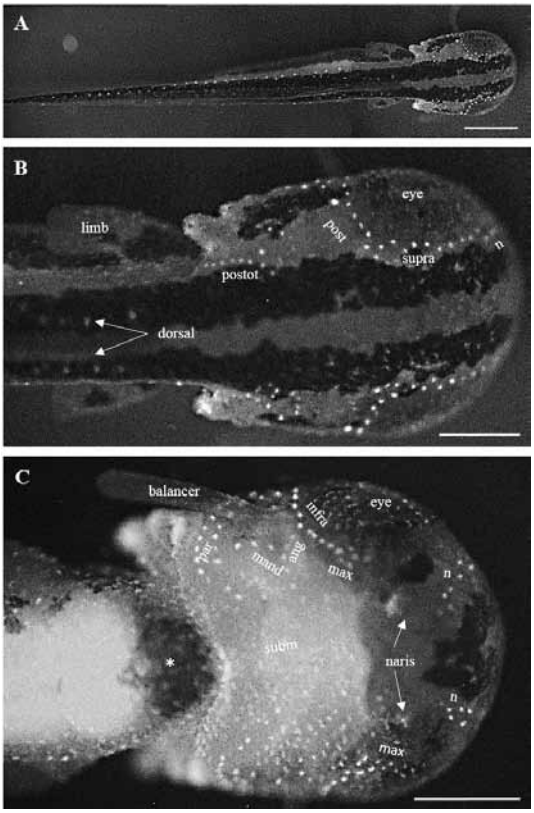

Figure 1: The neuromasts of a larva of the Italian crested newt *T. carnifex* before hatching. Fluorescent DiASP staining. **A**–Dorsal view of the whole body. **B**–Dorsal view of the head. **C**–Ventral view of the head. ang – angular group, asterisk – heart, infra – infraorbital group, max – maxillary group, mand – mandibular group, n – nasal group, par – parietal group, post – postorbital group, postot – postotic group, subm – submandibular group, supra – supraorbital group. Scale bar: 1 mm (A), 0,5 mm (B and C).

Slika 1: Nevromasti ličinke velikega pupka *T. carnifex* pred izleganjem. Fluorescentno barvanje z DiASP. **A**–Dorzalna stran telesa. **B**–Glava dorzalno. **C**–Glava ventralno. ang – angularna skupina, infra – infraorbitalna skupina, man – mandibularna skupina, max – maksilarna skupina, n – nasalna skupina, par – parietalna skupina, post – postorbitalna skupina, postot – postotična skupina, subm – submandibularna skupina, supra – supraorbitalna skupina, zvezdica – srce. Merilo: 1 mm (A), 0,5 mm (B in C).

anterior to the infraorbital group (Fig. 2B), mostly one medial and two lateral rows of neuromasts which are oriented with their long axis perpendicular to adjacent neuromasts (Fig. 2B). Both maxillary and nasal neuromasts continue in one line to the pre-maxilla part of the upper jaw. The postorbital group has fewer neuromasts that are located in one row behind the circumorbital row (supra and infraorbital) and perpendicular to the circumorbital row (Figs. 2B, 4A). The neuromasts in the postotic group form a loose pattern on the caudal portion of the dorsal part of the head and appear to be continuous with the medial line of the body neuromasts (Figs. 2A, 4A). The neuromasts in the postotic group are oriented parallel as well transverse to body axis (Fig. 4A). The

parietal group consists of two adjacent curved rows of neuromasts which transition into the submandibular group (Figs. 2B, 3). The rows of neuromasts in the parietal group are perpendicular to each other. The angular row lies posterior to the jaw angle, vertically to the infraorbital and continues to the mandibular row of neuromasts (Figs. 2B). On the ventral side of the head the submandibular group of neuromasts is arranged along the hyoid skeletal elements in two rows at the rostral part which converge to one row that extends to the caudal part of the head and joins the parietal group (Fig. 3). The rows of neuromasts at the rostral part of the submandibular group are oriented perpendicular to each other.

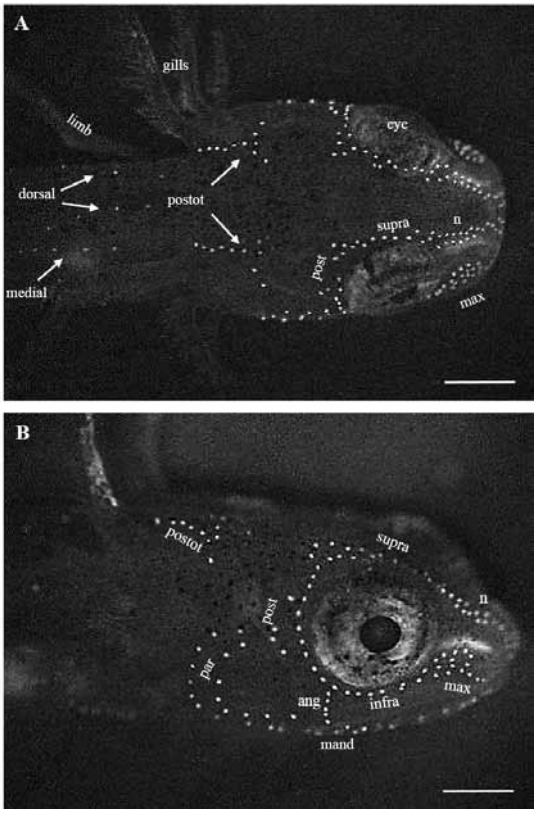

Figure 2: The head of post-hatched larva of the Italian crested newt *T. carnifex* with labeled neuromasts. Fluorescent DiASP staining. **A**–Lateral view. **B**–Dorsal view. The anterior part of trunk dorsal and medial line of neuromasts is also visible in A. ang – angular group, dorsal – dorsal line of neuromasts, infra – infraorbital group, max – maxillary group, mand – mandibular group, medial – medial line of neuromasts, n – nasal group, par – parietal group, post – postorbital group, postot – postotic group, subm – submandibular group, supra – supraorbital group. Scale bar: 1 mm.

Slika 2: Glava izležene ličinke velikega pupka *T. carnifex* z označenimi nevromasti. Fluorescentno barvanje z DiASP. **A**–Dorzalni pogled. **B**–Lateralni pogled. Na sliki A je viden tudi sprednji del dorzalne in mediane linije nevromastov trupa, ang – angularna skupina, dorsal - dorzalna linija nevromastov, infra – infraorbitalna skupina, man – mandibularna skupina, max – maksilarna skupina, medial – osrednja linija nevromastov linija nevromastov, n – nasalna skupina, par – parietalna skupina, post – postorbitalna skupina, postot – postotična skupina, subm – submandibularna skupina, supra – supraorbitalna skupina. Merilo: 1 mm.

The neuromasts on the trunk occur along three distinct lines: the dorsal, medial, and ventral line (Figs. 2A, 3). All three lines begin slightly rostral to the front limb and the medial line continues up to the tip of the tail, while the other two lines (dorsal and ventral) are shorter, and extend only to the hind limb. The dorsal line neuromasts are also very sparse and at the middle part of the trunk approach the medial line (not shown). The neuromasts in the medial and ventral lines lie parallel with the major body axis, although the rostral part of the ventral line also follows the curve of the pectoral girdle. (Figs 3, 4B). The dorsal line neuromasts are oriented dorso-ventrally, inclined at 45˚, and they are less numerous than those in the other two lines.

In young post-hatching larvae the neuromasts are situated individually in the rows on the head and along the body, but later approximately after one month after hatching the neuromasts occur in pairs (Fig. 4A-B). The total number of neuromasts on one side of the head in pre-hatched larvae is lower (75.5 ± 2.1) than in post-hatched larvae (137 ± 6.8) (Table 1) mostly due to lower number of neuromasts in the maxillary, nasal and submandibular group. The total number of neuromasts doubles in older larvae (277 ± 10.3) due to the formation of neuromast pairs. The largest numbers of neuromasts are found in the circumorbital, maxillar, nasal and submandibular group (Table 1). Along one side of the trunk, newly hatched larvae begin with approximately 56 single neuromasts but this number later doubles due to the formation of neuromast pairs. A rough estimate of the total number of neuromasts on the whole surface of the body in older larvae is approximately 750 to 800 neuromasts.

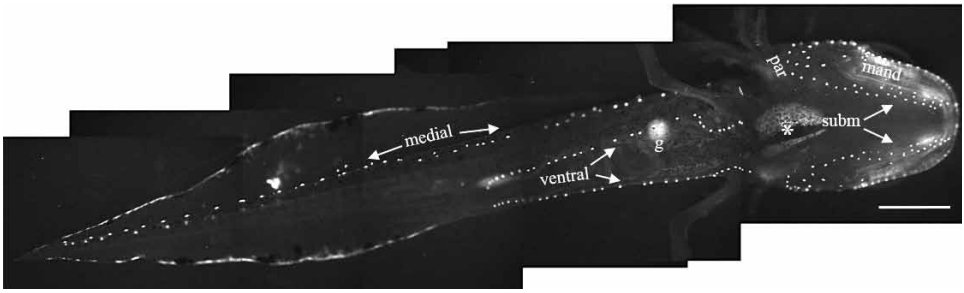

Figure 3: Fluorescent imaging of labeled neuromasts of the ventral side of the body of the Italian crested newt *T. carnifex* larva. asterisk – heart, g – gall blader, mand – mandibular neuromasts, medial – medial line of neuromasts, par – parietal neuromasts, subm – submandibular neuromasts, ventral – ventral lines of neuromasts. Scale bar: 1 mm

Slika 3: S fluorescenčnim barvilom označeni nevromasti ventralne stani telesa ličinke velikega pupka *T. carnifex.* g – žolčnik, medial – osrednja linija nevromastov, mand – mandibularni nevromasti, medial – osrednja linija nevromastov, par - parietalni nevromasti, subm – submandibularni nevromasti, ventral – ventralni liniji nevromastov, zvezdica – srce. Merilo: 1 mm.

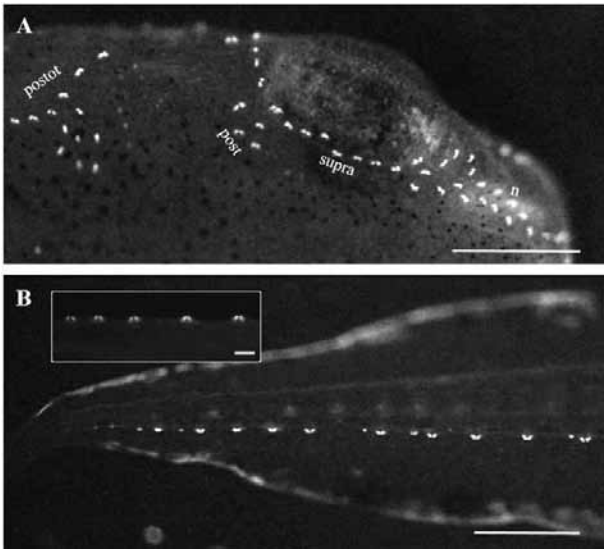

Figure 4: Older larva of the Italian crested newt *T. carnifex* with double neuromasts. Fluorescent DiASP staining. **A**–Head region with postotic (post), postorbital (post), supraorbital (supra), and nasal (n) group of neuromasts. **B**–Tail region with the medial line neuromasts. **Insert in B**–Double neuromasts of tail under higher magnification. Scale bar: 1 mm (A and B), 100 µm (insert in B).

Slika 4: Starejša ličinka velikega pupka *T. carnifex* z dvojnimi nevromasti. Fluorescentno barvanje z DiASP. **A**–Glavina regija s postotično (post), postorbitalno (post), supraorbitalno (supra) in nasalno (n) skupino nevromastov. **B**–Repna regija z mediano linijo nevromastov. **Manjša slika na B**–Dvojni nevromasti repa pod večjo povečavo. Merilo: 1 mm (A in B), 100 µm (manjša slika na B).

Table 1: The number of neuromasts on the head of larvae of Italian crested newt *T. carnifex* at different ages. The given number of neuromasts is for one side of the head.

Tabela 1: Število nevromastov na glavi pri različno starih ličinkah velikega pupka *T. carnifex*. Podano število nevromastov je za eno stran glave.

	Number of neuromasts			
Neuromast group	Before hatching (N=2)	Hatched larvae (N=2)	Post-hatched larvae (N=7)	Older larvae with double neuromasts in stitches (N=6)
angular	2.5 ± 0.7	2.5 ± 0.7	3.6 ± 0.8	7.3 ± 1.6
circumorbital	19.0 ± 1.4	20.5 ± 0.7	22.1 ± 2.7	45.7 ± 4.3
mandibular	7.0 ± 0.0	8.5 ± 0.7	9.9 ± 1.1	19.7 ± 2.3
maxillar	6.0 ± 1.4	7.0 ± 1.4	20.0 ± 1.0	40.0 ± 2.2
nasal	7.0 ± 1.4	11.0 ± 1.4	20.1 ± 2.0	42.7 ± 4.3
parietal	12.5 ± 0.7	12.5 ± 0.7	16.4 ± 1.9	34.0 ± 2.5
postorbital	5.0 ± 1.4	8.5 ± 0.7	8.4 ± 0.8	17.0 ± 1.7
postotic	9.0 ± 1.4	11.5 ± 0.7	13.6 ± 2.4	27.0 ± 5.3
submandibular	7.0 ± 0.0	21.0 ± 1.4	21.9 ± 1.9	44.3 ± 3.7
Total	75.5 ± 2.1	103.0 ± 7.1	137.0 ± 6.8	277.7 ±10.3

Discussion

The goals of this preliminary study of neuromasts of Italian crested newt *Triturus carnifex* larvae were two-fold, first to optimize a non-destructive method and to confirm that the DiASP fluorescent dye has no harmful effects on live larvae and, secondly, to perform a detailed description of neuromast distribution on the head and on the rest of the body and to compare with the available data for other urodele species.

Our work is the first non-destructive study of neuromast topography in urodele amphibians using the hair cell-specific fluorescent dye DiASP. Previous studies of neuromast topography in urodeles utilized destructive approaches that necessitated killing the animals for conventional light and electron scanning microscopy on whole specimens (review in Fritzsch 1981, Lannoo 1985, 1987). All newt larvae used in our study survived and no teratogenic effects of DiASP on their further development were observed. DiASP therefore presents an alternative, non-destructive method for studies of the ontogeny and evolution of distribution patterns of this functionally important sensory system in live amphibians.

Among aquatic amphibians, the arrangement of neuromasts in the lateral line system varies among and within the different orders (Lannoo 19987, 1988): caecilians have single rows of mechanoreceptive neuromast organs; generalized anurans have single rows of neuromasts that divide to form secondary neuromasts or "stitches"; generalized urodeles have transverse stitches, and double or triple rows of neuromasts. The general arrangement of neuromasts in *T. carnifex* larvae is similar to that described for other aquatic urodeles with three rows (dorsal, medial and ventral) on the trunk and many distinctive rows on the head divided into nasal, maxillary, supraorbital, infraorbital, postorbital, parietal, postotic, mandibular and submandibular groups (Lannoo 1987, 1988, Smith et al. 1988, Mali 1990). The neuromasts may be single, or clustered in parallel to form stitches which in turn, are organized into groups that are arranged in different orientations in a pattern that maximizes directionality (Lannoo 1985, 1987). Topographically similar neuromasts are oriented in the same direction (Lannoo 1987, 1988), which is consistent with what we found in *T. carnifex* larvae. The arrangement of circumorbital neuromasts is tangent to the eyes. The rest of the other groups of neuromasts on the head and body are oriented either parallel or perpendicular with the body axis making them sensitive to water displacements in all directions along a plane across the body surface (Lannoo 1987). Three groups of head neuromasts near the snout (nasal, maxillary and submandibular) have a more complex arrangement than the rest of the other groups: the rows are perpendicular to each other, thus allowing high resolution in prey detection (Dijkgraaf 1962, Russell 1976, Lannoo 1987).

The lateral-line system in *T. carnifex* is completely formed just before hatching, as is common for larvae of other urodelan species (Sato 1976, Smith et al. 1988, Northcutt et al. 1994), but the number of neuromasts in individual groups is lower than in post-hatching larvae of *T. carnifex.* This is especially prominent in the maxillary, nasal, and submandibular groups of neuromasts due to different head morphology which is rounder and smaller in younger larvae but then gradually elongates as it grows larger with age. Likewise, as in other post-hatching larvae of urodeles (Lannoo 1987), the neuromasts are single in younger larvae of *T. carnifex*, but later (in one month old larvae) are doubled in short stitches. The early division of individual (primary) neuromasts to form secondary neuromasts and stitches is a common characteristic for the older larvae of the salamander family Salamandridae to which *T. carnifex* belongs, and is also seen in the families Ambystomatidae, Cryptobranchidae, and Proteidae (Lannoo 1987). In most other salamander families (Hynobiidae, Dicamptodontidae, Plethodontidae, Amphiumidae and Sirenidae) the neuromasts remain single in older larvae as well in permanently aquatic adults (Russell 1976, Lanoo 1987). In all urodeles, the final number of neuromasts per stitch is variable and increases with age while the total number of stitches remains constant (Lannoo 1985). Some urodeles retain only two neuromasts per stich while others develop three or more and the number of neuromasts per stitch can even differ among the species of the same genus. For example, stitches with three neuromasts are predominant in *Ambystoma mexicanum,* while *A. triginum* has seven (Lannoo 1985). Because we did not analyze the larvae of *T. carnifex* older than three months, we

do not know how many neuromasts are found in fully formed stitches.

The total number of neuromasts not only varies among taxa, but even between the left and rights sides of the same individual (Lannoo 1987). Lannoo (1987) compared the total numbers of neuromasts from one side of the head of larvae in different species of seven urodeles families (Ambystomatidae, Amphiumidae, Dicamptodontidae, Hynobiidae, Plethodontidae, Proteidae, Salamandridae) that ranged from a mean of 94 in the smallest larvae in *Hynobius nebulosus* (with snout-vent length 14.5 mm) to a mean of 150 in larger sized larvae in *Necturus maculosus* (with snout-vent length 210 mm). The total number of single neuromasts on one side of the head in post-hatched larvae of *T. carnifex* (with snout-vent length 16.5 mm) was approximately 137 (range 126 to 148) and this is similar to that described by Lannoo (1987) for larve of *Notophthalmus viridescens* (with snout-vent length 17.5 mm). This number of neuromasts doubles in older larve of *T. carnifex* when stitches contain two neuromasts. Consistent with the literature (Lannoo 1985, 1987), the number of neuromasts in different groups on the head of *T. carnifex* varies and the groups with the largest number of neuromasts are the circumorbital, maxillar, nasal and submandibular groups. These observed patterns are important since neuromast density affects the mechanosensory ability of the neuromast system as a whole and the more neuromasts an animal has, the greater will be its ability to perceive water displacements (Lannoo 1985).

The increase in the number of neuromasts per stitch with age is accompanied by an increase in the number of hair cells in individual neuromasts (Lannoo 1985, Mali 1990). Mali (1990) described the progressive changes in the number of hair cells per neuromast in larvae of the alpine newt *Ichthyosaura alpestris*, with 2-5 hair cells in neuromasts at hatching, 3-10 hair cells after 25 days post-hatching and 11 -16 hair cells in 60-day old post-hatching larvae. The number of sensory cells per neuromast in older larvae of different species of newts (e. g. *Ichthyosaura alpestris, Lissotriton vulgaris, Triturus cristatus*) range from 12 to 20 (Fritzsch and Wahnschaffe 1983, Mali 1990). A similar number of sensory cells per neuromast was described for larvae of the fire salamander *Salamandra salamandra* (Fritzsch and Wahnschaffe 1983), adult axolotls *Ambystoma mexicanum* (Jørgensen and Flock 1973), as well as for adult European blind cave salamanders *Proteus anguinus* (Bulog 1988, Mali 1990). We did not quantify the number of hair cells in individual neuromasts because the specimens were examined under stereo microscope where individual hair cells could not be discerned. Nevertheless, it seems likely that the number of neuromast hair cells in *T. carnifex* is similar to that seen in other newts. However, further study should focus on the ontogeny of the stitches and numbers of hair cells per neuromast in *T. carnifex* larvae.

In conclusion, the DiASP fluorescent staining method is a safe and completely non-destructive method for studies of neuromast ontogeny and detailed arrangement in live urodele amphibians. Comparison of the general topography of neuromasts in *T. carnifex* larvae with other urodelan larvae confirms neuromast distribution to be a conservative trait in urodele amphibians.

Povzetek

Nevromasti so mehanosenzorični organi sistema bočne linije primarno vodnih vretenčarjev, vključno z dvoživkami. Pri slednjih so zastopani pri ličinkah in pri odraslih permanentno vodnih repatcih in brezjezičnicah. Nameščeni so v vrhnjici kože na glavi in vzdolž telesa in imajo pomembno vlogo v lokalizaciji plena in prostorski orientaciji. Razporeditev nevromastov v bočni liniji dvoživk je bila predmet mnogih raziskav, ki pa so bile izvedene na destruktiven način, saj je bilo potrebno živali evtanazirati. Alternativna nedestruktivna metoda, ki je v uporabi pri ribah in pri dvoživkah še ni bila preizkušena, je uporaba vitalnih fluorescentnih barvil, npr. kationskega barvila DiASP, ki specifično barva čutnice znotraj nevromastov. Namen naše raziskave je bil: i) preizkusiti uporabnost barvila DiASP na dvoživkah in ii) opisati ontogenijo razporeditve nevromastov pri ličinkah velikega pupka *Triturus carnifex* (Laurenti, 1768) in primerjati s podatki iz literature za druge vrste repatcev.

Za raziskavo smo uporabili v ujetništvu zležene ličinke velikega pupka v razponu starosti pred izleganjem do 10 tednov po izleganju. S pomočjo

fluorescentnega vitalnega barvila DiASP smo lahko natančno lokalizirali razporeditev nevromastov, saj je barvilo selektivno barvalo nevromaste, ki so se jasno razlikovali od morebitnih avtofluorescentih delov telesa. Vse z DiASP tretirane ličinke so preživele, prav tako nismo zasledili nikakršnih teratogenih učinkov na njihov nadaljni razvoj. Osnovna razporeditev nevromastov pri ličinkah velikega pupka je podobna razporeditvi pri ostalih predstavnikih iz družine pupkov in močeradov (Salamandridae). Nevromasti so urejeni v specifičnem vzorcu na glavi in treh paralelnih linijah na trupu. Glede na pozicijo in kolokalizacijo s skeletnimi elementi lobanje lahko nevromaste na glavi razdelimo v 8 glavnih skupin: nazalna, maksilarna, cirkumorbitalna (supra- in infraorbitalna), postorbitalna, parietalna, postotična, mandibularna in submandibularna. Najbolj kompleksna ureditev nevromastov je v predelu gobca (v nazalni, maksilarni in submandibularni skupini), saj so le-ti razporejeni v več linijah, ki potekajo pravokotno ena na drugo. Takšna ureditev omogoča zaznavanje premikov vode v različnih smereh, kar naj bi pripomoglo k učinkovitejšemu zaznavanju plena. Na trupu so nevromasti razporejeni v tri paralelne linije (dorzalna, mediana in ventralna linija), ki se pričnejo nekoliko pred sprednjimi okončinami. Medtem ko mediana linija poteka vse do konice repa, sta ostali dve liniji krajši in segata le do zadnjih okončin. Tipičen vzorec razporeditve nevromastov je pri velikem pupku izoblikovan tik pred izleganjem ličink, kar je značilno tudi za ostale repatce. Nevromasti so nameščeni posamično, kasneje (en mesec po izleganju) pa zasledimo dvojne nevromaste oz. nevromaste v parih, ki tvorijo kratke "šive" ali "stitches". Število nevromastov znotraj posameznih "šivov" je vrstno specifično, žal pa ne moremo trditi, da so dvojni nevromasti tudi končna oblika "šiva", saj ličink starejših od treh mesecev nismo vključili v študijo. Celokupno število nevromastov pred izleganjem je tudi bistveno nižje v primerjavi s starejšimi ličinkami. Slednje je še posebej izrazito na glavinem delu, predvsem na račun manjšega števila nevromastov v maksilarni, nazalni in submandibularni skupini, kar je v korelaciji s samo obliko glave, ki je pri mlajših ličinkah majhna in okrogla in se s starostjo ličink postopoma podaljšuje.

Naša raziskava je ena prvih nedestruktivnih pristopov za analizo topografije nevromastov pri dvoživkah. Potrdili smo, da je uporaba fluorescenčnega barvila DiASP varna, in da omogoča natančno analizo ontogenije nevromastov in njihove razporeditve na živih dvoživkah. Medvrstna primerjava kaže na splošno topografijo nevromastov kot konzervativno lastnost repatih dvoživk.

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References

Bulog, B., 1988. Surface ultrastructure of lateral line sensory receptors in *Proteus anguinus* Laur (Urodela, Amphibia). Inst. Phys. Conf. Ser., 93 (3), 151-152. EUREM 88, York, England.

Collazzo, A., Fraser, S.E., Mabee, P.M., 1994. A dual embryonic origin for vertebrate mechanoreceptors. Science, 264 (5157), 426-431.

Coombs, S., Janssen J.J., Webb, J.C., 1988. Diversity of lateral line system: evolutionary and functional consideration. In: Atema, J., et al. (eds): Sensory biology of aquatic animals. Springer Verlag, New York, pp. 553-554.

Coombs, S., Bleckmann, H., 2014. The germs of the past: a brief history of lateral line research in the context of the hearing sciences. In: Coombs, S., et al. (eds): The lateral line system. Springer Science & Business Media, New York, pp. 1-16.

Dawson, A.B., 1936. Changes in the lateral-line organs during the life of the newt, *Triturus viridescens*. A consideration of the endocrine factors involved in the maintenance of differentiation. JEZ Ecological Genetics and Physiology, 74 (2), 221-237.

Dijkgraaf, S., 1962. The functioning and significance of the lateral-line organs. Biol. Reel., 38, 51-105.

Duellman, W.E., Trueb, L., 1986. Lateral – line system. In: Duellman, W.E., Trueb, L. (eds): Biology of amphibians. McGraw-Hill, Inc., New York, pp. 378-379.

Flock, Å., Wersäll, J., 1962. A study of the orientation of the sensory hairs of the receptor cells in the lateral line organ of fish, with special reference to the function of the receptors. J. Cell Biol., 15, 19-27.

Flock, Å., Duvall, A.J., 1965. The ultrastructure of the kinocilium of the sensory cells in the inner ear and lateral line organs. J. Cell. Biol., 25, 1-8.

Flock, Å., Jørgensen, J.M., 1974. The ultrastructure of lateral line sense organs in the juvenile Salamander *Ambystoma mexicanum*. Cell Tiss. Res., 152, 283-292.

Fritzsch, B., 1981. The pattern of lateral-line afferents in urodeles: A horseradish-peroxidase study. Cell Tiss. Res., 218 (3), 581-594.

Fritzsch B., Wahnschaffe U., 1983. The electroreceptive ampullary organs of urodeles. Cell Tiss. Res., 229, 483–503.

Hong, C., Guo-Hua, Y., Heatwole, H., 2000. Ultrastructure of the skin mechanoreceptors of the chinese giant salamander, *Andrias davidianu.* J. Morph., 245, 80–85.

Jørgensen, J.M., Flock, Å., 1973. The ultrastructure of lateral line sense organs in the adult salamander *Ambystoma mexicanum*. J. Neurocytol., 2, 133-142.

Lannoo, M.J., 1985. Neuromast topography in *Ambystoma* larvae. Copeia, 3, 535-539.

Lannoo, M.J., 1987. Neuromast topography in urodele amphibians. J. Morphol., 191, 247-263.

Lannoo, M.J., 1988. The evolution of the amphibian lateral line system and its bearing on amphibian phylogeny. J. Zool. Syst. Evol. Res., 26 (2), 128-134.

Mali, L., 1990. Ultrastructural analysis and functional-morphological orientation of lateral-line neuromasts in alpine newt (*Triturus alpestris*) and proteus (*Proteus anguinus* Laur.). University of Ljubljana. Graduate thesis.

Northcutt, R.G., Catania, K.C., Criley, B.B., 1994. Development of lateral line organs in the axolotl. J. Comparative Neurology, 340, 480-514.

Russell, I.J., 1976. Amphibian lateral line receptors. In: Llinas, R., Precht, W. (eds): Frog neurobiology. Springer, Berlin, Heidelberg, pp. 513-550.

Sato, A., 1976. Electron microscopic study of the developing lateral-line organ in the embryo of the newt, *Triturus pyrrhogaster.* Anat. Rec., 186*,* 565-584.

Schuster, K., Ghysen, A., 2011. Imaging the development of the fish lateral-line system. In: Sharpe, J., Wong R.O. (eds): Imaging in developmental biology: a laboratory manual. Cold Spring Harbor, New York, pp. 282–283.

Shelton, P.M.J., 1970. The lateral line system at metamorphosis in *Xenopus laevis* (Daudin). J. Embryol. Exp. Morph., 24 (3), 511-524.

Smith, S.C., Lannoo, M.J., Armstrong, J.B., 1988. Lateral-line neuromast development in *Ambystoma mexicanum* and a comparison with *Rana pipiens.* J. Morph., 198, 367-379.

Webb, J.F., 2014. Morphological diversity, development, and evolution of the mechanosensory lateral line system. In: Coombs, S., et al. (eds): The lateral line system. Springer Science & Business Media, New York, pp. 17-72.

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Analiza dosežkov osnovnošolcev na nacionalnem preverjanju znanja iz biologije

Analysis of elementary school pupils' achievements on the national assessment of knowledge in biology

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Izvleček: Namen nacionalnega preverjanja znanja (NPZ) je dobiti dodatne povratne informacij o tem, kako deluje vzgojno-izobraževalni sistem in kakšna je stopnja doseganja ciljev in standardov znanja, ki jih določajo učni načrti. Biologija je bila doslej petkrat eden izmed predmetov na NPZ. V našo analizo smo zajeli 119 nalog z zadnjih štirih preverjanj (2009, 2011, 2014 in 2017), pri katerih je sodelovalo 18730 učencev. Zanimalo nas je, katere so šibke in katere močne strani biološkega znanja naših učencev ob koncu osnovnošolskega izobraževanja. Ugotovili smo, da so učenci v splošnem pokazali zadovoljivo temeljno znanje. Kakovost znanja vseh preverjanih vsebinskih enot (celica, dedovanje in biotehnologija, človek, ekologija, rastline, živali, raziskovanje in poskusi ter branje tabel, grafov ali slik) je bila podobna, pri načrtovanju pouka pa bi bilo kljub temu treba več pozornosti posvetiti področjem celice, dedovanja in biotehnologije. Po pričakovanjih so učenci pokazali več znanja pri nalogah izbirnega tipa, manj pa pri nalogah polodprtega tipa. Prav tako pričakovano so pokazali več znanja pri nalogah prve taksonomske ravni, manj pri nalogah druge in še manj pri nalogah tretje taksonomske ravni. V prihodnosti bi bilo smiselno v NPZ iz biologije vključiti še več nalog, ki bi lahko učiteljem pomagale pri načrtovanju in izvajanju pouka.

Ključne besede: osnovna šola, dosežki, znanje, biologija, nacionalno preverjanje znanja

Abstract: The purpose of the national assessment of knowledge (NAK) in Slovenia is to gather feedback information about how the national educational system works and what level of educational aims and standards that are prescribed in the national curriculum is being reached. Thus far, biology has been one of the subjects on the NAK five times. In our survey, we analysed 119 items taken from the last four NAK in biology (2009, 2011, 2014, and 2017) in which 18,730 pupils took part. We were interested in weak and strong sides of the biological knowledge of our pupils at the end of their primary education. We determined that pupils, in general, possess satisfactory basic knowledge of biology. The quality of knowledge of all six tested content units (cell, heredity, and biotechnology; human body; ecology; plants; animals; research, experiments, reading of tables, graphs, and figures) was not significantly different.

However, pupils showed the least knowledge about cell, heredity, and biotechnology; therefore, in planning lessons in the future, there should be more emphasis on these three topics. Pupils showed significantly more knowledge on multiple-choice items in comparison to half-open-ended items, which was expected. As also expected, they showed significantly more knowledge on items of the first cognitive level, less on items of the second cognitive level, and the least on items of the third cognitive level. The role of the NAK in biology should remain helping teachers in planning their lessons and, through this, positively influencing pupils' knowledge.

Keywords: primary education, achievements, knowledge, biology, national assessment of knowledge

Uvod

Nacionalno preverjanje znanja (NPZ) ob koncu tretjega vzgojno-izobraževalnega obdobja poteka v Sloveniji od šolskega leta 2000/2001 (Izhodišča nacionalnega … 2005) in je za učence 9. razreda obvezno (Zakon o osnovni šoli, 64. člen). Od šolskega leta 2005/2006 se preverja znanje treh predmetov, in sicer poleg učnega jezika in matematike še tretji predmet. Leta 2006 je bil za vse učence samo en tretji predmet preverjanja, in sicer biologija, od leta 2007 pa so kot tretji predmet po navadi štirje predmeti (leta 2011 le trije). Kateri štirje predmeti bodo izbrani za preverjanje kot »tretji« predmet, vsako leto septembra izmed obveznih predmetov 8. in 9. razreda s sklepom določi minister za šolstvo. Na katerih šolah bo preverjen kateri od teh štirih predmetov, pa se določi z naključnim razporejanjem šol znotraj statističnih regij v skupine za določen predmet, pri čemer se upošteva tudi velikost šol (Cankar 2014; Letno poročilo … 2017). Biologija je bila doslej petkrat tretji predmet na NPZ (2006, 2009, 2011, 2014 in 2017).

Zunanji preizkusi znanja pri predmetih ob koncu tretjega vzgojno-izobraževalnega obdobja so revizijski preizkusi (evalvirajo program) in imajo tudi nekatere lastnosti diagnostičnih preizkusov (diagnosticirajo napredek ter šibke in močne strani posameznikovega znanja) (Izhodišča nacionalnega … 2005). S tega stališča se NPZ razlikuje od mature, ki je selekcijsko preverjanje, saj se v visokošolski študij prve stopnje lahko vpiše, kdor je opravil maturo (Zakon o visokem šolstvu, 38. člen), in ker se pri izbiri kandidatov za vpis v visokošolski študij prve stopnje upošteva tudi splošni uspeh, dosežen pri maturi, lahko pa tudi uspeh pri posameznih, s študijskim programom določenih predmetih mature (Zakon o visokem šolstvu, 41. člen). Je pa NPZ podobno raziskavi PISA (program mednarodne primerjave dosežkov učencev; http://www.pei.si/Sifranti/ResearchProgramPresentation.aspx?id=2), ki »zbira pomembne podatke o dosežkih učencev, s pomočjo katerih lahko preučujemo izobraževalne politike in prakse in dolgoročno spremljamo trende dosežkov s področja bralne, matematične in naravoslovne pismenosti učencev« (Masters 2008: iii). NPZ je podobno tudi raziskavi TIMSS (Mednarodne raziskave trendov v znanju matematike in naravoslovja; http://www.pei.si/Sifranti/ResearchProgramPresentation.aspx?id=1), ki daje »poglobljene analize dosežkov učencev v povezavi z nacionalnimi dokumenti, ki navajajo, kaj na bi se učenci naučili, kaj so cilji in standardi izobraževanja« in »so lahko vir informacij za izboljšave kurikula, poteka in rezultatov izobraževanja« (Štraus 2005: 19). Temeljni namen NPZ iz biologije je dobiti dodatne informacije o tem, kako deluje vzgojno-izobraževalni sistem in kakšna je stopnja doseganja ciljev in standardov znanja, ki jih določajo učni načrti za biologijo v osnovni šoli. S temi podatki je mogoče ustrezno načrtovati nadaljnje vzgojno-izobraževalno delo, da bi tako izboljšali kakovost poučevanja in učenja in s tem tudi znanje učencev (Izhodišča nacionalnega … 2005; Informacije za učence in starše 2017).

Dosežki nacionalnega preverjanja znanja dajejo dodatno informacijo o znanju učencev (Zakon o osnovni šoli, 64. člen). Ta informacija je namenjena učencem in njihovim staršem (daje informacijo o znanju učenca ob zaključku

osnovnega šolanja), učiteljem (daje informacijo o dejanskem znanju njegovih učencev, omogoča kritično ovrednotenje njegovega poučevanja, usklajevanje njegovih kriterijev vrednotenja znanja s kriteriji drugih učiteljev, pomoč pri uporabi učnih načrtov, pomoč pri preverjanju doseganja standardov), šolam (ravnateljem in drugim strokovnim delavcem šole) in celotnemu sistemu vzgoje in izobraževanja na nacionalni ravni (omogoča evalvacijo učnih načrtov in kakovosti izobraževanja na nacionalni ravni ter spreminjanje in razvoj učnega sistema (Izhodišča nacionalnega … 2005).

Pričakuje se, da šole dobljene podatke analizirajo, ovrednotijo in v skladu s kakovostjo dosežkov tudi ukrepajo. Šole naj bi tudi primerjale dosežke svojih učencev s povprečnim dosežkom vseh učencev, ki so tisto leto opravljali NPZ. Niso pa ti podatki namenjeni razvrščanju šol in učencev po dosežkih učencev na NPZ *(*Zakon o osnovni šoli, 64. člen), saj tako razvrščanje lahko pripelje do diskriminacije šol in pomeni »izrabo NPZ v namene, ki so popolnoma v nasprotju z njegovo vlogo in pomenom v slovenskem šolskem sistemu*«* (Vogrinc 2014).

Od šolskega leta 2005/2006 dosežki učenca na NPZ ne vplivajo na šolske ocene ali uspeh ob koncu osnovne šole niti ne odločajo o prehodu na srednješolsko raven izobraževanja (razen izjemoma). To omogoča, da naloge lahko preverjajo širši spekter standardov znanja, ki jih določajo učni načrti, in s tem dajejo tudi kakovostnejše podatke o doseganju teh standardov.

Na NPZ se preverja standarde iz učnih načrtov (Izhodišča nacionalnega … 2005). Delež, ki ga neka vsebinska enota zajemajo v preizkusu, je določen v skladu z njenim deležem v učnih načrtih in pomenom, ki ga ima za splošno izobrazbo (Letno poročilo … 2017). Vključeni so cilji iz naslednjih vsebinskih enot učnih načrtov: naravoslovje v 6. razredu (vsebinski enota živa narava), naravoslovje v 7. razredu (vsebinski enoti živa narava in vplivi človeka na okolje), biologija v 8. razredu (vse), biologija v 9. razredu (ne preverja se enot biotska pestrost (L), biomi in biosfera (M) ter vpliv človeka na naravo in okolje (N) (Informacije o preizkusu znanja … 2018). Leta 2011 sta stopila v veljavo nova učna načrta za naravoslovje in biologijo v osnovni šoli (Učni načrt. Program osnovna šola. Naravoslovje 2011, Učni načrt. Program osnovna šola. Biologija 2011), zato sta v NPZ iz biologije od leta 2014 vključeni tudi genetika in biotehnologija, ki ju v predhodnih učnih načrtih ni bilo.

V naši raziskavi smo želeli poiskati močne in šibke strani biološkega znanja slovenskih devetošolcev, izkazanega na NPZ, ter na teh temeljih dobiti in dati povratno informacijo o biološkem znanju slovenskih učencev ob koncu osnovnošolskega izobraževanja. Ta informacija je pomembna tako za učitelje biologije in šole kot tudi za institucije, ki se ukvarjajo z izobraževanjem učiteljev biologije. Zanimalo nas je, ali učenci teme, ki jih predpisujejo temeljni standardi učnih načrtov, zadovoljivo obvladajo. Poleg tega smo želeli preveriti, ali na dosežke učencev vplivajo tip naloge, taksonomska raven naloge in preverjana vsebina.

Material in metode

Dosežki učencev

Biologija je del nacionalnega preverjanja znanja ob koncu 3. obdobja osnovne šole, torej ob koncu 9. razreda. Je eden izmed predmetov, ki se ne preverja vsako leto, temveč vsakih nekaj let. Doslej je bila biologija vključena v NPZ petkrat, in sicer leta 2006, 2009, 2011, 2014 in 2017. V našo analizo smo vključili dosežke vseh 18730 učencev, ki so sodelovali na zadnjih *štiri*h zaporednih preverjanjih (Tab. 1). Povprečni dosežek učencev pri biologiji se je v teh letih gibal med 47,46 in 52,23 odstotnimi točkami, standardni odklon pa med 15,94 in 16,57.

Preizkus znanja

Preizkus znanja iz biologije je v letih 2009, 2011, 2014 in 2017 sestavljalo po 32 ali 35 nalog (Preg. 1, Tab. 1), ki so preverjale različno biološko vsebino v skladu z učnimi načrti za naravoslovje (Učni načrt. Program osnovnošolskega izobraževanja. Naravoslovje 7 1998, Učni načrt. Program osnovna šola. Naravoslovje 2011) in za biologijo (Učni načrt. Program osnovnošolskega izobraževanja. Biologija 1998, Učni načrt. Program osnovna šola. Biologija 2011) v osnovni

Tabela 1: Osnovni podatki o učencih, nalogah in dosežkih na NPZ iz biologije v letih 2009-2017 in v naši raziskavi.
Table 1: The basic information on pupils, items, and achievements on NAK in biology in 2009-2017 and in our analysis

		NPZ			Naša analiza			
		Dosežek učencev		Naloge	Dosežek učencev		Naloge	
Leto	Št. učencev	M	SD	f	M	SD	f	f (%)
2009	4736	52,23	15,94	32	49,67	24,63	30	25,2
2011	5683	47,74	16,22	32	49,82	21,69	28	23,5
2014	4210	47,46	16,57	32	46,45	22,44	29	24,4
2017	4101	50,92	16,23	35	48,69	24,69	32	26,9
Skupaj	18730			131			119	100,0

šoli ter v skladu z vsakoletno strukturo preizkusa (Informacije o preizkusu znanja … 2018). Vsega skupaj je bilo v teh štirih letih 131 bioloških nalog, od katerih smo jih v analizo vključili 119 (90,1 %) (Tab. 1). Vključene naloge so preverjale šest vsebinskih enot, v vsaki je bilo od 13 do 31 nalog (Tab. 2). Izključenih 12 nalog je preverjalo raznoliko drugo vsebino, zato jih je bilo v vsaki vsebinski enoti premalo, da bi bila ta reprezentativno predstavljena. Analizirane naloge so bile dveh tipov, in sicer 42 (35,3 %) nalog izbirnega tipa z enim pravilnim odgovorom ter 77 (64,7 %) nalog polodprtega tipa, pri katerih je učenec samostojno napisal kratek odgovor (Tab. 3). Naloge so bile treh taksonomskih ravni po prirejeni Bloomovi lestvici, ki jo uporablja Državni izpitni center za nacionalno preverjanje znanja in za maturo (Informacije o preizkusu znanja … 2018) (Tab. 4). Vsaka naloga na NPZ je bila ovrednotena z eno točko (https://www.ric.si/preverjanje_znanja/predmeti/ostali_predmeti/2011120910493238/).

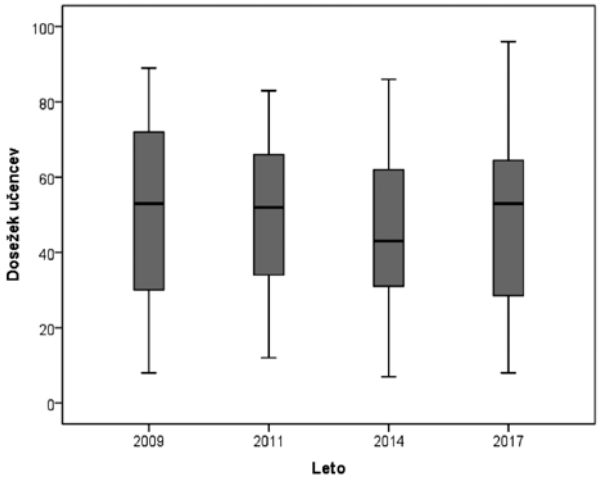
Statistična analiza

Podatke o povprečnem številu točk, ki so jih učenci dosegli pri vsaki od 119 nalog, smo statistično obdelali s programom SPSS (21.0). S preizkusom Kolmogorov-Smirnov smo ugotovili, da so bili podatki za vse postavke razporejeni normalno ($p > 0,001$). Z Levenovim preizkusom smo ugotovili, da so bile variance dosežkov učencev za *šolska* leta, vsebinske enote in tipe nalog homogene ($p > 0,001$), za kognitivne ravni pa ne ($p < 0,01$). Za ugotavljanje statistične značilnosti razlik med odgovori učencev na naloge različnih šolskih let in vsebine smo zato uporabili preizkus ANOVA, za ugotavljanje značilnosti razlik med odgovori na naloge različnih tipov smo uporabili preizkus t, za ugotavljanje značilnosti razlik med odgovori na naloge različnih taksonomskih ravni pa preizkus Kruskal-Wallis.

Rezultati

Dosežki učencev na NPZ iz biologije glede na šolsko leto

V analizo smo zajeli 119 nalog z zadnjih *štirih* zaporednih NPZ iz biologije in ugotovili, da so bili v letih 2009, 2011 in 2017 dosežki učencev podobno visoki (povprečna vrednost M = 48,69–49,82; standardni odklon SD = 21,69–24,69; mediana Me = 52–53), medtem ko je bil dosežek leta 2014 nižji (M = 46,45; SD = 22,44; Me = 43) (Sl. 1). Ta razlika ni bila statistično značilna (ANOVA; df = 3, F = 0,127, p = 0,944).



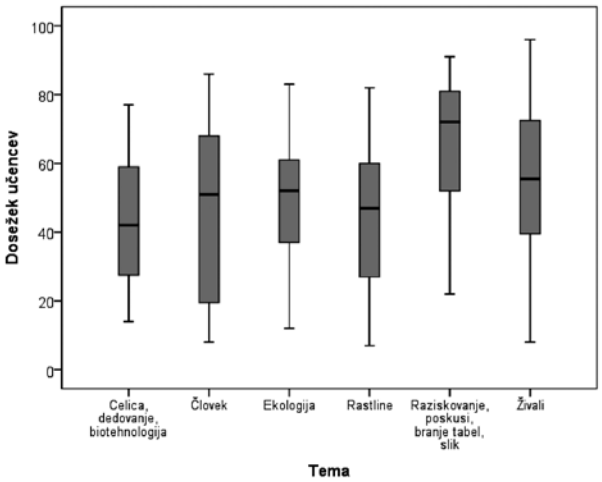
Slika 1: Mediane, kvartili in skrajne vrednosti dosežkov učencev na NPZ iz biologije v letih 2009-2017.
Figure 1: Medians, quartiles and extreme values of the achievements of pupils on NAK in biology in 2009-2017.

Dosežki učencev na NPZ iz biologije glede na vsebino nalog

Analizirali smo 119 nalog, ki so preverjale naslednjih šest vsebinskih enot iz učnih načrtov za osnovno šolo: celica, dedovanje, biotehnologija; človek; ekologija; rastline; raziskovanje in poskusi, branje tabel, grafov, slik; živali. Učenci so imeli najnižje dosežke pri vsebinski enoti, ki je vključevala celico, dedovanje in biotehnologijo (povprečna vrednost M = 42,91; standardni odklon SD = 19,23; mediana Me = 42) (Preg. 2, Tab. 2, Sl. 2, Fig. 2), nekoliko višji dosežki so bili pri nalogah o rastlinah (M = 44,58; SD = 22,05; Me = 47) ter zgradbi in delovanju človeka (M = 44,71; SD = 25,54; Me = 51). Še višji dosežki so bili pri nalogah o ekologiji (M = 50,59; SD = 19,98; Me = 52) in živalih (M = 55,13; SD = 25,05; Me = 55,5). Največ znanja so učenci pokazali pri nalogah, ki so zajemale raziskovanje in poskuse ter branje tabel, grafov ali slik (M = 63,69; SD = 22,41; Me = 72). Razlike med odgovori na naloge različnih vsebinskih enot niso bile statistično značilne (ANOVA; df = 5, F = 2,029, p = 0,080).

Tabela 2: Dosežki učencev na NPZ iz biologije v letih 2009-2017 po vsebinskih enotah analiziranih nalog.
Table 2: Achievements of pupils on NPZ in biology in 2009-2017, based on the content area of analysed items.

	Število nalog		Dosežek učencev	
Vsebinska enota	f	f (%)	M	SD
Celica, dedovanje, biotehnologija	23	19,3	42,91	19,23
Rastline	19	16,0	44,58	22,05
Človek	31	26,1	44,71	25,54
Ekologija	17	14,3	50,59	19,98
Živali	16	13,4	55,13	25,05
Raziskovanje in poskusi, branje tabel, grafov, slik ipd.	13	10,9	63,69	22,41
Skupaj	119	100,0		



Slika 2: Mediane, kvartili in skrajne vrednosti dosežkov učencev na NPZ v letih 2009-2017 po vsebinski enoti nalog.

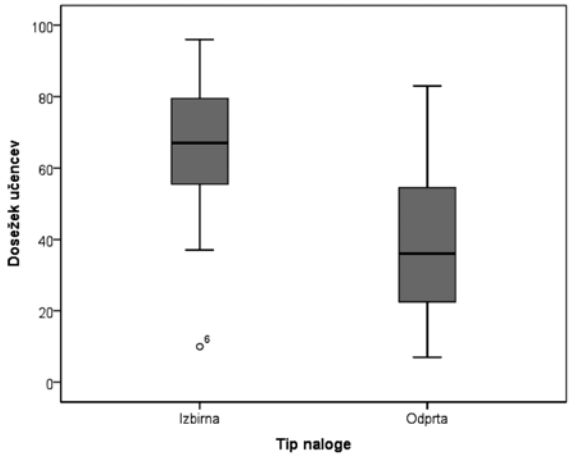
Figure 2: Medians, quartiles and extreme values of the achievements of pupils on NAK in biology in 2009-2017, based on the content area of analysed items.

Dosežki učencev na NPZ iz biologije glede na tip naloge

Naloge, ki smo jih analizirali, so bile dveh tipov - polodprtega in izbirnega. Dosežki u*čenc*ev pri nalogah polodprtega tipa so bili nižji (povprečna vrednost M = 39,10; standardni odklon SD = 20,41; mediana Me = 36) (Tab. 3, Sl. 3) kot pri nalogah izbirnega tipa (M = 66,17; SD = 17,05; Me = 67,5). Ta razlika je bila statistično značilna (preizkus t; df = 117, t = 7,309, p < 0,001).

Tabela 3: Dosežki učencev na NPZ iz biologije v letih 2009-2017 po tipu analiziranih nalog.
Table 3: Achievements of pupils on NPZ in biology in 2009-2017, based on the type of analysed items.

	Število nalog		Dosežek učencev	
Tip naloge	f	f (%)	M	SD
Polodprta	77	64,7	39,10	20,41
Izbirna	42	35,3	66,17	17,05
Skupaj	119	100,0		



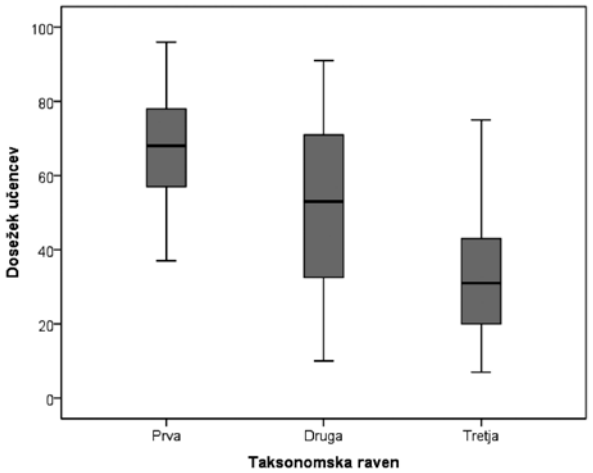
Slika 3: Mediane, kvartili in skrajne vrednosti dosežkov učencev na NPZ v letih 2009-2017 po tipu nalog.
Figure 3: Medians, quartiles, and extreme values of the achievements of pupils on NAK in biology in 2009-2017, based on the type of analysed items.

Dosežki učencev na NPZ iz biologije glede na taksonomsko raven naloge

Analizirane naloge so bile treh taksonomskih ravni. Učenci so imeli najnižje dosežke pri nalogah tretje taksonomske ravni, torej pri samostojnem reševanju novih problemov, samostojni interpretaciji in vrednotenju (povprečna vrednost M = 31,37; standardni odklon SD = 16,02; mediana Me = 31) (Tab. 4, Sl. 4). Nekoliko višji dosežki so bili pri nalogah razumevanja in uporabe, ki so druge taksonomske ravni (M = 50,71; SD = 22,64; Me = 53), najvišji dosežki pa so bili pri nalogah prve ravni, ki preverjajo znanje in poznavanje (M = 67,62; SD = 14,46; Me = 68). Razlike med odgovori na naloge različnih taksonomskih ravni so bile statistično značilne (Kruskal-Wallis; χ^2 = 42,145, df = 2, p < 0,001). S preizkusom Mann-Whitney smo ugotovili, da so bile statistično značilne tudi razlike med nalogami prve in druge ravni (p = 0,001), prve in tretje ravni (p < 0,001) ter druge in tretje ravni (p < 0,001).

Tabela 4: Dosežki učencev na NPZ iz biologije v letih 2009-2017 po taksonomskih ravneh analiziranih nalog.
Table 4: Achievements of pupils on NPZ in biology in 2009-2017, based on the taxonomic level of analysed items.

	Število nalog		Dosežek učencev	
Taksonomska raven	f	f (%)	M	SD
Samostojno reševanje novih problemov, samostojna interpretacija, vrednotenje (TR 3)	38	31,9	31,37	16,02
Razumevanje in uporaba (TR 2)	52	43,7	50,71	22,64
Znanje in poznavanje (TR 1)	29	24,4	67,62	14,46
Skupaj	119	100,0		



Slika 4: Mediane, kvartili in skrajne vrednosti dosežkov učencev na NPZ v letih 2009-2017 po taksonomski ravni nalog.

Figure 4: Medians, quartiles, and extreme values of the achievements of pupils on NAK in biology in 2009-2017, based on the taxonomic level of analysed items.

Razprava

Dosežki učencev na NPZ iz biologije glede na šolsko leto

Ugotovili smo, da se splošni dosežki učencev pri 119 analiziranih nalogah na štirih NPZ iz biologije v letih od 2009 do 2017 med seboj ne razlikujejo značilno. To ni presenetljivo, saj komisija za biologijo, ki pripravlja naloge, kot izhodišče pri tem uporablja tako imenovano specifikacijsko tabelo. V tej so določeni deleži nalog glede na tip, taksonomsko raven in vsebinsko enoto, zato so preizkusi znanja v teh pogledih med seboj usklajeni (Letno poročilo … 2017). Kljub temu so še vedno možne razlike v zahtevnosti, ki so posledica drugih dejavnikov, na primer ožje vsebine znotraj predvidenih vsebinskih enot. Iz rezultatov sklepamo, da naloge tudi v tem pogledu dosegajo zadovoljivo podobno raven zahtevnosti.

Povprečna vrednost dosežkov učencev, ki so izraženi v odstotnih točkah, se je v teh letih gibala med 46,45 in 49,82, kar je na prvi pogled razmeroma nizko in bi iz tega lahko napačno sklepali na nezadovoljivo znanje učencev. Dejstvo pa je, da dosežki učencev na NPZ od leta 2005/2006 ne vplivajo več na njihov šolski uspeh ali nadaljnje izobraževanje, temveč naj v skladu z namenom NPZ kažejo kakovost poučevanja in učenja (Izhodišča nacionalnega … 2005). Zato so naloge, ki morajo biti usklajene z učnimi načrti za naravoslovje in biologijo, oblikovane tako, da preverjajo širše standarde in morajo dosegati tudi ustrezen nivo težavnosti, če naj pokažejo, kaj učenci dobro znajo in kje imajo težave. Z nalogami, ki bi bile ozko usmerjene in prenizke zahtevnosti, preverjanje ne bi pokazalo šibkih in močnih strani znanja učencev.

Iz rezultatov analize bi lahko sklepali, da NPZ svojega namena, torej kakovostnejšega znanja učencev, ne dosega, saj se splošni dosežki učencev od leta 2009 do 2017 ne izboljšujejo, temveč ostajajo na podobni ravni. Dejstvo je, da je biologija vsebinsko široka in da z vsakim preverjanjem lahko zajamemo samo določeno število ciljev iz učnih načrtov. Naloge se praviloma ne ponavljajo iz leta v leto, zato je večinoma nemogoče spremljati znanje učencev pri posameznem učnem cilju. Mogoče pa je pripraviti take naloge, da s povratno informacijo o dosežkih učitelje nato opozorimo na dobro znanje, predvsem pa na morebitna napačna pojmovanja in pomanjkljivo razumevanje pri učencih. Učitelji te podatke lahko upoštevajo

pri pripravi in izvedbi pouka in tako postopoma vplivajo na izboljševanje znanja učencev.

Dejstvo je tudi, da z NPZ dobimo povratno informacijo o znanju, ki ga učenci pokažejo, ne vemo pa, koliko znanja učenci dejansko imajo, pa se ga ne potrudijo pokazati, ker dosežki NPZ zanje nimajo nikakršnih posledic (razen v izjemnih primerih ob omejitvi vpisa). Zato je že ves čas slišati predloge, da bi morali dosežene točke spremeniti v ocene, ki bi imele posledice za učence (se upoštevale ali že v osnovni šoli ali pa za vpis v srednjo šolo). Tukaj je treba opozoriti na raziskavi TIMSS in PISA, katerih dosežki prav tako v nobenem pogledu ne vplivajo na uspeh učencev in njihovo napredovanje v izobraževalni vertikali, kar pomeni, da jih učenci verjetno rešujejo s podobno stopnjo odgovornosti kot NPZ. In vendar sta raziskavi TIMSS in PISA splošno sprejeti kot dobra pokazatelja stanja naravoslovnega izobraževanja v sodelujočih državah. Tudi z NPZ dobimo podatke, iz katerih lahko zadovoljivo sklepamo, kje so šibke in kje močne strani biološkega znanja naših osnovnošolcev, ne glede na to, da se pri reševanju NPZ morda ne potrudijo vsi učenci po svojih najboljših močeh.

Dosežki učencev na NPZ iz biologije glede na vsebino nalog

V 119 analiziranih nalogah smo preverili šest vsebinskih enot iz učnih načrtov za naravoslovje in biologijo v osnovni šoli. Ugotovili smo, da razlike med znanjem posameznih enot niso statistično značilne, kar pomeni, da učenci podobno kakovostno obvladajo vso preverjano vsebino. Pri analizi so nas še posebej zanimale naloge o biotehnologiji, dedovanju in celici, torej sklop, ki je s prenovo učnih načrtov leta 2011 doživel največ novosti. Pri tej enoti so učenci pokazali najnižje dosežke (povprečni dosežek = 42,91). Biotehnologijo so v učni načrt (v 9. razred) vključili šele s prenovo učnih načrtov leta 2011 (Učni načrt. Program osnovna šola. Biologija 2011). Dedovanje je bilo v učnih načrtih iz leta 1998 uvrščeno v 9. razred, torej za učence, stare približno 14 let. Z učnim načrtom leta 2011 je bila ta tema prestavljena v 8. razred, kar pomeni, da se je učijo eno leto mlajši. Raziskave kažejo, da je zelo malo učencev sposobnih povezati konceptualno znanje o mejozi s proceduralnim znanjem, torej s fazami procesa mejoze (Cavallo 1996). Po drugi strani pa sta Smith in Sims (1992) ugotovila, da učenci posedujejo kognitivne spretnosti, ki so potrebne za reševanje najznačilnejših problemov v klasični genetiki. Tudi podrobnejša analiza odgovorov naših učencev pri nalogah na NPZ, ki so zahtevale uporabo Punnettovega kvadrata, je pokazala, da učenci to zmorejo, saj so bili njihovi dosežki dobri (Letno poročilo … 2017). To potrjuje ugotovitve, do katerih je prišla Cavallo (1996), da namreč učenci z uporabo algoritmov nimajo težav, ne razumejo pa dobro, kako so posamezne sestavine Punnettovega kvadrata dejansko povezane s procesom mejoze. Da so bili med vsemi preverjenimi vsebinskimi enotami najnižji dosežki prav pri celici, dedovanju in biotehnologiji, verjetno lahko pripišemo tudi dejstvu, da so prva leta od ponovne uvedbe dedovanja v učni načrt imeli težave tudi učitelji, saj mlajši genetike sploh še niso poučevali, starejši pa je niso od leta 1998, ko je bila izločena iz učnega načrta za biologijo. Podobno velja tudi za biotehnologijo, ki je bila uvedena povsem na novo. Kar se tiče celice, sta se s prenovo učnih načrtov leta 2011 spremenili poglobljenost učnih ciljev in razred, v katerem se jo poučuje (zdaj predvsem v 6 in 7. razredu).

Učni cilji o rastlinah, živalih, človeku in ekologiji so v prenovljenih učnih načrtih iz leta 2011 nekoliko drugačni, kot so bili pred tem. Predhodni učni načrt je izhajal iz ekosistemov, ki so jih učenci spoznavali najprej v bližnji okolici, potem pa širše. V novih učnih načrtih je izhodišče celica, od katere se prek organizmov gradi navzgor do ekosistemov (Učni načrt. Program osnovna šola. Biologija 2011). Človeka zdaj obravnava snov 8. razreda. Povprečni dosežek učencev pri nalogah, ki so preverjale znanje o ekologiji in živalih, je bil 50,59 in 55,13 kar je zadovoljivo. Pri temah o rastlinah in človeku pa so učenci pokazali manj znanja (povprečni vrednosti = 44,58 in 44,71). Rastline so snov 6. razreda, zato je tu treba upoštevati faktor pozabljanja, vzroke za slabše znanje o človeku pa bi bilo treba še nadalje raziskati.

Največ znanja (povprečna vrednost = 63,69) so učenci pokazali pri nalogah, ki so preverjale znanje o raziskovanju in poskusih, kamor spada načrtovanje preprostih poskusov, postavljanje hipotez, razumevanje pomena kontrole v poskusu ipd. V tej skupini so bile tudi naloge, pri katerih

so morali učenci znati brati tabele, grafe in slike, da so jih lahko rešili. Tudi ti cilji so pomemben del naravoslovne pismenosti, rezultati pa kažejo, da je poučevanje na tem področju zadovoljivo. Višji dosežki pri tej enoti so verjetno tudi posledica dejstva, da se v skladu z učnimi načrti učenci s to vsebino zdaj spoznavajo vse od začetka izobraževanja in jo postopoma nadgrajujejo in poglabljajo, poleg tega so ti cilji vključeni v različne predmete in jih lahko dobro utrdijo.

Povzamemo naj, da je temeljno znanje biologije na NPZ pri večini vsebinskih enot zadovoljivosti. Razlike med znanjem posameznih vsebinskih enot niso tako velike, da bi bile statistično značilne. Ker je raziskava pokazala najnižje dosežke pri vsebinskem sklopu o celici, dedovanju in biotehnologiji, bi bilo na tem področju treba več pozornosti nameniti izobraževanju bodočih učiteljev in dodatnemu usposabljanju učiteljev praktikov. Watts in Jofili (1998) ugotavljata, da učitelji pri poučevanju genetike posegajo predvsem po tradicionalnih učnih metodah in tradicionalnem zaporedju učne snovi ter večinoma uporabljajo podobne učne strategije, kar ni dovolj učinkovito. Bodoči učitelji biologije imajo primanjkljaj pri znanju celične biologije (Dikmenli 2010), morali pa bi biti prav na tem področju visoko usposobljeni. Dejstvo je namreč, da je v sodobnem svetu razumevanje temeljnih konceptov celične biologije bistvenega pomena za naravoslovno pismenost državljanov (Venville in sod. 2005). Učitelji morajo upoštevati, da učenci težko razumejo mehanizme delovanja v celični biologiji, ker jih je težko predstaviti brez posebnih instrumentov (Mbajiorgu in sod. 2007) in ker zahtevajo določeno raven abstraktnega mišljenja (Banet in Ayuso 2000, Smith in Sims 1992).

Dosežki učencev na NPZ iz biologije glede na tip naloge

Ugotovili smo, da imajo učenci statistično značilno višje dosežke pri nalogah izbirnega tipa (povprečna vrednost = 66,17) kot pri polodprtih nalogah (povprečna vrednost = 39,10). To je bilo pričakovano, saj učenci pri izbirnem tipu nalog izberejo enega med že danimi odgovori, medtem ko ga morajo pri polodprtih nalogah sami oblikovati. Poleg tega polodprte naloge pogosto zahtevajo celovit večstopenjski razmislek na višjih taksonomskih ravneh (Izhodišča nacionalnega … 2005).

Dosežki učencev na NPZ iz biologije glede na taksonomsko raven naloge

Našli smo statistično značilne razlike med dosežki pri nalogah vseh treh kognitivnih ravni, ki se uporabljajo pri NPZ. Najvišji dosežki so bili pri nalogah znanja in razumevanja (povprečna vrednost = 67,62), ki preverjajo temeljno raven znanja. Pri tem je treba omeniti, da so te naloge večinoma naloge izbirnega tipa, ki so že same po sebi manj zahtevne za reševanje, kar je pokazala tudi naša analiza. Ne moremo namreč z vsemi tipi nalog enako dobro preverjati vseh vrst znanja oziroma taksonomskih ravni (Izhodišča nacionalnega … 2005). Pri nalogah druge taksonomske ravni so bili dosežki nižji (povprečna vrednost = 50,71). Te naloge so bile večinoma polodprtega tipa, samo nekaj je bilo izbirnih. Pri tretji taksonomski ravni so bile vse naloge polodprtega tipa, dosežki pa najnižji (povprečna vrednost = 31,37). To ne preseneča, saj naloge polodprtega tipa zahtevajo povezovanje znanja in samostojno oblikovanje odgovora. Dejstvo, da imajo naši učenci dobro temeljno znanje biologije, manj uspešno pa to znanje uporabljajo v novih situacijah, je pokazala že raziskava PISA (Strgar 2010, Štraus in sod. 2007).

Zaključki

- Ugotovili smo, da so učenci v splošnem pokazali zadovoljivo temeljno znanje.
- Na dosežke učencev ni vplivala preverjana vsebina, saj razlike med dosežki pri šestih vsebinskih enotah niso bile statistično značilne.
- Na dosežke učencev je vplival tip naloge, saj so bili dosežki pri nalogah izbirnega tipa statistično značilno višji kot pri nalogah polodprtega tipa.
- Na dosežke učencev je vplivala taksonomska raven naloge, saj so bile razlike med dosežki pri nalogah vseh treh taksonomska ravni statistično značilne. Po pričakovanju so učenci dosegli najboljše rezultate pri nalogah najnižje,

najslabše rezultate pa pri nalogah najvišje taksonomske ravni.

- Kljub temu, da je bila kakovost znanja vseh preverjanih vsebinskih enot podobna, bi bilo pri načrtovanju pouka treba v prihodnje več pozornosti posvetiti področjem celice, dedovanja in biotehnologije.
- Eden izmed namenov NPZ bi moral tudi v prihodnosti ostati pomoč učiteljem, saj tako lahko pozitivno vplivajo na kakovost znanja učencev. Zato bi bilo smiselno v NPZ iz biologije vključiti še več nalog, ki bi učitelje usmerjale pri načrtovanju in izvajanju pouka.

Summary

The purpose of the national assessment of knowledge (NAK) is to gather feedback information about how the educational system works and what level of educational aims and standards that are prescribed in the national curriculum is being reached. Thus far, biology has been one of the subjects on the NAK five times. In our survey, we analysed 119 items taken from the last four assessments (2009, 2011, 2014, and 2017) in which 18,730 pupils took part. Items covered six content units (cell, heredity, and biotechnology; human being; ecology; plants; animals; research, experiments, reading of tables, graphs, and figures); each unit was represented by 13 to 31 items. There were two types of items: 35% of multiple-choice items and 65% of half-open-ended items. Items also differed by the cognitive level according to an adjusted Bloom's scale that is in use for the NAK. Included were 24% of items of the first cognitive level, 44% of the second, and 32% of the third cognitive level.

The data were analysed using SPSS for Windows statistical software. Distribution of data was normal, so we used t-test and ANOVA to identify statistically significant differences between answers of pupils to different groups of items (content unit, type, and cognitive level of the item).

We were interested in the weak and strong sides of the biological knowledge of our pupils at the end of their primary education. We determined that the general achievements of pupils on the NAK in biology in the years 2009 to 2017 do not differ significantly. This is not surprising because in the preparation of the NAK specification tables are used, which prescribe the share of items of each group (content unit, type, and cognitive level). Means of achievements in these years were between 46.45 and 49.82. This could lead to the unjustified conclusion that pupils' knowledge is weak. Achievements are in fact the result of the complexity of the items that test broader standards included in the curriculum. In this manner, we can gather data about what pupils know well and what topics are problematic.

We determined that pupils, in general, possess satisfactory basic knowledge of biology. The quality of knowledge of all six tested content units (cell, heredity, and biotechnology; human being; ecology; plants; animals; research, experiments, reading of tables, graphs, and figures) does not differ significantly (mean = 42.91–63.69). However, pupils showed the least knowledge about cell, heredity, and biotechnology (mean = 42.91); therefore, in planning lessons in the future, there should be more emphasis on these three topics. Pupils' knowledge about plants, the human body, ecology, and animals was approximately similar (mean = 44.58–55.13) while they achieved better knowledge on topics that included research, experiments, reading of tables, graphs, and figures (mean = 63.69).

Pupils showed significantly more knowledge of multiple-choice items (mean = 66.17) in comparison to half-open-ended items (mean = 39.10), which was expected. As also expected, they showed significantly more knowledge on items of the first cognitive level (mean = 67.62), less on items of the second cognitive level (mean = 50.71), and the least on items of the third cognitive level (mean = 31.37).

One of the aims of the analysis of the NAK results is to provide support to teachers in planning and executing their lessons and, through this, positively influence pupils' knowledge of biology. For this reason, we think that future NAK in biology should include more items that would help teachers deal with problematic biology topics.

Literatura

Banet, E., Ayuso, E., 2000. Teaching Genetics at Secondary School: A Strategy for Teaching about the Location of Inheritance Information. Journal of Science Education, 84(3), 313–351.

Cankar, G., 2014. O izbiranju tretjih predmetov na nacionalnem preverjanju znanja.
https://www.ric.si/preverjanje_znanja/splosne_informacije/ (junij 2018)

Cavallo, A.M.L., 1996. Meaningful Learning, Reasoning Ability, and Students' Understanding and Problem Solving of Topics in Genetics. Journal of Research in Science Teaching, 33(6), 625–656.

Dikmenli, M., 2010. Misconceptions of Cell Division Held by Student Teachers in Biology: a Drawing Analysis. Scientific Research and Essay, 5(2), 235–247.

Informacije o preizkusu znanja za leto 2018.
https://www.ric.si/preverjanje_znanja/predmeti/ostali_predmeti/2011120910493238/ (junij 2018)

Informacije za učence in starše. 2017. Državni izpitni center, Ljubljana.
https://www.ric.si/preverjanje_znanja/splosne_informacije/ (junij 2018)

Izhodišča nacionalnega preverjanja znanja v osnovni šoli. 2005. Državni izpitni center, Državna komisija za vodenje nacionalnega preverjanja znanja v osnovni šoli, Ljubljana.
https://www.ric.si/mma/izhodi%C5%A1%C4%8Da%20npz%20v%20o%C5%A1/2006070611531042/ (junij 2018)

Letno poročilo o izvedbi nacionalnega preverjanja znanja v šolskem letu 2016/2017. 2017. Državni izpitni center, Državna komisija za vodenje nacionalnega preverjanja znanja v osnovni šoli, Ljubljana.
https://www.ric.si/preverjanje_znanja/statisticni_podatki/ (junij 2018)

Masters, G.N., 2008. Predgovor k izvirniku. V: Štraus, M. (ur.). Program mednarodne primerjave dosežkov učencev PISA : zbornik prispevkov o metodoloških vidikih raziskave PISA. Pedagoški inštitut, Ljubljana, str. i–iii.

Mbaijorgu, N.M., Ezechi, N.G., Idoko, E.C., 2007. Addressing Nonscientific Presuppositions in Genetics Using a Conceptual Change Strategy.
www.interscience.wiley.com (junij 2018)

Smith, M.U., Sims, S.O., 1992. Cognitive Development, Genetics Problem Solving, and Genetics Instruction: A Critical Review. Journal of Research in Science Teaching, 29, 701–713.

Strgar, J., 2010. Biological Knowledge of Slovenian Students in the Living Systems Content Area in PISA 2006. Acta Biologica Slovenica, 53(2), 99–108.

Štraus, M., 2005. Izvedbeni in doseženi kurikulum za matematiko v višjih razredih osnovne šole med 1995 in 2003. Šolsko polje, 16(3/4), 19–40.

Štraus, M., Repež, M., Štigl, S., 2007. Nacionalno poročilo PISA 2006: naravoslovni, bralni in matematični dosežki slovenskih učencev, Pedagoški inštitut, Ljubljana, 223 strani.

Učni načrt. Program osnovna šola. Biologija., 2011. Ministrstvo za šolstvo in šport : Zavod RS za šolstvo, Ljubljana, 43 strani.

Učni načrt. Program osnovna šola. Naravoslovje., 2011. Ministrstvo za šolstvo in šport : Zavod RS za šolstvo, Ljubljana, 37 strani.

Učni načrt. Program osnovnošolskega izobraževanja. Biologija., 1998. Ministrstvo za šolstvo, znanost in šport : Zavod Republike Slovenije za šolstvo, Ljubljana, 51 strani.

Učni načrt. Program osnovnošolskega izobraževanja. Naravoslovje 7., 1998. Ministrstvo za šolstvo, znanost in šport : Zavod Republike Slovenije za šolstvo, Ljubljana, 34 strani.

Venville, G., Gribble, S., Donovan, J., 2005. An Exploration of Young Children's Understandings of Genetics Concepts from Ontological and Epistemological Perspectives. Science Education, 89, 614–633.

Vogrinc, J., 2014. Dopis 2. DK NPZ_marec2014.
https://www.ric.si/preverjanje_znanja/splosne_informacije/ (junij 2018)

Watts, M., Jofili, Z., 1998. Toward Critical Constructivistic Teaching. International Journal of Science Education, 20, 159–170.

Zakon o osnovni šoli (Uradni list RS, št. 81/06 – uradno prečiščeno besedilo, 102/07, 107/10, 87/11, 40/12 – ZUJF, 63/13 in 46/16 – ZOFVI-K), 64. člen.
http://www.pisrs.si/Pis.web/indexSearch?search=Zakon+o+osnovni+%C5%A1oli (junij 2018)

Zakon o visokem šolstvu (Uradni list RS, št. 32/12 – uradno prečiščeno besedilo, 40/12 – ZUJF, 57/12 – ZPCP-2D, 109/12, 85/14, 75/16, 61/17 – ZUPŠ in 65/17)
http://www.pisrs.si/Pis.web/pregledPredpisa?id=ZAKO172 (junij 2018)

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Within-weed bed architectural adaptation of branching pattern in *Myriophyllum spicatum* L.

Prilagoditve razvejanosti rastlin vrste *Myriophyllum spicatum* L. v različnih delih sestoja

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Abstract: Regarding architectural adaptations in aquatic plants caused by the velocity of water flow only scarce, older information is available. When studying different *Myriophyllum spicatum* L. specimen architecture in the same water body differences in individual main axes and branching pattern were detected at the upstream and at the downstream end of individual plant beds. Samples from these two locations showed significant differences in architectural composition. At the downstream parts of the water body individual plants were longer and the number of branches was higher, which is contributed to flow velocity.

Keywords: *Myriophyllum spicatum*, architecture of plants, water flow velocity

Povzetek: Večina podatkov o prilagoditah vodnih rastlin, ki jih povzroča hitrost toka vode, je redkih in zastarelih. Pri proučevanju razrasti različnih primerkov vrste *Myriophyllum spicatum* L. v istem vodnem telesu, smo ugotovili razlike med dolžino glavnega poganjka in vzorci razvejanja rastlin v zgornjem in spodnjem delu sestoja rastlin. V spodnjem delu so bili poganjki daljši, njihova razvejanost pa je večja, kar pripisujemo hitrosti vodnega toka.

Ključne besede: *Myriophyllum spicatum*, razvejanost rastlin, hitrost vodnega toka

Introduction

The effect of water current on aquatic plants has been a topic of interest for many authors. Aspects getting highest attention were *i.a*. occurrence and distribution of species related to water flow conditions, leaf di-/polymorphism or physiological adaptations (nutrients, photosynthesis), and many more (for additional information see Neuhold et al. 2016). As aquatic plant beds form obstacles in the water channel, water flow increases around the beds, whereas the flow within beds is attenuated to a considerable extent (Sand-Jensen and Mebus 1995, Wenninger and Janauer 1991).

The publications dealing with this topic differ in methods regarding accuracy, spatial dimension of the approach, the measuring devices used, and even in studying either natural weed beds or experimental set-ups (e.g. Wilson 2007). A quite comprehensive picture of the interaction of aquatic

plant beds with water movement and sedimentation processes is given by Madsen et al. (2001).

This phenomenon namely the effect of plant beds on water flow velocity is also documented by other authors (Carter et al. 1988, Marshall and Westlake 1990, Chambers et al. 1991, Machata-Wenninger and Janauer 1991, Sand-Jensen and Mebus 1996, Sand-Jensen and Pedersen 1999). Especially the two last-cited contributions included detail of the flow conditions found upstream, within and downstream of the macrophyte beds.

In the documentation provided in publications listed above the longitudinal form of the studied weed beds can only be delineated indirectly by the shape of the isopleths in successive cross-sections. In the present brief study we were interested in two different weed bed features: (i) describing the general shape of the longitudinal profile of flow velocity developed along the mid-line of the *M. spicatum* beds present in the Wiener-Neustädter-Canal, (ii) assessing architectural differences of *M. spicatum* bed between the upstream end, affected by the direct impact of the flow, and the trailing end of *M. spicatum* bed. (iii) comparing this within-weed bed architectural adaptation of branching pattern and with that in locations with slow and fast flow velocity.

Site and methods

Individual plants (ramets) of a *Myriophyllum spicatum* L. clone were sampled from a historic canal (Wiener Neustädter Canal, WNC) near the towns Laxenburg/Biedermannsdorf. The selected weed bed was located at the coordinates: 48°04'44.79"N / 16°21'29.09"E. The canal is characterised by regulated constant discharge, providing an ideal environment for macrophyte development. Regarding current velocity at this site see Table 1.

Individuals of *M. spicatum* were sampled from the upstream end of the weed bed (N = 4), where the full force of the current acts on the plants. At the downstream end of the weed bed the sampled individuals (N = 4) were sheltered by a protective canopy of stems and branches growing upstream. Axes and branches sampled from the very upstream and the very downstream end were spread on a plastic sheet, straightened, counted and their length was determined with a tape measure. For comparison samples from additional two locations in the same channel, namely a location with fast water flow and one with slow water flow were taken into account (Neuhold et al. 2016).

Statistics

Data on characteristic parameters of the plant architecture 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 the normally-distributed data together with Duncan Post-Hoc mean separation test, testing for significance between means. Methods followed Untersteiner (2007).

Results

We present a few architectural features of the sampled plants on a percentage basis, as the simplest way of comparison. Since *Myriophyllum* plants had been sampled from other locations in the Wiener-Neustädter-Canal, too (Neuhold et al. 2016), yet growing under considerably different flow conditions, we used the data from the 'up'-location in our sampled weed bed to set the benchmark for comparing common architectural features.

Table 1: Means of flow velocity in the canal cross section free of *Myriophyllum spicatum* growth ("fast" and "slow" water velocity: see Neuhold et al. 2016) and at the location "up" at the beginning of the stand.
Tabela 1: Hitrost vodnega toka na delu kanala brez prisotnosti vrste *Myriophyllum spicatum* (hitra ("fast") in počasna ("slow") hitrost vodnega toka: glej Neuhold in sod. 2016) in na mestu "up"na začetku sestoja

Flow velocity (ms^{-1})	fast	up	slow
Mean value	1.44	0.92	0.39
Standard deviation	0.20	0.26	0.05

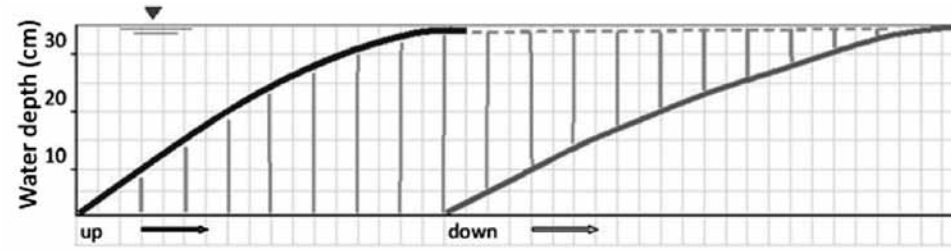

Figure 1: Weed bed of ramets of *Myriophyllum spicatum* - longitudinal section; the beginning of black line indicates sampling point for individual ramets at the upstream end of the weed bed ("up"), while the beginning of dark grey line indicates sampling point for individual ramets at the downstream end of the weed bed ("down"). The presence of plants within the water column is shown by vertical lines. Arrow: water surface indicator.

Slika 1: Sestoj vrste *Myriophyllum spicatum* - vzdolžni presek; začetek črne črte označuje točko vzorčenja posameznih rastlin na začetku sestoja, medtem ko začetek temno sive črte označuje vzorčevalno točko za posamezne rastline na spodnjem delu sestoja. Prisotnost rastlin v vodnem stolpcu je prikazana z navpičnimi črtami. Puščica kaže na vodno gladino.

Figure 1 shows the longitudinal section of a weed bed of *M. spicatum*. Maximum lengths of individuals sampled from the two parts of the weed bed were 90 cm at "up" and 120 cm at "down" location, respectively.

Figure 2 shows the relation of all branches recorded for the sampled ramets to the flow velocity. The branching at 'up' in Fig. 1 is rather similar to that in fast flow and that in 'down' (Fig. 1) is similar to that in slow flowing water ('slow' in Table 1).

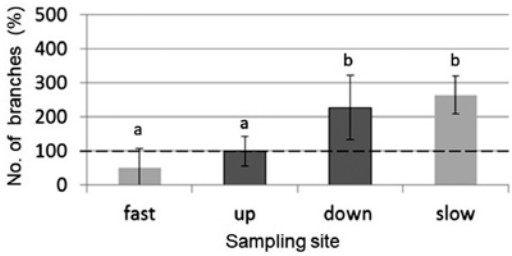

Figure 2: Number of all branches recorded for the sampled ramets. The value 100% relates to the mean number of all branches sampled from ramets at the "up" location. It provides the benchmark for the other locations. "Down" indicates the downstream end of the plant bed. The dark grey bars refer to "up" and "down" sampling locations. Light grey bars: data based on samples from other locations in the same channel, published by Neuhold et al. (2016). "Fast" indicates the same parameter for plants growing in very fast flow, while "slow" the number of branches for plants growing under lower flow velocity. Statistical differences are indicated by different characters.

Slika 2: Število stranskih poganjkov pri vzorčenih posameznih rastlinah. Vrednost 100 % se nanaša na povprečno število vseh poganjkov na začetku sestoja – "up". Vse ostale vrednosti so % glede na referenčne vrednosti na začetku sestoja. "Down" označuje lokacijo na koncu sestoja. Temno sivi stolpci se nanašajo na lokacije za vzorčenje na začetku in koncu sestoja. Svetlo sivi stolpci označujejo sestoje, ki so se razvili na lokacijah z hitrim ("fast") oziroma počasnim ("slow") tokom (Neuhold et al. 2016). Statistično značilne razlike so označene z različnimi črkami.

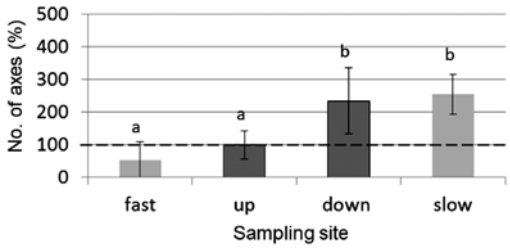

Figure 3: Number of terminal axes (as % of benchmark "up") in plants with no furcation. Detailed legend: see Fig. 2.
Slika 3: Število glavnih osi (% glede na referenčne vrednosti na začetku sestoja - "up") pri rastlinah, ki nimajo stranskih poganjkov. Podrobna legenda: glej sliko 2.

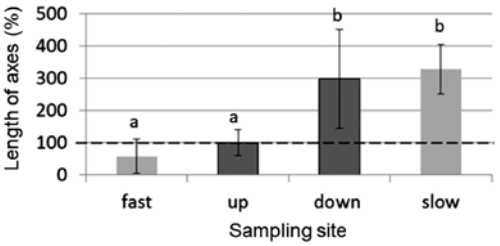

Figure 4: Length of terminal axes (as % of benchmark "up"). Detailed legend: see Fig. 2.
Slika 4: Dolžina glavnega poganjka (% glede na referenčne vrednosti na začetku sestoja – "up"). Podrobna legenda: glej sliko 2.

Aside from just looking at the total set of sampled ramets and their branches we also checked the group of ‚terminal axes', i.e. axes which had not developed any additional branching ('branches without furcation'). Fig. 3 and Fig. 4 show the number and length of terminal axes, respectively.

The difference of the means between ramets from "up" and "down" locations is smaller for all branches (Fig. 2), than that of the terminal axes (Fig. 3).

Discussion

During our work with *Myriophyllum spicatum* in the Wiener Neustädter Canal, we noticed a marked difference in the longitudinal composition of the weed beds. At the upstream end the axes and branches showed a quite compact arrangement, forming the main body of the plant bed. Close to the middle part of the bed the ramets lifted from the sediment, forming the trailing and waving axes of the downstream end. This triggered our interest in looking for architectural differences, and a related environmental parameter causing this variation.

The graphs and tables show clear and significant differences in the architecture between samples from the upper, and from the lower end of the weed bed, respectively. It is striking to see that in the trailing axes (i) the number of all branches of ramets, (ii) the number of 'terminal' (not branched) axes, and (iii) the length of the terminal axes always reach higher values than those recorded for the samples from the upstream end of the weed beds.

As regards the longitudinal mid-line section of the weed bed, ramets at the upstream end receive full light intensity over their whole length, whereas ramets following downstream reach full light only with their uppermost branches. The trailing ramets start their upward growth at

the canal bottom covered by the thickest layer of ramets located upstream, which results in maximum light attenuation. Further downstream the shading canopy gets less dense and the trailing ramets finally reach up to full light conditions with a longer part of their stems and branches near the water surface.

The well-known effect of increased growth of internodes and sometimes petioles in low light conditions (Nultsch 2001, p.527) could account for the longer length of the trailing terminal axes. Since higher numbers of ramets with and without branches were also recorded, this could provide an additional indication of a shading effect caused by ramets in the upstream part of the weed bed. In a recent study of shallow Lake Taihu (China) and its regions of turbid and clear water, Guan et al. (2018) showed that *Potamogeton malaianus* Miq. developed larger shoots, more and longer leaves and more biomass near the water surface. But the leaf morphology of the 'Bamboo-leaved Pondweed' is much different from that of *M. spicatum* and in lakes the plants grow quite straight up with elongated lower internodes, whereas in running waters the stems bend in reaction to flow conditions. This causes a close packing of the ramets of submersed species, and in *M. spicatum* sampled in our study, the leaf whorls were not bleached, missing the typical signs of 'etiolation'.

In general, a marked increase in the intensity of an environmental parameter affects plant growth, as exemplified *i.a.* by Bartélémy and Caraglio (2007; p.390), showing the impact of full light in *Araucaria araucana* (Molina) K. Koch growth, whereas UV radiation is a strong factor scaling down plant size (Caldwell 1968; Caldwell et al. 1982).

The same holds true for the movement of the ambient medium: wind impact shapes alpine vegetation clumps by upwind erosion and downwind regeneration (Costin et al. 2000; p.50). Also strong winds in alpine regions and near sea shores cause stunted growth (Natur erleben 2018) in many plants, and other morphological changes (Wind effect 2018).

Enhanced flow velocities, based on suitable minimum flow conditions, successfully controlled nuisance aquatic plant growth, but the results differed among architectural types, like *Sparganium erectum* L., *Potamogeton crispus* L. and *Myriophyllum aquaticum* (Vell.) Verdc. (Ochs et al. 2018), with the latter one showing the strongest effect of eradication. Water flow velocity also determined the competitive process between *M. spicatum* and *Elodea nuttallii* (Planch.) H.St. John, revealing a higher sensitivity to flow and turbulence, for the latter (Mazej and Germ 2013). Plant architecture (growth form) also determines water flow within weed beds, as shown by Wolters et al. (2018), who compared *Sparganium emersum* Rehmann, *Potamogeton natans* L. and *Callitriche obtusangula* Le Gall ex Hegelm.

For reaching a final decision between 'shading of light' and 'sheltering from water flow' as the cause for different architectural forms detected for upstream and downstream ramets the flume-like constant discharge in the Wiener Neustädter Canal provided ideal preconditions.

Along the central line of the weed bed upstream and downstream ramets are significantly different. According to Neuhold et al. (2016) ramets sampled at the 'fast' location, as well as ramets at the 'slow' location, both taken from the upstream end of their plant stands, were exposed to full light, but experienced very different flow speed. Therefore one can conclude that the internal differences in architectural features of *M. spicatum* weed beds are, at least mainly, caused by different water flow conditions, affecting the individuals at the very upstream end of the weed bed more than those trailing and waving at the very downstream end.

Conclusion

Our results show that *Myriophyllum spicatum* ramets, branched as well as those without additional furcation ('Terminal axes'), sampled from the upstream, and the downstream end of the same plant bed, respectively, are characterised by statistically significant differences in architectural features. Upstream samples, affected by the full force of the water flow develop an architecture related to that of samples taken from plant beds located in much faster flow sections, whereas architecture of the sheltered downstream samples was closely related to that of samples collected from slower flow passages at a different reach of the canal.

Based on our results testing other common aquatic plant species for the adaptation of architectural features caused by water current impact would deserve progressing in future studies.

Povzetek

Rezultati raziskave kažejo, da so za razvejane kot tudi nerazvejane ramete vrste *Myriophyllum spicatum,* rastoče na začetku in na koncu sestoja, značilne razlike v zgradbi rastlin. Na rastline na začetku sestoja deluje močan vodni tok, ki se odraža v zgradbi rastline, ki je značilna za predele kanala z hitrim vodnim tokom, medtem ko je bila zgradba rastlin na koncu sestoja podobna zgradbi rastlin, ki uspevajo na območjih s počasnim vodnim tokom.

Rezultati nakazujejo, da bi bilo v prihodnjih študijah potrebno raziskati tudi arhitekturne značilnosti drugih rastlinskih vrst v odvisnosti od vodnega toka.

References

Barthélémy, D., Caraglio, Y., 2007. Plant architecture: A dynamic, multilevel and comprehensive approach to plant form, structure and ontogeny. Annals of Botany, 99, 375-407.

Caldwell, M.M., 1968. Solar ultraviolet radiation as an ecological factor for alpine plants. Ecological Monographs, 38, 243-268.

Caldwell, M.M., Robberecht, R., Nowak, R.S., Billings, W.D., 1982. Differential photosynthetic inhibition by unltravilet radiation in species form the arctic-alpine life zone. Arctic and Alpine Research, 14, 195-202.

Machata-Wenninger, C., Janauer, G.A., 1991. The measurement of current velocities in macrophyte beds. Aquatic Botany, 39, 221–230.

Madsen, J.D., Chambers, P.A., James, W.F., Koch, E.W., Westlake, D.F., 2001. The interaction between water movement, sediment dynamics and submersed macrophytes. Hydrobiologia, 444, 71-84.

Marshall, W.P.J., Westlake, D.F., 1990. Water velocities around water plants in chalk streams. Folia Geobotanica et Phytotaxonomica, 25, 279-289.

Mazej, Z., Germ, M., 2013. Spatial pattern of native species *Myriophyllum spicatum* and invasive alien species *Elodea nuttallii* after introduction of the latter one into the Drava River (Slovenia). Biologia, 68, 202-209.

Neuhold, B., Janauer, J.D., Janauer, G.A., 2016. Architectural adaptation in *Myriophyllum spicatum* L. in a lotic environment: is it caused by current velocity? Acta Biologica Slovenica, 59, 73-87.

Nultsch, W., 2001. Allgemeine Botanik. Georg Thieme, Stuttgart. 663 pp.

Sand-Jensen, K., Mebus, J.R., 1996. Fine-scale patterns of water velocity within macrophyte patches in streams. OIKOS, 76, 169-180.

Sand-Jensen, K., Pedersen, O., 1999. Velocity gradients and turbulence around macrophyte stands in streams. Freshwater Biology, 42, 315-328.

Wilson, C.A.M.E., 2007. Flow resistance models for flexible surberged vegetation. Journal of Hydrology, 342, 213-222.

Wolters, J.-W., Verdonschot, R.C.M., Schoelynck, J., Verdonschot, P.F.M., Meire, P., 2018. The role of macrophyte structural complexity and water flow velocity in determining the epiphytic macroinvertebrate community composition in a lowland stream. Hydrobiologia, 806, 157-173.

Web sources

Natur erleben: http://www.naturerleben.net/category/im-gebirge/fruhling-im-gebirge/zwergwuchs-eine-erfolgsstrategie/; accessed 20180505 – 18:59.

Wind effect: http://agriculture-aajtak.blogspot.co.at/2013/09/13-important-effects-of-wind-on-crop.html; accessed 20180505 – 19:26.

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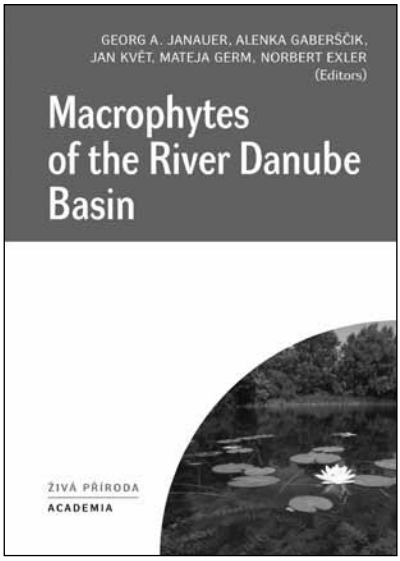
LJUBLJANA 2018 Vol. 61, Št. 1: 67–68

Makrofiti porečja reke Donave

Macrophytes of the river Danube basin



JANAUER, A. Georg, GABERŠČIK, Alenka, KVĚT, Jan, GERM, Mateja, EXLER, Norbert (uredniki). Macrophytes of the River Danube Basin, (Živá příroda). 1. izdaja, Praga: Academia, 2018, 407 str., ISBN 978-80-200-2743-6.

Monografijo Makrofiti porečja reke Donave je izdala založba Academia iz Prage v nizu publikacij Živa narava (Živá příroda). Monografijo so uredili Georg A. Janauer, Alenka Gaberščik, Jan Květ, Mateja Germ in Norbert Exler. Avtorji teksta so poleg urednikov še številni vodilni strokovnjaki s področja vodnih rastlin iz različnih držav donavskega porečja. V knjigi so predstavljeni vodni makrofiti v reki Donavi, s poudarkom na prisotnosti makrofitov v glavni strugi, v nekaterih pritokih in umetnih kanalih, kot tudi v nekaterih drugih tipih vodnih teles.

Publikacija omogoča bralcu vpogled v svet vodnih rastlin na način, kakršen do sedaj še ni bil predstavljen. Knjiga je osredotočena predvsem na makrofite, nekaj pa je tudi podatkov o okoljskih parametrih njihovih habitatov. Popis rastlin je narejen v zaporednih odsekih, dolgih do 1 km vzdolž celotne reke. Rezultati so prikazani z ustreznimi diagrami za posamezen rečni odsek. V knjigi so poleg znanstvenih poimenovanj vrst rastlin, tudi njihova imena v jezikih, ki se govorijo v državah, skozi katere teče reka. Vpogled v makrofitsko vegetacijo predstavlja pomemben doprinos k poznavanju biocenoze reke Donave, ki je po svoji dolžini na 18. mestu na svetu in s svojim porečjem združuje največje število držav med vsemi rekami.

Knjiga je sestavljena iz 19 poglavij. V uvodnem poglavju je obravnavana ekologija vodnih makrofitov, njihova vloga v vodnih ekosistemih in pomembni evropski pravni predpisi, ki zajemajo makrofite kot bistven biološki element.

V drugem poglavju so predstavljene metode s katerimi so strokovnjaki zbirali in obdelovali podatke. V sledečih poglavjih pa so predstavljene habitatne značilnosti in makrofiti glavne struge reke, njenih pritokov ter nekaterih ribnikov in kanalov, od njenega izvira v Nemčiji do delte v Romuniji, kjer se Donava izliva v Črno morje. Na začetku vsakega poglavja je grafična umestitev pregledanega območja. V knjigi najdemo tudi predstavitev mesojedih vodnih rastlin, ki predstavljajo posebno skupino makrofitov. Knjigo zaokroža seznam makrofitskih vrst, najdenih na pregledanem območju, vključno s sinonimi.

Knjiga Makrofiti porečja reke Donave je namenjena različnim strokovnjakom, ki z reko upravljajo in želijo izboljšati poznavanje biodiverzitete vodnih rastlin reke in spremembe njihove pogostosti in pojavljanja vzdolž njenega toka, pa tudi širši javnosti, ki se lahko pobliže seznani z vodnimi rastlinami v različnih delih porečja reke Donave.

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Univerza v Ljubljani

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 hyphen and followed by the text (A - 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 full name of 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.