ACTA BIOLOGICA SLOVENICA

VOL. 48 ŠT. 1 LJUBLJANA 2005

prej/formerly BIOLOŠKI VESTNIK

ISSN 1408-3671
UDK 57(497.4)

izdajatelj/publisher
Društvo biologov Slovenije

ABS ACTA BIOLOGICA SLOVENICA
LJUBLJANA 2005 Vol. 48, Št. 1 : 3-48

Acta Biologica Slovenica
Glasilo Društva biologov Slovenije – Journal of Biological Society of Slovenia

Izdaja – Published by
Društvo biologov Slovenije – Biological Society of Slovenia

Naslov uredništva – Adress of Editorial Office
Acta Biologica Slovenica, Večna pot 111, SI-1001 Ljubljana, Slovenija
http://bijh.zrc-sazu.si/abs/

Oblikovanje – Design
Žare Vrezec

ISSN 1408-3671 UDK 57(497.4)

Natisnjeno – Printed on: 2005
Tisk – Print: Tiskarna Pleško d.o.o., Ljubljana
Naklada: 500 izvodov

Cena letnika (dve številki): 3.500 SIT za posameznike, 10.000 SIT za ustanove

Številka poslovnega računa pri Ljubljanski banki: 02083-142508/30

Publikacijo je sofinancirala Agencija za raziskovalno dejavnost Republike Slovenije.

Acta Biologica Slovenica je indeksirana v – is indexed in: Biological Abstracts, Zoological records

ACTA BIOLOGICA SLOVENICA
LJUBLJANA 2005 Vol. 48, Št. 1: 3-12

Sprejeto (accepted): 2005-09-27

The influence of zinc on the accumulation of cadmium and copper in the terrestrial isopod *Porcellio scaber* (Crustacea, Isopoda)

Vpliv cinka na akumulacijo kadmija in bakra pri kopenskem enakonožcu *Porcellio scaber* (Crustacea, Isopoda)

Primož ZIDAR

Department of Biology, Biotechnical Faculty, University of Ljubljana, Večna pot 111, SI-1000 Ljubljana, Slovenia; e-mail: primoz.zidar@bf.uni-lj.si

Abstract. Relative to data derived from single-metal exposure not much is known about metal kinetics in terrestrial isopods exposed to a mixture of metals. In the work presented the accumulation pattern of Zn, Cd and Cu were studied in *Porcellio scaber*, one of the most investigated isopods. Animals were fed with hazel leaves dosed with single metals or their binary mixtures. After twenty-one days of exposure, food consumption and metal accumulation were measured. Results revealed that the accumulation of Zn is not affected by a decreased food consumption rate or by possible interactions between the mixture constituents. In contrast, the accumulation of Cd and Cu is significantly reduced when food is also contaminated with Zn. The lower accumulation of Cd and Cu can be ascribed to interactions with Zn that affect uptake and/or loss of metals.

Keywords: Isopods, *Porcellio scaber*, metal mixtures, zinc, copper, cadmium, food consumption, accumulation

Izvleček. Kinetika kovinskih ionov pri kopenskih rakih enakonožcih, ki so izpostavljeni zmesi kovin je, v nasprotju z izpostavitvijo le eni kovini, malo poznana. V predstavljenem delu smo spremljali akumulacijo cinka, kadmija in bakra pri kopenskem raku enakonožcu vrste *Porcellio scaber.* Poskusne živali smo hranili z listi leske, ki smo jim dodali raztopino ene ali pa zmes dveh kovin. Po enaindvajsetih dneh izpostavitve smo izmerili količine zaužite hrane in količino akumuliranih kovinskih ionov. Rezultati so pokazali, da na akumulacijo cinka ne vpliva upad prehranjevanja kakor tudi ne interakcije med kovinami v zmesi. Nasprotno je akumulacij kadmija in bakra značilno nižja ob sočasni prisotnosti cinka v hrani. Nižja akumulacija kadmija in bakra je posledica njune interakcije s cinkom, kar vpliva na manjši privzem ali pa večje izločanje kadmija in bakra iz telesa.

Ključne besede: raki enakonožci, *Porcellio scaber*, zmesi kovin, cink, baker, kadmij, prehranjevanje, akumulacija kovin

Introduction

Terrestrial isopods have been successfully adopted as monitor organisms in assessing the bioavailability of metals (WIESER & AL. 1976, COUGHTREY & AL. 1977, HOPKIN & AL.1986, DALLINGER & AL. 1992, HOPKIN & AL.1993, CORTET & AL. 1999, PAOLETTI & HASSALL 1999). Many investigations have proved that they accumulate the highest tissue concentrations of zinc, copper, lead, and cadmium known for any invertebrate (HOPKIN 1989, CORTET & AL. 1999, HEIKENS & AL. 2001). Their main metal storage organ is the hepatopancreas (WIESER 1961, COUGHTREY & AL. 1980, HOPKIN & MARTIN 1982) where metals are bound to specific low-molecular-weight peptides (DONKER & AL 1990, ŽNIDARŠIČ 2003), or stored as undissolved metal granules (WIESER & KLIMA 1969, PROSI & DALLINGER 1988, HOPKIN 1989). The hepatopancreas of terrestrial isopods consists of two cell types in which two types of intracellular granules have been observed; B granules in S cells and C granules in B cells (WIESER & KLIMA 1969, HOPKIN & MARTIN 1982, PROSI & DALLINGER 1988, HOPKIN 1989, HOPKIN & AL. 1989). In isopods collected from contaminated sites both types of granules contain Zn whereas only B granules contain Cu and Cd (HOPKIN 1989). Zn stored in B cells can be excreted as these cells show a daily cycle of apocrine secretion whereas Cd and Cu stored in S cells cannot be excreted because S cells have primarily a storage function and were never observed to secrete material into the gland lumen (HOPKIN 1990, HAMES & HOPKIN 1991a).

The digestive system is the main route for metal intake in terrestrial isopods. Metal uptake from food depends on the availability of metals (MARTIN & AL. 1976), the number of micro-organisms in the gut and food (COUGHTREY & AL. 1980), the nutritional status (FARCAS & AL. 1996), the rate of food consumption (DROBNE & HOPKIN 1995, BIBIČ & AL. 1997), the temperature (DONKER & AL. 1998), pH inside the gut (HOPKIN 1989), and some other factors.

Relative to data derived from single-metal exposures not much is known about the metal uptake in terrestrial isopods exposed to mixtures of metals. Interactions between metals in a mixture may affect the bioavailability of a single metal (VAN GESTEL & HENSBERGEN 1997) and the accumulation and excretion kinetics may differ when animals are exposed to various metals simultaneously (WITZEL 2000, ODENDAAL & REINECKE 2004). As isopods in an industrially polluted environment are usually exposed to several metals at the same time (BEYER & AL. 1984, HOPKIN 1989), some further investigations with metal mixtures are needed.

In the present work, we studied the accumulation of Zn, Cd and Cu in one of the most investigated isopods, *Porcellio scaber*, after exposure to single metals or their mixtures. We aimed to show the importance of such investigations for better understanding of metal accumulation and excretion kinetics in isopods and for proper interpretation of biomonitoring data.

Materials and methods

Experimental animals and procedure

Specimens of *Porcellio scaber* raised in the laboratory were used in the experiments. The parental population was collected in an unpolluted environment in the vicinity of Ljubljana, Slovenia. Juvenile isopods of 15-25 mg fresh weight were placed in plastic Petri dishes (diameter 9 cm), three animals per dish (N=36 for each concentration). A filter paper moistened with water was added to each Petri dish to assure constant humidity. Animals were fed with hazel leaves (*Corylus avellana*) dosed with a single metal (Zn, Cd, Cu) or binary mixtures (Zn+Cd, or Zn+Cu) for 21 days. The leaves were collected from an uncontaminated area in autumn. Solutions of $ZnCl_2$ (>98 % pure Merck, Darmstad, Germany), $CuCl_2*2H_2O$ (≥99 % pure Merck, Darmstad, Germany) and $CdCl_2*H_2O$ (>98 % pure Merck, Darmstad, Germany) were applied to the leaves by spraying. The amount of solution applied

to each leaf was adjusted to give the following nominal concentrations: Zn – 1000 and 3500 mg kg^{-1} dry weight; Cu – 500 and 2000 mg kg^{-1} dry weight; Cd – 250 and 500 mg kg^{-1} dry weight. Actual concentrations were analysed and did not differ of those desired by more than 5%. The base concentrations of Zn, Cu and Cd in the control leaves from the collection site were 40 mg Zn kg^{-1} dry weight, 20 mg Cu kg^{-1} dry weight and 0.15 mg Cd kg^{-1} dry weight. Control animals were fed with untreated food. The experiment was performed in a climate chamber at a relative humidity of 100% and at 16 hours light and 8 hours dark regime. Temperature was kept constant at 21°C (±1°C).

Food consumption was measured as the difference in the weight of leaves at the beginning and at the end of the experiment. Food consumption rates (CR) were calculated as the absolute consumption of food per week divided by the dry weight of the animals.

Metals concentration analyses

After exposure to the metal treated food, animals were food deprived for 24 hours to empty their guts. Then they were lyophilised, weighed and completely digested in a nitric/ perchloric acid mixture (7:1). After evaporation of the acid, the residue was taken up in 0.1% HNO_3. Total Zn, Cu and Cd concentrations in whole animals were determined by flame atomic absorption spectrometry (Perkin Elmer AAnalyst 100). As certified reference material a Dogfish Liver (DOLT-2, National Research Council Canada) was used. The leaves were analysed for metals in the same way.

Statistical analyses of data

Data on metal accumulation and food consumption rates were analysed using two-way ANOVA with the factors Zn concentration in food (3 levels), Cd concentration in food (3 levels), Cu concentration in food (3 levels), and the food consumption rate as the covariate (the criteria for significance: $p<0.05$). For each metal concentration or combination used accumulation of a single metal was correlated with food consumption rate by using Pearson's correlation coefficient (the criteria for significance: $p<0.05$). For all statistical analyses the SPSS 12.0 for Windows software was used.

Results

Accumulation of cadmium and zinc

The concentration of Cd in control animals and animals fed with only Zn-dosed food did not exceed 2 mg kg^{-1} dry body weight. Animals exposed to food contaminated with 250 and 500 mg Cd kg^{-1} dry food weight accumulated up to 400 mg Cd kg^{-1} dry body weight (Fig. 1). Cadmium accumu-

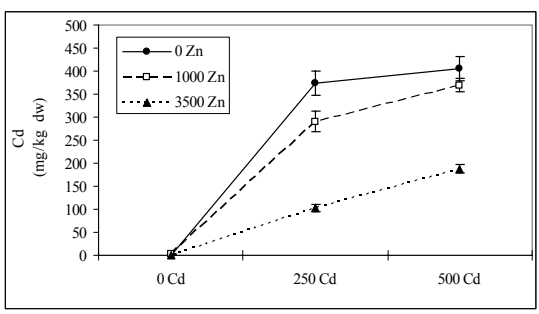


Figure 1: Concentration of Cd in *Porcellio scaber* after three weeks exposure to food contaminated with Zn or Cd or their mixture (AVR±SE).

lation from food was significantly lower where Cd in food was combined with Zn (Tab. 1). Cadmium accumulation was in general correlated with the food consumption rate which decreased with the increased concentration of both Cd and Zn in food (Fig. 3). No correlation between accumulated Cd and accumulated Zn was found.

Table 1: ANOVA (p values) for Zn, Cd and Cu accumulation in *Porcellio scaber* after 21 days of exposure to food dosed with a single metal (Zn, Cd, or Cu) or binary metal mixtures (Zn+Cd or Zn+Cu).

	Dependent variable			
	Zn accumulation		*Cd accumulation*	*Cu accumulation*
Factors:	*(Zn+Cd)*	*(Zn+Cu)*	*(Zn+Cd)*	*(Zn+Cu)*
Zn in food	*0.000*	*0.000*	*0.007*	*0.001*
Cd in food	*0.007*		*0.000*	
Cu in food		*0.897*		*0.000*
*Two way interaction Zn*Cd*	*0.136*		*0.000*	
*Two way interaction Zn*Cu*		*0.614*		*0.000*
Covariate: *Food cons. rate*	*0.098*	*0.602*	*0.000*	*0.136*

Accumulation of Zn (Fig. 2) was not dependent on food consumption rate (Tab. 1 and Fig. 3). In contrast to Cd, the accumulation of Zn from food even slightly increased when offered in a mixture with Cd (Fig. 2).

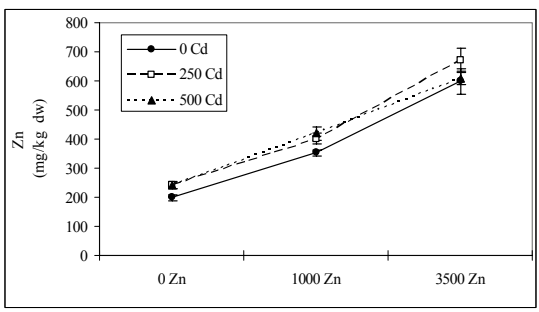


Figure 2: Concentration of Zn in *Porcellio scaber* after three weeks exposure to food contaminated with Zn, Cd, or their mixture (AVR±SE).

Accumulation of copper and zinc

The concentration of Cu in control animals and animals fed with Zn-dosed food was around 200 mg kg^{-1} dry body weight (Fig. 4). The copper concentration in Cu treated animals increased with increasing Cu concentration in food. Animals exposed to food contaminated with 3500 mg Zn kg^{-1} food in a mixture with 500 or 2000 mg Cu kg^{-1} food accumulated significantly less Cu compared to animals fed with only Cu-contaminated food. 1000 mg Zn kg^{-1} food did not affect the accumulation

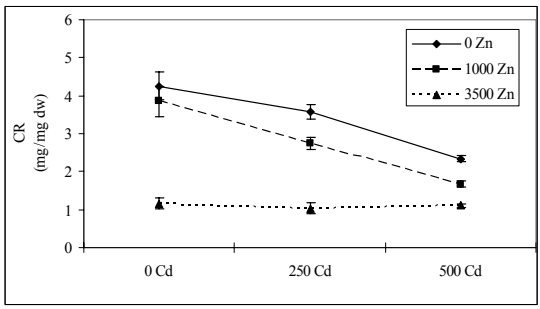


Figure 3: Food consumption rate (CR) in *Porcellio scaber* after three weeks exposure to food contaminated with Zn, Cd, or their mixture (AVR±SE).

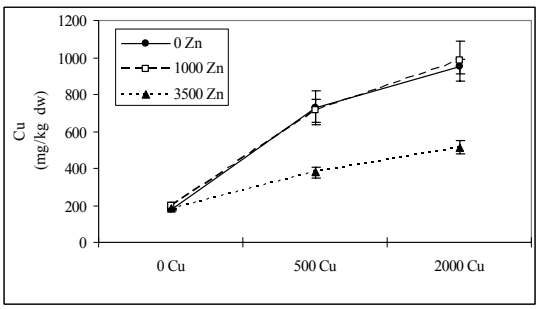


Figure 4: Concentration of Cu in *Porcellio scaber* after three weeks exposure to food contaminated with Zn, Cu, or their mixture (AVR±SE).

of Cu. In general, the food consumption rate had no significant effect on the accumulation of Cu, although the consumption rate decreased with increasing concentration of Zn and Cu in food (Fig. 6, Tab. 1). On the other hand, in animals exposed to 500 mg Cu kg^{-1} + 3500 mg Zn kg^{-1} food, Cu accumulation significantly correlated with food consumption rate.

The accumulation of Zn was affected neither by the Cu concentration in food nor by the food consumption rate (Tab. 1) and increased with increasing concentration of Zn in food (Fig. 5).

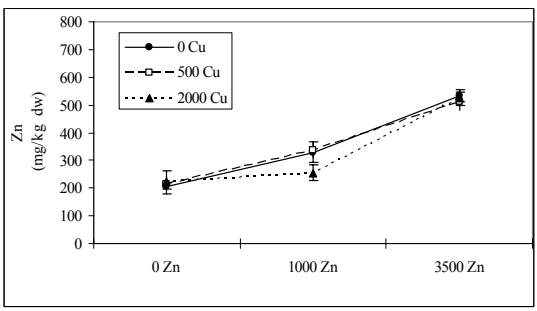


Figure 5: Concentration of Zn in *Porcellio scaber* after three weeks exposure to food contaminated with Zn, Cu, or their mixture (AVR±SE).

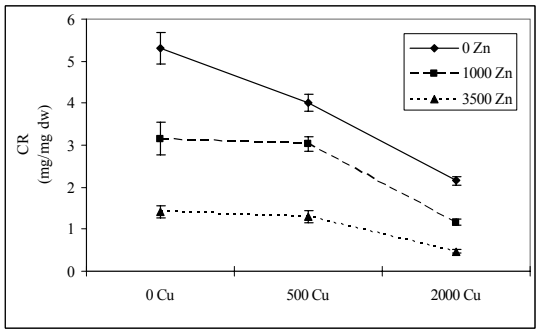


Figure 6: Food consumption rate (CR) in *Porcellio scaber* after three weeks exposure to food contaminated with Zn, Cu, or their mixture (AVR±SE).

Discussion

Our results demonstrated lower accumulation of Cd and Cu in *Porcellio scaber* after exposure to food dosed with Zn+Cd or Zn+Cu mixtures compared to single metal exposure. In contrast, the accumulation of Zn was nearly unaffected by the presence of Cd or Cu. The changed accumulation pattern for Cd and Cu can be attributed to a changed food consumption rate or to interactions between the mixture constituents.

Metal intake may be avoided by regulation of the food consumption rate (DROBNE & HOPKIN 1995, ZIDAR & AL. 2003b) or by food selection (KASCHL & AL. 2002, ZIDAR & AL. 2003a, 2004, 2005). The two selected concentrations of Zn, Cd, and Cu used in our experiment were previously determined as the concentrations with no major impact on food consumption rate and as concentrations that decrease the food consumption rate in short term exposure to a single metal (ZIDAR 2000). In contrast to our previous finding, 1000 mg Zn /kg and 500 mg Cu /kg were recognised as concentrations that already affect food consumption rate. In a mixture, all metal concentrations decreased food consumption. At all concentrations used the food consumption rate was correlated with accumulation of Cd, but not with Cu or Zn accumulation, although the latter had the strongest effect on food consumption rate. This proved that Cd accumulation could be reduced by the regulation of food consumption rate, as found in our previous studies (KASCHL & AL. 2002, ZIDAR & AL. 2003ab, ZIDAR & AL. 2005).

Beside food consumption rate, chemical and physiological interactions between food constituents might also affect metal bioavailability. It is known that Cd has a higher affinity for chloride ions than Zn (reviewed in RAINBOW 1997) which affects the uptake of Cd from the substrate in collembolans (VAN GESTEL & HENSBERGEN 1997). In isopods Cd complexation with chloride most probably does not affect the uptake of Cd. HAMES & HOPKIN (1991b) found an even higher assimilation rate for Cd compared to Zn when both metals were offered in a mixture as chloride salts (100 mg Zn/ kg + 100 mg Cd/ kg dry food weight). In *Porcellio laevis* exposed to food contaminated with a Zn and Cd sulphate mixture, lower accumulation of Cd was observed (ODENDAAL & REINECKE 2004). Copper is not known to complex with chloride but it has stronger affinity for organic ligands than Zn (HERTZ & AL. 1990). POSTHUMA & AL. (1997) reported that Cu reduced the sorption of Zn to soil whereas Cu sorption was inert toward Zn addition. In contrast to our findings, Cu also stimulates the uptake of Zn in *Enchytraeus crypticus* (POSTHUMA & AL.1997).

Elevated concentrations of Zn might either reduce Cd and Cu uptake or increase their loss from storage sites in *P. scaber*. The only significant route by which metals can be assimilated or excreted

in terrestrial isopods is via the digestive system: via the cells of the hepatopancreas and papilate region of the gut (HOPKIN & MARTIN 1984, HAMES & HOPKIN 1989). There are two potential major mechanisms for the uptake of dissolved trace metals in crustaceans; binding to metal-specific membrane carriers, or entry via routes used in the uptake of major ions like Ca (RAINBOW 1997). The later route is known for Cd (WRIGHT 1977), whereas Zn uptake does not appear to follow any route for major ion uptake (RAINBOW & DALLINGER 1993). Evidence from studies on invertebrates suggests that assimilation of Zn into cells is regulated metabolically whereas Cu transport is strictly passive, dependent solely on the extracellular-intracellular gradient (summarised in HOPKIN 1993). Once in the cell, Cu and Cd are bound to a sulphur-containing ligand whereas Zn is bound to phosphate (HOPKIN 1993). DONKER (1992) found that the more Zn is stored in the hepatopancreas of *P. scaber* the lower is the proportion of Cd accumulated in this organ, while the concentration of Cd in the remainder of the body increased. DONKER (1992) assumed a limited storage capacity for metals in the hepatopancreas. The study of WITZEL (2000) revealed a significant increase in uptake and excretion of Cd and Zn when exposed to Zn and Cd simultaneously. WITZEL (2000) assumed that combined contamination with Cd and Zn inhibits the complete translocation of the assimilated metals into the hepatopancreas. Probably a fraction of Zn and/or Cd remains in the haemolymph or in other tissues, which can be excreted more easily (WITZEL 2000). This means that Zn might compete with Cu or Cd for metal-specific membrane carriers or for binding sites in metal binding proteins when entering the cell.

Conclusions

In *Porcellio scaber* the accumulation of Zn is not affected by decreased food consumption rate or by possible interactions between mixture constituents. In contrast, the accumulation of Cd and Cu is significantly reduced when food is also contaminated with Zn. The lower accumulation of Cd and Cu can be ascribed to metal interactions that affect uptake and/or loss of metals and which should be taken into account in biomonitoring data evaluation.

Acknowledgements

This work was financially supported by the Slovenian Ministry of Education, Science and Sport (projects No. Z1-3189 and PO-0525).

Povzetek

Kopenski raki enakonožci so pomembni pokazatelji obremenjenosti okolja s kovinami, saj kovine v telesu kopičijo. Kovine sprejemajo pretežno s hrano, skozi prebavilo, skladiščijo pa zlasti v celicah prebavnih žlez, vezane na nizko molekularne beljakovine ali pa v netopnih granulah. Količina privzetih kovin je odvisna od količine zaužite hrane, količine mikroorganizmov v hrani in prebavilu, dostopnosti kovin v okolju, pH prebavnih sokov, temperature v okolju in drugih dejavnikov. Interakcije med sestavinami hrane in kovinami lahko vplivajo na dostopnost, akumulacijo in izločanje kovin. Podatkov o medsebojnem vplivu različnih kovin v zmesi na privzem posamezne kovine je malo. Kopenski enakonožci so v industrijsko onesnaženem okolju običajno izpostavljeni visokim koncentracijam več kovin hkrati, zato so raziskave o medsebojnem vplivu kovin potrebne.

V predstavljenem delu smo spremljali akumulacijo cinka, kadmija in bakra pri kopenskem raku

enakonožcu vrste *Porcellio scaber* po izpostavitvi eni kovini ali pa dvem kovinam hkrati. Tovrstne raziskave so pomembne za razumevanje kinetike kovin pri rakih enakonožcih ter za pravilno vrednotenje okoljskih podatkov.

V poskusih smo uporabili mladiče kopenskih enakonožnih rakov vrste *Porcellio scaber*, ki so bili vzgojeni v laboratoriju. Živali smo 21 dni hranili z listi leske (*Corylus avellana*), ki smo jim dodali vodno raztopino cinka, bakra ali kadmija (Zn – 1000 in 3500 mg kg^{-1} suhe teže hrane; Cu – 500 in 2000 mg kg^{-1} suhe teže hrane; Cd – 250 in 500 mg kg^{-1} suhe teže hrane) ali pa zmesi cinka in kadmija oziroma cinka in bakra. Primerjali smo količino akumuliranih kovin v odvisnosti od koncentracije kovin v hrani in količine zaužite hrane v času poskusa.

Pri živalih, ki so bile izpostavljene le eni od kovin je telesna vsebnost kovin naraščala s koncentracijo kovine v hrani. Z naraščajočo koncentracijo kovin je upadala količina zaužite hrane, kar je vplivalo na privzem kadmija, na privzem cinka in bakra pa ni imelo značilnega vpliva. Akumulacija bakra in kadmija je bila značilno nižja ob prisotnosti cinka v hrani, medtem ko na akumulacijo cinka prisotnost bakra ali kadmija ni vplivala. Rezultati so tako pokazali, da med cinkom in kadmijem ter cinkom in bakrom verjetno prihaja do interakcij, zaradi česar se privzem kadmija in bakra zmanjša. Prav tako interakcije med cinkom in kadmijem ter cinkom in bakrom lahko vplivajo na kinetiko asimiliranega kadmija in bakra zaradi česar je izločanje presežnih koncentracij slednjih uspešnejše. Omenjene ugotovitve je potrebno upoštevati pri vrednotenju okoljskih podatkov.

Literature:

BEYER W.N., G.W. MILLER & E.J. CROMARTIE 1984: Contamination of the O2 soil horizon by zinc smelting and its effect on woodlouse survival. J. Environ. Qual. 13 (2): 247-251.

BIBIČ A, D. DROBNE, J. ŠTRUS & A.R. BYRNE 1997: Assimilation of zinc by *Porcellio scaber* (Isopoda, Crustacea) exposed to zinc. Bull. Environ. Contam. Toxicol. 58: 814-821.

CORTET J., A. GOMOT-DE VAUFLERY, N. POINSOT-BALAGUER, L. GOMOT, C. TEXIER & D. CLUZEAU 1999: The use of invertebrate soil fauna in monitoring pollutant effects. Eur. J. Soil. Biol. 35 (3): 115-134.

COUGHTREY P.J., M.H. MARTIN & E.W. YOUNG 1977: The woodlouse, *Oniscus asellus*, as a monitor of environmental cadmium levels. Chemosphere 12: 827-832.

COUGHTREY P.J., M.H. MARTIN, J. CHARD & S.W. SHALES 1980: Micro-organisms and metal retention in the woodlouse *Oniscus asellus*. Soil. Biol. Biochem. 12: 23-27.

DALLINGER R., B. BERGER & S. BIRKEL 1992: Terrestrial isopods: Useful biological indicators of urban pollution. Oecologia 89: 32-41.

DONKER M.H 1992: Energy reserves and distribution of metals in populations of the isopod *Porcellio scaber* from metal-contaminated sites. Funct. Ecol. 6: 445-454.

DONKER, M.H., KOEVOETS, P., VERKLEIJ, J.C.C., VAN STRAALEN, N. 1990. Metal binding compounds in hepatopancreas and haemolymph of *Porcellio scaber* (Isopoda) from contaminated and reference areas. Comp. Biochem. Physiol. 97: 119-126.

DONKER M.H., H.M ABDEL-LATEIF, M.A. KHALIL, B.M. BAYOUMI & N.M. VAN STRAALEN 1998: Temperature, physiological time, and zinc toxicity in the isopod *Porcellio scaber*. Environ. Tox. Chem. 17/8: 1558-1563.

DROBNE D. & S.P. HOPKIN 1995: The toxicity of zinc to terrestrial isopods in a standard laboratory test. Ecotox. Environ. Safe. 31: 1-6.

FARKAS S., E. HORNUNG & E.FISCHER 1996: Toxicity of copper to *Porcellio scaber* Latr. (Isopoda) under different nutritional status. Bull. Environ. Contam. Toxicol. 57: 582-588.

HAMES C.A.C. & S.P. HOPKIN 1989: The structure and function of the digestive system of terrestrial isopods. J. Zool. 217: 599-627.

HAMES C.A.C. & S.P. HOPKIN 1991a: A daily cycle of apocrine secretion by the B cells in the hepatopancreas of terrestrial isopods. Can. J. Zool. 69: 1931-1937.

HAMES C.A.C. & S.P. HOPKIN 1991b: Assimilation and loss of ^{109}Cd and ^{65}Zn by the terrestrial isopods *Oniscus asellus* and *Porcellio scaber*. Bull. Environ. Contam. Toxicol. 47: 440-447.

HEIKENS A., W.J.G.M. PEIJNENBURG & A.J. HENDRIKS 2001: Bioaccumulation of heavy metals in terrestrial invertebrates. Environ. Pollut. 113: 385-393.

HERTZ J., C. ANGEHRN-BETTINAZZI & H. STÖCKLI 1990: Distribution of heavy metals in various litter horizons and forest soil. Inter. J. Environ. Anal. Chem. 39: 91-99.

HOPKIN S.P, G.N. HARDISTY & M.H. MARTIN 1986: The woodlouse *Porcellio scaber* as a žbiological indicator'of zinc, cadmium, lead and copper pollution. Environ. Pollut. 11: 271-290.

HOPKIN S.P. & M.H. MARTIN 1982: The distribution of zinc, cadmium, lead and copper within the hepatopancreas of a woodlouse. Tiss. Cell 14: 703-715.

HOPKIN S.P. & M.H. MARTIN 1984: Heavy metals in woodlice. Symp. zool. Soc. Lond. 53: 143-166.

HOPKIN S.P. 1989: Ecophysiology of metals in terrestrial invertebrates. Elsevier Applied Science, Barking, U.K..

HOPKIN S.P. 1990: Critical concentrations, pathways of detoxification and cellular ecotoxicology of metals in terrestrial arthropods. Funct. Ecol. 4: 321-327.

HOPKIN S.P. 1993: Deficiency and excess of copper in terrestrial isopods. In: DALLINGER R. & P.S. RAINBOW (Eds.): Ecotoxicology of Metals in Invertebrates. SETAC Spec. Publ. Ser., Lewis Publishers, pp. 359-382.

HOPKIN S.P., C.A.C. HAMES & A. DRAY 1989: X-ray microanalytical mapping of the intracellular distribution of pollutent metals. Microsc. Anal. 23-27.

HOPKIN S.P., D.T. JONES & D. DIETRICH 1993: The isopod *Porcellio scaber* as a monitor of the bioavailability of metals in terrestrial ecosystems: towards a global žwoodlouse watch' scheme. The Science of the Total Environment, Supplement 1993, Elsevier Science Publishers B.V., Amsterdam, 357-365.

KASCHL U.I., P. ZIDAR, J. ŠTRUS & V. STORCH 2002: Food choice experiments with cadmium nitrate dosed food in terrestrial isopod *Oniscus asellus* (Crustacea). Acta Biol. Slov. 45 (2), 3 -14.

MARTIN M.H, P.J. COUGHTREY & E.W. YOUNG 1976: Observations on the availability of lead, zinc and cadmium by the woodlouse, *Oniscus asellus*. Chemosphere 5: 313-318.

ODENDAAL J.P. & A.J. REINECKE 2004: Evidence of metal interaction in the bioaccumulation of cadmium and zinc in *Porcellio laevis* (Isopoda) after exposure to individual and mixed metals. Water Air Soil Poll. 156: 145-161.

PAOLETTI M.G. & M. HASSALL 1999: Woodlice (Isopoda: Oniscidea): their potential for assessing sustainability and use as bioindicators. Agr. Ecosyst. Environ. 74: 157-165.

POSTHUMA L., R. BAERSELMAN, R.P.M. VAN VEEN & E.M. DIRVEN-VAN BREEMEN 1997: Single and joint toxic effects of copper and zinc on reproduction of *Enchytraeus crypticus* in relation to sorption of metals in soil. Ecotox. Environ. Safe. 38: 108-121.

PROSI F. & R. DALLINGER 1988: Heavy metals in the terrestrial isopod *Porcellio scaber* Latreille. I. Histochemical and ultrasructural characterisation of metal-containing lysosomes. Cell. Biol. Toxicol. 4: 81-96.

RAINBOW P.S. & R. DALLINGER 1993: Metal uptake, regulation, and excretion in freshwater invertebrates. In: DALLINGER R. & P.S. RAINBOW (Eds.): Ecotoxicology of Metals in Invertebrates. SETAC Spec. Publ. Ser., Lewis Publishers, pp. 119-131.

RAINBOW P.S. 1997: Ecophysiology of trace metals uptake in Crustaceans. Estuar. Coast. Shelf. S. 44: 169-175.

VAN GESTEL C.A.M. & P.J. HENSBERGEN 1997: Interaction of Cd and Zn toxicity for *Folsomia candida* Willem (Collembola: Isotomidae) in relation to bioavailability in soil. Envir. Tox. Chem. 16: 1177-1186.

WIESER W. & J. KLIMA 1969: Compartmentalization of copper in the hepatopancreas of isopods. Microscopie 24: 1-9.

WIESER W. 1961: Copper in isopods. Nature 191:1020.

WIESER W., G. BUSCH & L. BÜCHEL 1976: Isopods as indicators of copper content of soil and litter. Oecol. 23: 107-114.

WITZEL B: 2000: The influence of zinc on the uptake and loss of cadmium and lead in the woodlouse, *Porcellio scaber* (Isopoda, Oniscidea). Ecotox. Environ. Safety. 47: 43-53.

WRIGHT D.A. 1977: The effect of calcium on cadmium uptake by the shore crab Carcinus maenas. J. Exp. Biol. 67: 163-173.

ZIDAR P. 2000: Effects of metal ions on food consumption of terrestrial isopod *Porcellio scaber* (Crustacea: Isopoda). Ph.D thesis. Ljubljana

ZIDAR P., D. DROBNE, J. ŠTRUS & A. BLEJEC 2003b: Intake and assimilation of Zn, Cu and Cd in the terrestrial isopod *Porcellio scaber* Latr. (Crustacea, Isopoda). Bull. Environ. Contam. Toxicol. 70: 1028 – 1035.

ZIDAR P., D. DROBNE, J. ŠTRUS, C.A.M. VAN GESTEL & M. DONKER 2004: Food selection as a means of Cu intake reduction in the terrestrial isopod *Porcellio scaber* (Crustacea, Isopoda). Appl. Soil Ecol. 25 (3): 257-265.

ZIDAR P., I.U. KASCHL, J. ŠTRUS, D. DROBNE & J. BOŽIČ 2003a: Avoidance behaviour in *Oniscus asellus* (Crustacea, Isopoda) as an indicator of food quality. Arch. Ind. Hyg. Toxicol. 54, 177-181.

ZIDAR P., J. BOŽIČ & J. ŠTRUS 2005: Behavioural response in the terrestrial isopod *Porcellio scraber* (Crustacea) offered a choice of uncontaminated and cadmium-contaminated food. Ecotox. (Lond.) 14: 1-10.

ŽNIDARŠIČ N. 2003: Copper and zinc dynamics and related cellular mechanisms in the digestive system of isopod crustaceans *Porcellio scaber* (Crustacea: Isopoda). Ph.D thesis, Ljubljana.

ACTA BIOLOGICA SLOVENICA
LJUBLJANA 2005 Vol. 48, Št. 1: 13-20

Sprejeto (accepted): 2005-09-27

Analysis of Cell Motility with a Successive Sequence of Images

Analiza gibanja celic s serijo zaporednih fotografij

Katarina ZLATOLAS, Peter VERANIČ*, Kristijan JEZERNIK

Institute of cell biology, Lipičeva 2, Medical Faculty of Ljubljana, Ljubljana
* corresponding author

Abstract. Analysis of cell motility usually requires several hours of continuous video recording of a single view field. Consequently, the method is very much time consuming and only a small number of cells is usually included into analysis. To increase the number of cells monitored in an experiment we developed a simple and expeditious method for analysing the cell motility. A combination of successive series of images of individual cells relocated on CELLocate coverslips and vector graphic editing software Adobe Illustrator 9.0 enabled simultaneous monitoring of locomotion for up to ten times more cells as with previous methods.

Keywords: Cell motility, digital camera, successive sequence of images, software Adobe Illustrator 9.0.

Izvleček. Analiza celičnega gibanja ponavadi zahteva večurno snemanje enega vidnega polja z video kamero. Metoda je zato zamudna in le malo celic se lahko vključi v analizo. Z namenom povečanja števila celic v določenem poizkusu, smo razvili preprosto in hitro metodo za analizo celičnega gibanja. S kombinacijo zaporednega slikanja istega vidnega polja, kar nam je omogočila uporaba CELLokate krovnih stekelc, in vektorskega grafičnega programa Adobe Illustrator 9.0, smo lahko v analizo gibanja celic vključili do desetkrat več celic, v primerjavi z dosedanjimi metodami.

Ključne besede: Celično gibanje, digitalni fotoaparat, zaporedna serija posnetkov, Adobe Ilustrator 9.0

Introduction

Monitoring of cell motility provides information about the level of cell differentiation, its responsiveness to growth factors and can determine at which phase of cell cycle the cell is (BONNETON & al. 1999). The motility of cells is involved in many physiological (embryonic development) and pathological processes (invading malignant cells, immune response and wound repair). Epithelial cells are very much restricted in their motility, because of their attachment to the neighbouring cells and to the extracellular matrix. In order to move from their original place to the final destination, the

epithelial cells have to go through epithelio-mesenchymal transformation (EMT). Cells, that undergo EMT, become motile and assume fibroblast appearance. Mesenchymal cells have a specialized ability to move through the extracellular matrix, they have front-to-back end polarity and form only transient contacts with their neighbours and the extracellular matrix (HAY 1995).

For the measurement of cell motility, various methods have been used recently, mainly using video microscopy and analysis of a sequence of images taken at specific intervals (MORTON & TCHAO 1994, WICK & al. 2003). The common problem for all these methods is that they require monitoring of the same view field for a prolonged period of time due to slow motility of cells. Recently, cover slips with a microgrid became available. They enable relocation of individual cells and consecutive monitoring of several view fields within the time frame of a single experiment.

We have developed a method for measurement of cell motility, where a series of consecutive shots with digital camera can be taken on several view fields within the time frame of a single experiment. Series of images, analysed with vector graphic editing software, precisely show the path, direction and speed of individual cell.

Materials

Cell culture

Cultures of mice urothelial cell line (g/G) were maintained in 1 : 1 mixture of cell culture medium DMEM and HAM F12 with 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenit, 100 ng/ml hydrocortisone in 10 % fetal calf serum. All chemicals were obtained from SIGMA Chemical Co. (St. Louis, MO). Cells were grown on microgrid cover slips (Eppendorf CELLocate Coverlips, square 55 µm) in plastic dishes (radius = 2 cm). Motility was stimulated with FGF1 (20 ng / ml) for 48 hours.

Between photographic expositions, the cell cultures were maintained in incubator at 37 °C. The cells were outside the incubator, during taking images, for the maximum of 5 minutes. The room temperature was between 28 °C and 30 °C.

CELLocate coverslips

CELLocate is a round coverslip made of glass in accordance with ISO 8255. The diameter of the coverslip is 12 mm. CELLocate is suitable for light, fluorescence and electron microscopy. Coverslips are available in two different grid sizes: the 55 µm grid size (measured between the inner edges of the measuring square) is suitable for relocation of individual cells, while the 175 µm grid size is more suitable for relocation of cell groups. In this study, we have used coverslips with 55 µm grid size. The grid is composed of 4 x 6 squares and marked alphanumerically.

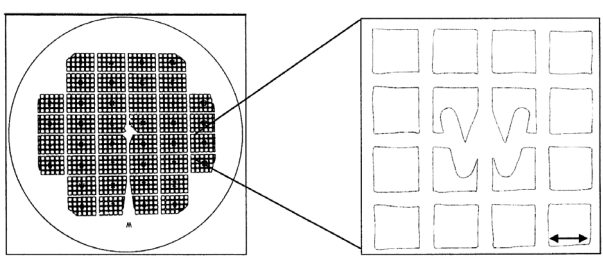


Figure 1: A microgrid on a CELLocate coverslip. The arrow indicates a 55 µm distance.
Slika 1: Mikromreža na CELLocate krovnem stekelcu. Puščica označuje 55 µm razdaljo.

Consecutive photography with digital camera

Digital camera NIKON COOLPIX 950 attached to inverted phase contrast microscope (NICON TE 300) was used.

A low-density culture of g/G cells grown on CELLocate coverslips, with the measuring grid, was used for the analysis of cell motility. The areas with 3 – 5 cells per square were selected. The same area on CELLocate coverslips was relocated every 30 minutes for 5 hours and 30 minutes and as a result, 12 images of an individual view field were obtained.

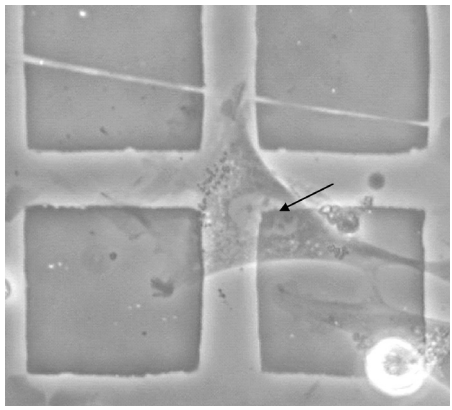


Figure 2: A cell grown on a microgrid of CELLocate coverslip. An arrow points to the nucleus.
Slika 2: Celica na mreži CELLocate krovnega stekelca. Puščica kaže jedro celice.

Results

The analysis of a sequence of consecutive images with Adobe Illustrator 9.0

The sequence of images, taken with digital camera, were copied to the hard disk of a personal computer. For the analysis of the motility a vector graphic editing software Adobe Illustrator 9.0 was used.

The analysis of cell motility was performed as follows.

The working sheet was resized to 55 x 55 mm in order to adjust it to the size of CELLocate microgirds square, which was 55 x 55 μm (Fig. 3)

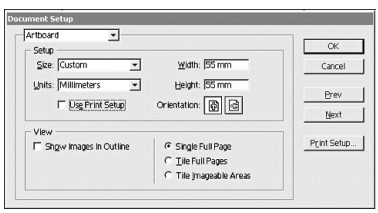


Figure 3: Resizing the working sheet: File > New > Document Set-up > Units = mm > Width = 55 mm > Height = 55 mm.
Slika 3: Umerjanje velikosti lista: Datoteka > Nova > Nastavitev dokumenta > Enote = mm> Širina = 55 mm > Višina = 55 mm

During the next step, a sequence of images were added using the »new layer« for each photograph in the sequence and borders of the microgrid square were aligned to the borders of the working sheet (Figs. 4 and 5).

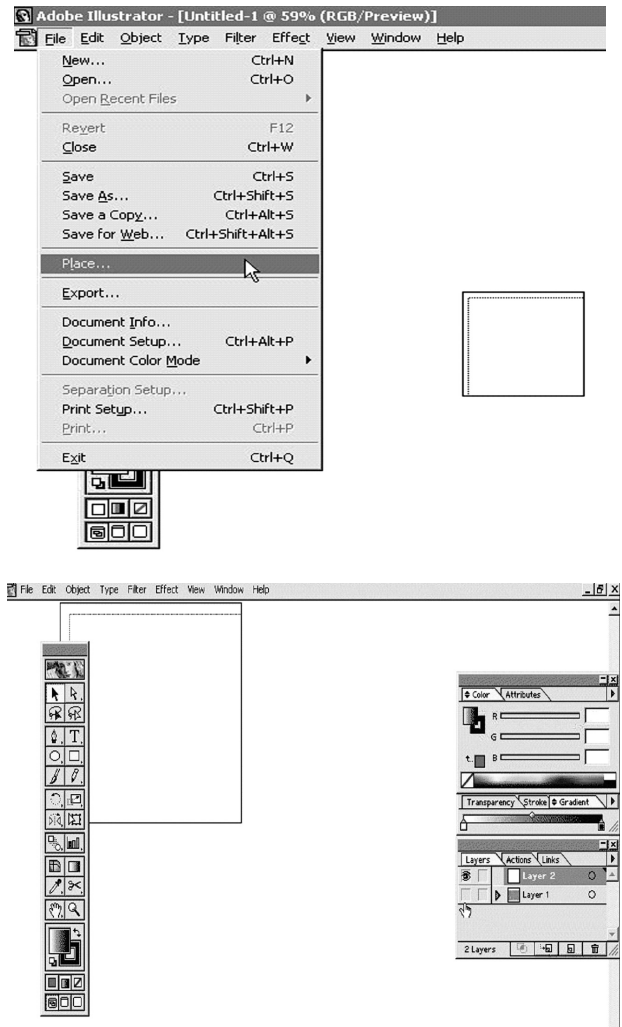


Figure 4 and 5: Serial addition of the pictures: Window > Show Layers > Layer 1 > File > Place (place 1st picture) (Fig. 4) > New Layer > Layer 2 (place second picture) (Fig 5).
Sliki 4 in 5: Zaporedno dodajanje slik: Okno> Pokaži plasti > Plast 1 > Datoteka > Postavi (postavimo prvo sliko) (Slika 5) > Nova plast > Plast 2 (postavimo drugo sliko) (Slika 5).

A contour of a nucleus of individual cell was made on each picture by using graphic tools (Fig.6). Each picture in a sequence was labelled and listed (Fig. 7 and 8).

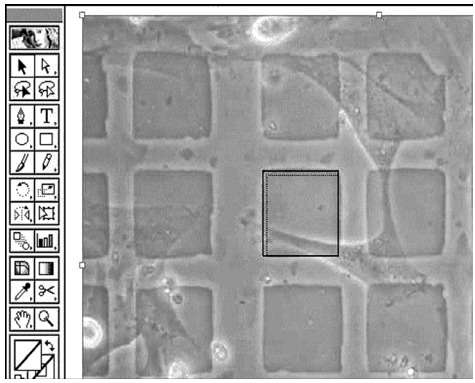
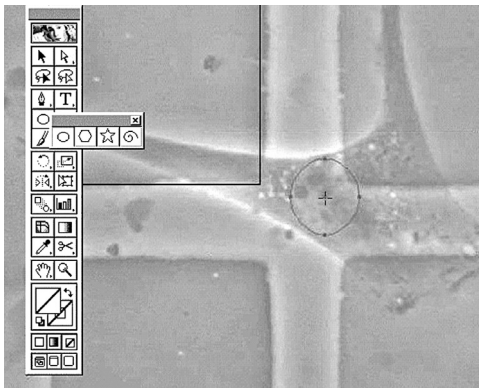
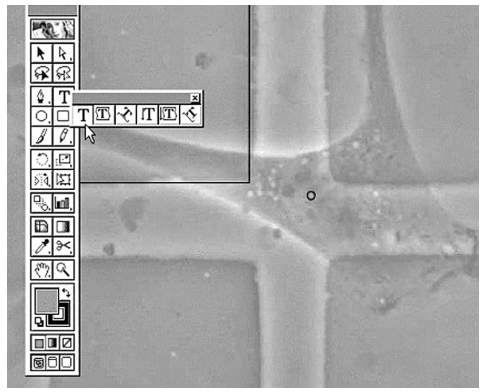


Figure 6: Borders adjustment: Window > Show Tools > Selection Tool (an arrow) > press and hold »shift« button and draw the corner of the picture with an arrow to adjust the borders of microgrid and working sheet.
Slika 6: Prilagoditev robov: Okno > Orodja > Izbrano orodje (puščica) > ob sočasnem držanju tipke »shift« s puščico vlečemo vogal fotografije, da se pokrijeta robova delovnega lista in kvadrata mikromreže





Figures 7 and 8: A contour of a nucleus: Tool > Ellipse Tool > (Fig.7) draw a contour of a nucleus > click in the centre of the ellipse > (Fig. 8) choose the width and length of the ellipse (2 x 2 mm) > Window > Show Colour > Selection Tool > choose the new ellipse (circle) > colour the circle > Type Tool > write the serial number of the picture
Sliki 7 in 8: Obrisovanje jedra: Orodja > Elipsa > (Slika 7) nariši obris jedra > klikni v sredino elipse > (Slika 8) izberi širino in višino elipse (2 x 2 mm) > Okno > Pokaži barve > Izbrano orodje > izberemo novo elipso > obarvamo elipso > Črkovno orodje > vpišemo zaporedno številko slike

The use of different colours (Window > Show Colour), transparency (Window > Show Transparency) and/or darker outlines (Window > Show Colour) is recommended to distinguishing partly overlapping nucleus positions in slow moving cells.

The procedure was repeated for all twelve images taken. Each photograph was deleted after contouring the nucleus and only contours were retained on the layers to reduce the size of the file (Fig. 9). In the end, all the layers were made visible on the monitor and the path of cells was analysed.

While the distance between the two points, showing position of the nucleus in 30 minutes intervals, was short (7-9 μm), the path between the points was estimated to be straight.

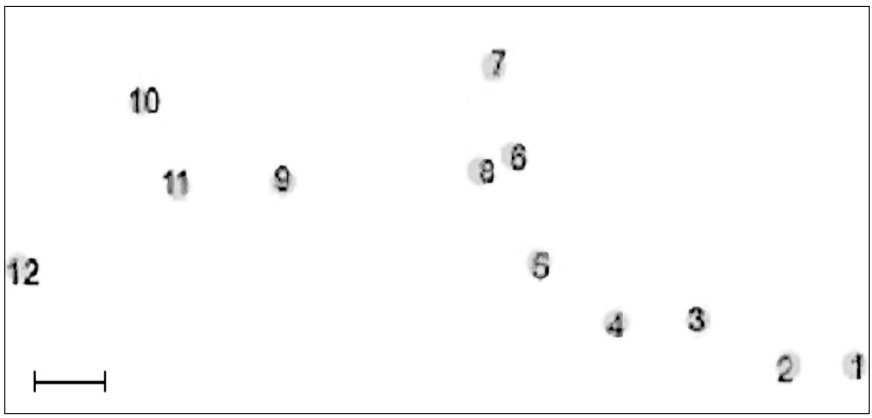


Figure 9: Result of 12 consecutive positions of a cell nucleus. Bar = 10μm
Slika 9: Prikazanih je 12 zaporednih položajev jedra gibajoče se celice. Merilna črtica = 10 μm

The velocity of an individual cell was calculated from the distance the cells reached in a definite time interval. The path was manually measured on a printed cumulative image, containing the positions of the nuclei at 30 minutes intervals. The system error was calibrated by 12 measurements of the distance between the same two points (from the centre to the centre of two points). The error in measurements of the path between the two points was ± 0.3 μm.

Discussion

The aim of the present study was to find an exact and quick method for the measurement of individual cell migration. The system of consecutive images and vector graphic editing software Adobe Illustrator 9.0 was used.

The problem in analysing epithelial cell migration is that an average speed of motility is approximately 20 μm per hour, so at least 5 hours of monitoring is required for the analysis of the motility of cell moving in a single view area (BRAY 1992). Thus, in most studies the number of monitored cells was limited less than eighth cells within an experiment (MORTON & TCHAO 1994, WICK & al. 2003). With our method, using series of successive images of cells on defined areas of a coverslip with a micro-grid, motility of more than 80 cells (20 cells per experimental group) was analysed within the time frame of a single experiment. The increased number of monitored cells makes the results of measurements more accurate.

In our experiments, where the velocity of urothelial cell line g/G was measured after stimulation with FGF1, we used 30 minutes long intervals, because we ascertained that this is the optimal interval for the velocities close to 20 μm / h. In 30 minutes, the centre of gravity of the fastest cells

changed their position for 10 μm, which is approximately half of the cell length. Therefore, the 30 minutes long interval between snap-shots is long enough for monitoring of five view fields with minimal error. Because of various velocities of cell motilities in different cell types and frequent changes of direction, shorter intervals are appropriate for the analysis of higher velocity. However, shorter intervals reduce the number of experimental groups, which can be monitored simultaneously.

During motility, cells undergo dramatic phenotype changes that are mainly based on the dynamic assembly and disassembly of actin filaments underlying the plasma membrane (BRAY 1992). Cells that become motile usually assume a fibroblastic appearance with lamellipodia adjusting the shape by each change of the motility direction (BONNETON & al. 1999, BRAY 1992). Such changes of the cell shape make motility of different parts of the cell inhomogeneous and so difficult to analyse. The problem is usually solved by determining nucleus as the centre of gravity. Because this most prominent organelle of eukaryotic cells, lies in the area, where usually most of the cell mass is concentrated (BONNETON & al. 1999). In our experiments, a contour of nucleus was drawn with Adobe Illustrator 9.0 software. The nuclei of our cells are usually round, which simplifies drawing a contour. The monitoring of an individual cell nucleus displacement was enabled by the use of CELLocate coverslips with a microgrid.

Until now, the displacement of each individual cell has usually been followed using recording tape of time-lapse video recorder, tracing paper and curvimeter. Due to better resolution compared to most video cameras, which were mainly used in such studies (BONNETON & al. 1999, MORTON & TCHAO 1994, WICK & al. 2003), makes digital camera better choice for the analysis of cell motility. Better resolution is important for the accurate localization of the nuclei taken as the centre of cell gravity.

During cell division, the nuclear envelopes become fragmented and reorganization of cytoskeleton appears. Cells become rounded, reduce the attachment surface and become immobile until the end of mitosis (BONNETON & al. 1999). Occasionally such dividing cells detach from the surface and become lost. Changing of cell morphology and motility during cell division represents an obstacle in measurements of velocity. For this reason, it is of benefit to monitor as many cells as possible.

Certain cell lines do not adhere well to glass and therefore, do not grow well on CELLocate. In such cases, Eppendorf Company recommends that CELLocate is coated with collagen. Using collagen is not appropriate for the coverslips with 55 μm grid size, because it reduces the clarity and visibility of cell nucleus. The margins of 175 μm grid size coverslips are deeper, which enhance the contrast and so enable the use of collagen coating.

This method for analysing the cell motility with a combination of successive series of images of individual cells and vector graphic editing software Adobe Illustrator 9.0 has revealed itself as very successful, while being quick and exact. Most of all it enables simultaneous monitoring of locomotion for much larger number of cells in comparison with previously used methods.

Povzetek

V dosedanjih raziskavah so za spremljanje gibanja celic uporabljali predvsem videomikroskopijo. Vse dosedanje metode omogočajo spremljanje gibanja celic le v enem vidnem polju za vsak eksperiment. Zaradi majhne hitrosti gibanja celic je spremljanje celic zamuden proces, zato je večina analiz omejena na majhno število celic, kar zmanjša verodostojnost dobljenih rezultatov. Z namenom, da bi lahko hkrati spremljali gibanje celic na več vidnih poljih, smo razvili metodo

zaporednega fotografiranja z digitalnim fotoaparatom in analizo hitrosti gibanja z grafičnim vektorskim programom Adobe Illustrator 9.0.

Med celičnim gibanjem se oblika celice spreminja, zato smo spremljali spremembe položaja težišča izbranih celic. Za težišče celice smo določili vedno bolj ali manj okroglo jedro, ki ponavadi leži v področju, kjer je zbrana glavnina mase celice.

Namesto videokamere smo uporabili digitalni fotoaparat, ki zaradi boljše ločljivosti omogoča natančnejše meritve. V določenih časovnih zamikih smo izbrana polja s celicami zaporedoma fotografirali in tako dobili serijo slik, ki ponazarjajo smer in hitrost gibanja določene celice. Ponovna lokalizacija celic in merjenje premika celice je omogočila uporaba CELLocate krovnih stekelc z jedkano mrežo. Serija dobljenih posnetkov individualnih celic predstavlja prepotovano pot celice, iz katere lahko izračunamo hitrost potovanja. Predstavljena metoda omogoča spremljanje celic na več vidnih poljih istočasno, kar lahko bistveno poveča število opazovanih celic in s tem relevantnost rezultatov merjenja hitrosti gibanja celic.

References

BONNETON C., J.B. SIBARITA & J.P. THIERY 1999: Relationship between cell migration and cell cycle during the initiation of epithelial to fibroblastoid transition. Cell Motility and the Cytoskeleton **43**: 288-295.

BRAY D. 1992: Cell movements. Garland publishing, INC., New York & London, pp. 17-45.

HAY E.D. 1995: An overview of epithelio-mesenchymal transformation. Acta Anat. **154**: 8-20.

MORTON D.M. & R. TCHAO 1994: Regulation of motility and cytoskeletal organization of rat bladder carcinoma cells by cyclic AMP. Cell Motility and the Cytoskeleton **29**: 375-382.

WICK N., S. THURNER, K. PAIHA, R. SEDIVY, I. VIETOR & L.A. HUBER 2003: Quantitative measurement of cell migration using time-lapse videomicroscopy and non-linear system analysis. Springer-Verlag: Histochemistry and Cell Biology, pp. 1-12.

ACTA BIOLOGICA SLOVENICA
LJUBLJANA 2005 Vol. 48, Št. 1: 21-31

Sprejeto (accepted): 2005-09-27

The macrophytes of lake Velenjsko Jezero, Slovenia – the succession of macrophytes after restoration of the lake

Makrofiti Velenjskega jezera, Slovenija – sukcesija makrofitov po restavraciji jezera

Zdenka MAZEJ[1], Mateja EPŠEK[2]

[1] ERICo Velenje; Environmental Research and Industrial Co-operation Institute, Koroška 58, 3320 Velenje, Slovenia; Tel.: +386 3 898 19 58; fax: +386 3 898 19 42; E-mail: zdenka.mazej@erico.si
[2] Biotechnical Faculty, Department of Biology, Večna pot 111, 1000 Ljubljana, Slovenia

Abstract. The macrophyte vegetation in the artificial lake Velenjsko jezero has been monitored since 1996. The pH of the lake was around 12 up to 1994, when it was remediated. After that macrophytes started to colonize a large proportion of the littoral very quickly. The pioneer species, which appeared in the first survey year (1996), were *Chara* sp., *Nuphar lutea*, *Potamogeton crispus* and *Myriophyllum spicatum*. *Potamogeton crispus* was the dominant species till 1997. In the following years *Najas marina* and *Potamogeton filiformis* prevailed over other species in the lake. Since the beginning of the colonization, the species composition had become more heterogeneous and the quantitative relationships between the species varied enormously. In the year 2004, 9 species were detected in the lake. While species *Najas marina*, *Potamogeton filiformis, Potamogeton lucens, Potamogeton nodosus* show positive development progress, in the last years minor appearance of *Chara* sp., *Potamogeton crispus* and *Najas minor* was observed.

Key words: lake Velenjsko jezero, succession of macrophytes, species composition and distribution, relative plant mass, maximal depth colonization

Izvleček. Makrofite v umetno nastalem Velenjskem jezeru spremljamo od leta 1996. Vrednost pH jezerske vode je bila vse do leta 1994, ko so v Termoelektrarni Šoštanj uvedli zaprti krog transportne vode, okoli 12. Vrednost pH je po tem ukrepu hitro padla na vrednost okoli 8. Makrofiti so postopoma pričeli naseljevati litoralno območje jezera. Pionirske vrste v letu 1996 so bile *Chara* sp., *Nuphar lutea*, in *Potamogeton crispus*. *Potamogeton crispus* je bila dominantna vrsta vse do leta 1997. V naslednjih letih sta prevladali vrsti *Najas marina* in *Potamogeton filiformis*. Od začetka naseljevanja je prihajalo do velikih kvalitativnih in kvantitativnih sprememb v vrstni sestavi in razporeditvi makrofitov. V jezeru je leta 2004

uspevalo 9 vrst. Medtem ko kažejo vrste *Najas marina*, *Potamogeton filiformis, Potamogeton lucens* in *Potamogeton nodosus* pozitiven trend v razvoju, pa v zadnjih letih opažamo manjše pojavljanje vrst *Chara* sp., *Potamogeton crispus* in *Najas minor.*

Key words: Velenjsko jezero, sukcesija makrofitov, vrstna sestava, distribucija makrofitov, relativna rastlinska biomasa, maksimalna globina naselitve

Introduction

The succession of submersed macrophytes in lakes is a complex process. Once they are established, macrophytes have significant feedback effects on lake water quality and ecosystem health, and are an important consideration in lake management (JEPPESEN & al. 1998). In lakes where conditions favour development of high biomass of submerged aquatic vegetation over large areas, there is potential for large-scale reductions in water column concentrations of nutrients and phytoplankton, and increase transparency (JEPPESEN & al. 1998). Factors that influence the biomass and distribution of submersed macrophytes both among and within lakes have been well studied. These factors include substratum character and water depth (WEISNER 1991), transparency (HUTCHINSON 1975, NICHOLS 1992, MIDDELBOE & MARKAGER 1997, MAZEJ & GABERŠČIK 1999, VAN DUIN & al. 2001, BEST & al. 2001), water chemistry (PIP 1984, HASLAM 1987, PRESTON 1995, GERM & GABERŠČIK 1996, GERM & al. 2003), temperature (PIP 1989, ROONEY & KALFF 2000), bottom slope (NICHOLS 1992), water level fluctuation and wave action (PRESTON 1995, ANDERSSON 2001), filamentous and planktonic algae (URBANC-BERČIČ & al. 2002), grazing (JACOBSEN & SAND-JENSEN 1992, WEISNER & al. 1997) and intra- and inter- species competition among macrophyte species (FOX 1992, AGAMI & WAISEL 2002). The underwater light regime is one of the most important determinants of submerged aquatic vegetation (HUTCHINSON 1975, NICHOLS 1992, BEST & al. 2001, VAN DUIN & al. 2001). DUARTE AND KALFF (1986) asserted that within lakes, the morphometric variables of bottom slope and community exposure are both negatively correlated with macrophyte biomass. In lakes, which shelve rapidly, the density of plants on the shore of the lake falls very quickly. On the contrary, if the shore is shallow and slopes gently, the belt overgrown by aquatic plants can be very wide (NICHOLS 1992).

Many macrophytes are tolerant to pH values between 10 and 11; higher values are destructive (BOWES 1987). The pH of Lake Velenjsko jezero was around 12 until 1994, when reconstruction of the fly ash system and introduction of a closed loop water cycle was made at the Šoštanj Thermal Power Plant. The pH declined to around 8 and biota started to colonize the lake. Our study was focused on aquatic macrophytes, an important group of primary producers, which provide conditions necessary for fish, zooplankton and benthos in the littoral zones of lakes. Information on the distribution and species composition of aquatic vegetation is necessary for management and understanding of aquatic systems. We monitored the succession of species composition and abundance of submersed macrophytes after restoration measures in Lake Velenjsko jezero. Our method was based on direct field observations along transects that enabled identification of the main environmental factors influencing the appearance of macrophytes.

Materials and Methods

Description of the site

Velenjsko jezero is located in the Šalek Valley, at an altitude of 366 m, with a surface 135000 m^2 and a maximal depth of 54 m. It is an artificial lake resulting from mining activity. As a result of subsidence, whole settlements, meadows and fields submerged and flooded. Up to 1983 fly ash slurry from the Šoštanj Thermal Power Plant was transported by pipeline and emptied into Velenjsko jezero. It brought ash and calcium hydroxide to the lake, raising the pH of lake water to 12. Since 1983 the ash was used to build in embankments, but effluent with a pH around 12 remained the predominant polluter of the lake until 1994. After reconstruction of the fly ash system and a closed loop water cycle (October 1994), biota appeared in the lake again. It was recolonized by phyto- and zooplankton, fish, macrophytes and other organisms. The pH of the lake is now around 8. The lake shore is sparsely overgrown with emergent plants (e.g. *Typha latifolia* L., *Phragmites australis* (Cav.) Trin. Ex Steud., *Schoenoplectus lacustris* (L.) Palla [*Scirpus lacustris* L.]); the major part of the lake shore consists of bare areas, meadows and allotments. The littoral zone of the lake can be divided into three main regions: the fairly steep western and south-western unstable shoreline, a north-eastern marshy area, and the eastern and southern part of the lakeshore, which is used for sunbathing, walking, riding, bicycling and other recreational activities. Near the shore are allotments.

Macrophyte cover

The method of surveying the entire littoral from a boat, using depthmeter, view box and sampling rake, was applied in the years 1996, 1997, 1999, 2000, 2003 and 2004. In the year 2003 surveying of macrophyte community was carried out in detail. The littoral was checked every month from June to September. The shoreline was divided into 25 sections of the length 200±2 m. Species abundance in each section was evaluated according to KOHLER & JANAUER (1995) on a five level descriptor scale (1 – very rare, 2 – infrequent, 3 – common, 4 – frequent, 5 – abundant, predominant). The evaluated quantity of plants is interpreted as mass index (MI), which is with »real biomass« (PM) related with the function PM = MI^3. Obtained data has been processed by standard methodology, made in co-operation between the University teams of Hohenheim (Germany) and Vienna /Austria) (KOHLEER AND JANAUER 1995). The Internet based description of the methodology is accessible on the internet side www.midcc.at. From the data we can calculated two mass indexes; MMO is the Mean Mass Index of the individual species with respect to the survey transects they occur, MMT is the Mean Mass Index of individual species with regard to the full length of the lake shoreline. The distribution ratio »d« of each species stands for MMT^3/MMO^3. Nearly similar and high values of MMT and MMO (»d« value is near 1) reveal that a species is abundant and that it occurred in almost all sections – the distribution of the species is homogeneous.

$$MMT = \sqrt[3]{\frac{\sum_{i=1}^{n} MI_i^{\,3} * AL_i}{GL}} \qquad MMO = \sqrt[3]{\frac{\sum_{i=x}^{n} MI_i^{\,3} * AL_i}{\sum_{i=x}^{n} AL_i}}$$

MI_i = Mass Index of species in transect i
Al_i = length of transect i, where species occurred
GL = total length of lake shoreline

The Relative Plant Mass (RPM %) was used to calculate the quantitative significance of individual species in a section (PALL & JANAUER 1995).

$$RPM_x[\%] = \frac{\sum_{i=1}^{n}(PM_{xi}*L_i)*100}{\sum_{j=1}^{k}\left(\sum_{i=1}^{n}(PM_{ji}*L_i)\right)}$$

$PM_{xi} = MI_{xi}^{3}$
RPM_x = relative plant mass of species x
MI_{xi} = for any transect i estimated mass of the species x
PM_{xi} = plant mass of species x in transect i
L_i = length of lake transect i
j = running index of the different species

Physical analyses of water:

Water transparency was measured with a Secchi disk. Temperature at 30 cm and pH were measured with a MultiLine P4.

Results

After restoration of the lake Velenjsko jezero, macrophytes, almost instantaneously, started to gradually overgrow large surface of the littoral. In the Tab. 1, presence and relative abundance of different macrophyte species in lake Velenjsko jezero for the period 1996 – 2004 is presented. The pioneer species that appeared in the year 1996 were *Chara* sp., *Nuphar lutea* (L.) Sibth. & Sm. and *Potamogeton crispus* L.. They colonized only a minor part of the littoral. Only three years later almost the whole littoral was overgrown and the species composition had become more heterogeneous. Changes in quantitative relationships between the species varied as well. *Potamogeton crispus* was the dominant species till 1997. In the following years *Najas marina* All. and *Potamogeton filiformis* Pers became the most abundant species in the lake.

Table 1: Presence and relative abundance of different macrophyte species in lake Velenjsko jezero for the period 1996 – 2004.
Presence: ♦ – present, – – not present. Relative abundance: a five level descriptor scale (1 – very rare, 2 – infrequent, 3 – common, 4 – frequent, 5 – abundant, predominant) (KOHLER & JANAUER 1995).
Tabela 1: Prisotnost in relativna abundanca posameznih makrofitskih vrst v Velenjskem jezeru v letih med 1996 in 2004.
Prisotnost: ♦ – prisotna – – ni prisotna. Relativna abundanca vrst: petstopenjska lestvica (1 = zelo redka; 2 = redka; 3 =zmerno zastopana; 4 = pogosta; 5 = prevladujoča vrsta) (KOHLER & JANAUER 1995).

Species / Year of survey	Abbrev.	1996*	1997			1999	2003			2004		
			Jun	Jul	Sept	Aug	Jun	Jul	Aug	Jun	Jul	Aug
Chara sp.	Cha sp	♦	1	-	-	-	-	-	-	1	1	1
Myriophyllum spicatum L.	Myr spi	-	2	2	2	2	2	1	2	2	2	2
Najas marina All.	Naj mar	-	-	-	3	4	1	3	4	-	2	4
Najas minor All.	Naj min	-	-	-	2	3	-	-	-	-	1	2
Nuphar lutea (L.) Sibth. Et Sm.	Nup lut	♦	1	1	1	1	1	1	1	1	1	1
Potamogeton crispus L.	Pot cri	♦	4	3	1	1	2	1	1	3	1	1
Potamogeton filiformis Pers.	Pot fil	-	1	-	-	1	2	3	3	3	3	3
Potamogeton lucens L.	Pot luc	-	-	-	-	2	1	2	2	1	1	2
Potamogeton nodosus Poir.	Pot nod	-	-	-	-	2	1	1	1	1	1	1

* Relative abundance of the species was not defined.

From the Fig. 1, where the distribution and relative abundance of different macrophyte species around lake Velenjsko jezero is presented for August 2003, it is evident that *Najas marina and Potamogeton filiformis* were the most abundant species in the lake in the late summer.

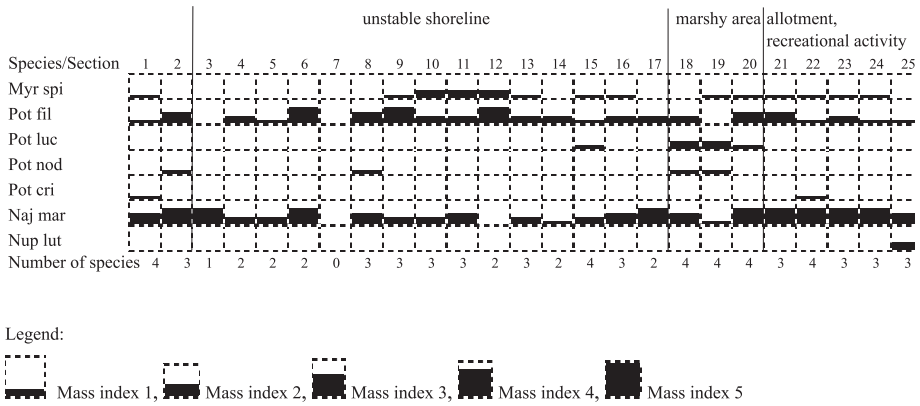


Figure 1. Distribution and abundance of different macrophyte species around lake Velenjsko jezero in August 2003. The Lake was divided into 25 section with the length of 200±2 m.
Slika 1: Distribucija in abundanca različnih makrofitskih vrst v Velenjskem jezeru avgusta 2003. Jezero smo razdelili na 25 transektov dolžine 200±2 m.

It can be seen from Fig. 2 that the quantitative relation among species during the growth season varied enormous. Species *Potamogeton filiformis* was dominant in June, while *Najas marina* was the predominant species in the lake in the late summer (RPM was 68.5% in August and 84.6% in September). Other species were much less abundant.

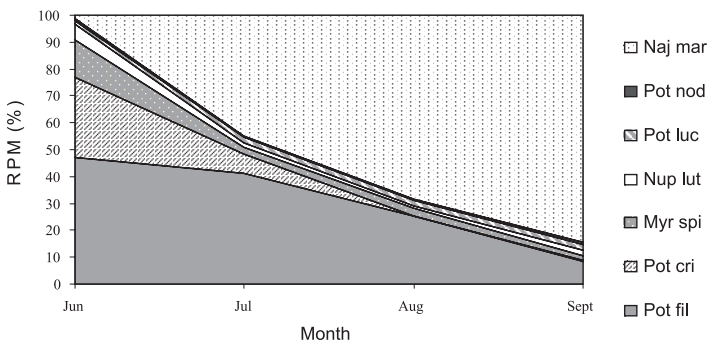


Figure 2. Relative plant mass (RPM %) throughout the season 2003 - Quantitative relation among species during the growth season.
Slika 2: Spreminjanje razmerja v relativni rastlinski biomasi (RPM %) med različnimi vrstami makrofitov od junija do septembra 2003.

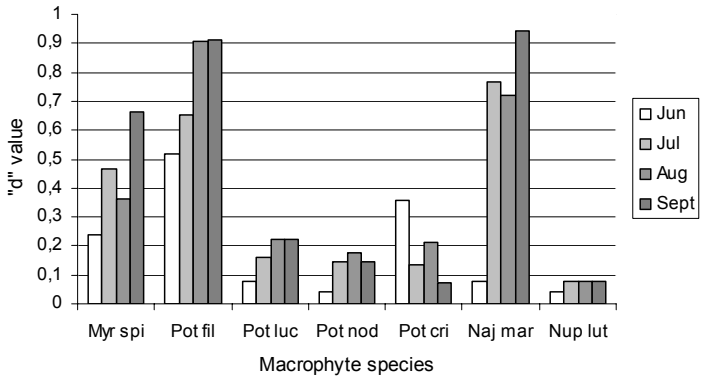


Figure 3. The distribution ratio - »d« values (the ratio between MMT^3 and MMO^3) for different species throughout the season 2003.
Slika 3: Distribucijski kvocient - »d« vrednost (razmerje med MMT^3 in MMO^3) za različne makrofitske vrste preko sezone 2003.

From the high »d« values, near 1 (Fig. 3) it is evident that *Najas marina* and *Potamogeton filiformis* overgrew most of the littoral in August and September, but the share of *Potamogeton filiformis* was much smaller than that of *Najas marina*. The RPM of *Potamogeton filiformis* was only 25.2 % in August and 8.2 % in September (Fig. 2).

Tab. 2 presents some physical characteristics of the water of lake Velenjsko jezero, in the years 1997, 2000, 2003 and 2004. The mean pH of the lake water was around 8 without any significant variations. The mean Secchi disk transparency (Zs) was the highest in 1997, but in the next years it decreased, while the mean temperature of the water increased.

Table 2: Mean physical and mean chemical characteristic of water in lake Velenjsko jezero averaged over the period May to September in the years 1997, 2000 and 2003; average value (minimum value/maximum value).
Tabela 2: Povprečni rezultati fizikalnih meritev in kemijskih analiz vode v Velenjskem jezeru v času od maja do septembra v letih 1997, 2000, 2003 in 2004; povprečje (minimalna/maksimalna vrednost).

Year	Sechi disc transparency (m)	Temperature (°C)	pH	P-tot. (mg/l)	N-tot. (mg/l)
1997	5.9 (4.2/9.0)	19.8 (17.8/21.3)	8.3 (8.2/8.7)	0.24 (0.13/0.44)	2.0 (1.47/2.57)
2000	4.5 (3.2/5.0)	22.2 (19.8/25.4)	8.4 (8.2/8.8)	-	-
2003	4.6 (4.0/5.5)	23.4 (19.4/25.8).	7.8 (7.4/8.3)	0.1 (0.1/0.1)	1.3 (0.9/2.1)
2004	3.7 (3.0/4.5)	21.7 (19.0/23.7)	8.2 (8.1/8.2)	0.1 (0.1/0.1)	-

Most macrophyte species colonized only shallow part of the littoral in 1997 (Tab. 3). This year, species *Potamogeton crispus* reached the maximum depth of 3.5 m. Four species (*Najas marina* – Zc = 4.0 m, *Myriophyllum spicatum* – Zc = 4.0 m, *Potamogeton lucens* – Zc = 4.0 m and

Potamogeton filiformis – Zc = 5.5 m) colonized the deepest part of the littoral in the year 2003. From the data of the Table 3, negative relationship between Zs and maximum colonization depth (Zc) was calculated – correlation coefficient Zs/Zc: r = – 0.59, n= 14.

Table 3: Maximum colonization depth (Zc) of seven macrophyte species in 1997 and in 2003; Zs (mean Secchi depth).
Tabela 3: Največja globina uspevanja (Zc) sedmih makrofitskih vrst v letu 1997 in v letu 2003; Zs (povprečna Secchi-jeva globina).

	Zs = 5.9 m	Zs = 4.6 m
Chara sp.	0.5	-
Myriophyllum spicatum L.	3.0	4.0
Najas marina All.	2.0	4.0
Najas minor All.	1.5	-
Nuphar lutea (L.) Sibth. Et Sm.	2.0	2.0
Potamogeton crispus L.	3.5	3.0
Potamogeton filiformis Pers.	0.3	5.5
Potamogeton lucens L.	-	4.0
Potamogeton nodosus Poir.	-	2.0

Discussion

Lake Velenjsko jezero could be classified as meso-eutrophic according to the level of total phosphorus (OECD 1982, MAZEJ & GABERŠČIK 1999), what offered good conditions for quick colonization of macrophytes after restoration of the lake. Lake Velenjsko jezero, because of its original basin shape, has a steep slope in its littoral along the majority of the lakeshore. This is the reason why only a narrow area of shoreline is colonized by submersed macrophytes. ANDERSSON (2001) asserted that the geomorphology of a lake basin is the primary factor affecting the structure of the interface zone between land and water. The steepness of the lake margins is decisive for sediment stability along slope gradients, which in turn influences the establishment of vegetation. The pioneer species, which colonized only a minor part of the littoral in the year 1996, were *Chara* sp., *Nuphar lutea* and *Potamogeton crispus* (MAZEJ 1998). Only three years later these species are not important components of the littoral vegetation anymore. *Chara* sp. disappeared from the lake when other macrophyte species overgrew the littoral in 1999, but it was detected in a very small quantity again in 2004. It is known that Charophytes are pioneers among macrophytes, and colonize a new habitat very quickly (PALMA-SILVA & al. 2002), but they are usually not competitive with angiosperms (BLINDOW 1992). *Nuphar lutea* has remained more or less at the same abundance at the same location throughout these years. Floating-leaved species may be expected to have a faster occupancy of areas with shallower water and smaller fetches, and the development of the floating-leaved macrophyte community may require years (REA & al. 1998). It has been reported (NICHOLS & SHOW 1986, BOLDUAN & al. 1994, JIAN & al. 2003) that *Potamogeton crispus* and *Myriophyllum spicatum* are very invasive species, which usually form a very dense population and establish large monospecific weed beds in many lakes. However, this was not the case in lake Velenjsko jezero. *Potamogeton crispus* was the dominant species till 1997. The life history of *Potamogeton crispus* differs from most other submersed

plants. Biomass production of *Potamogeton crispus* often reaches its maximum in early summer, allowing it to avoid competition from other species in the habitat primarily because they are still in the dormant state (TOBIESSEN & SNOW 1984). Young plants of *Potamogeton crispus* and *Myriophyllum spicatum* overwinter, and then grow rapidly in the spring. In this respect, the two species are similar (BOLDUAN & al. 1994). By early summer plants undergo senescence processes and then remain dormant until autumn. It is known that *Potamogeton crispus* grows well in eutrophic lakes, at low temperature and very low light intensities, being an important primary producer in freshwater ecosystems, providing a good food source for herbivorous fishes (JIAN & al. 2003). Grazing by fish, birds and invertebrates may be an important factor that limits development of plants. Grazing sensitivity differs among submersed macrophytes (JACOBSEN & SAND-JENSEN 1992, WEISNER & al. 1997), and it has been proposed that selective grazing affects species composition and even succession (CRAWLEY 1983). It was ascertained that species of genus *Potamogeton* were significantly more heavily grazed by invertebrates (mean 4.2 % of leaf area) than non-*Potamogeton* species (mean 0.8 % of leaf area) (JACOBSEN & SAND-JENSEN 1992). The grazing experiments made by Weisner & al. (1997) also showed that non-*Potamogeton* species like *Chara* sp. and *Myriophyllum spicatum* were not significantly affected by grazing. There are no data in the literature about herbivory on the species *Najas marina*, but we assume that this species is not tasteful food for herbivores due to its fragility and its prickly shoots. Fragile shoots are easily broken and spread around by the help of current, wind and birds. It might be the reason, that *Najas marina* predominates over other species in lake Velenjsko jezero, including *Myriophyllum spicatum*. *Myriophyllum spicatum* and *Najas marina* usually appear together in various natural habitats, where they compete with each other. The result of studies (AGAMI & WAISEL 2002) showed that *Myriophyllum spicatum* was more sensitive to intraspecific competition than *Najas marina*, and additionally the competitive effects of *Najas marina* on *Myriophyllum spicatum* were stronger than vice versa.

Light availability affects both the biomass and community structure of phytoplankton and submersed vegetation (VAN DUIN & al. 2001, BEST & al. 2001). Although a significant positive correlation between Secchi disc transparency and the maximum colonization depth of macrophytes has been found in several studies (HUTCHINSON 1975, CHAMBERS & KALFF 1985, NICHOLS 1992, MIDDELBOE & MARKAGER 1997, MAZEJ & GABERŠČIK 1999), our study showed that transparency was not the main factor which determined the depth distribution of macrophytes in lake Velenjsko jezero. The maximum colonisation depth of macrophytes is usually ascribed to light attenuation in the water column and the minimum light requirement for growth (BLINDOW 1992), although other parameters such as surface irradiance, hydrostatic pressure, water colour, temperature, grazing pressure, substrate type and epiphyte loading can also affect maximum colonization depth (SAND-JENSEN 1989, VAN DUIN & al. 2001). Tall macrophytes (caulescent angiosperms and charophytes) compensate for light limitation by shoot growth towards the water surface, so maximum colonization depth is therefore independent of transparency (MIDDELBOE & MARKAGER 1997). Although mean Secchi disc transparency was higher in 1997 than in 2003, the maximum colonization depth of macrophytes (*Potamogeton crispus*) was only 3.5 m in 1997 and 5.5 m in 2003 (*Potamogeton filiformis)*. The results of ROONEY AND KALFF (2000) showed that angiosperms colonized deeper parts during years characterized by an early warming of the water, independent of underwater irradiance. The year 2003 was very warm with high May air temperatures. The average temperature of lake water was 3.6 $^{\circ}$C higher than that in 1997. Since the majority of plants reproduce asexually, an earlier start to the growing season allowed plant communities more time for colonization.

Conclusion

The lake Velenjsko jezero has offered good conditions for development of macrophytes in less than ten years. In this short period, species composition and the quantitative relationships between the species varied enormously. Therefore further changes are expected in the coming years. We assume that plant interactions might constitute the main controlling factor regulating the distribution of macrophytes and coming projects should be oriented in studying plant interaction in grater detail.

Povzetek

Do leta 1983 je bilo Velenjsko jezero odlagališče pepela iz termoelektrarne Šoštanj. Od leta 1983 naprej so s sedimentacijo ločevali pepel od transportne vode. Pepel so odlagali na deponijo, vodo pa še naprej črpali v jezero, kjer se je pH dvignil na okoli 12 in iz jezera so izginile vse oblike življenja. Že eno leto po uvedbi zaprtega kroga transportne vode leta 1994, kjer vodo črpajo nazaj in jo ponovno uporabijo, se je pH na površini jezera zmanjšal na 9, leta 1997 pa pH vode po celotnem globinskem profilu ni presegal 8,7 in vanj se je po daljšem obdobju spet začelo vračati življenje.

Vrstno sestavo in razporeditev makrofitov v Velenjskem jezeru spremljamo bolj ali manj redno že od leta 1996. Makrofiti so pričeli po sanacijskih ukrepih zelo hitro naseljevati prazen litoral, saj so bile nudene ugodne razmere – dosti hranil, podlaga za ukoreninjenje in ugodne svetlobne razmere. Velenjsko jezero je nastalo z ugrezanjem, zaradi česar se njegova obala na mnogih mestih zelo hitro spusti v globino. To je tudi razlog, zakaj je z makrofiti poraščen le ozek pas litorala. Pionirske vrste, ki so se pojavile v prvem letu raziskav (1996) so bile *Chara* sp., *Nuphar lutea* in *Potamogeton crispus*. *Potamogeton crispus* je bila dominantna vrsta do leta 1997. V naslednjih letih pa sta vrsti *Najas marina* in *Potamogeton filiformis* prevladali nad ostalimi vrstami v jezeru. *Chara* sp. je bila prisotna v zelo majhni količini v jezeru le v letih 1996 in 1997, v naslednjih letih pa je v občasnih pregledih litorala nismo več zasledili. Zopet pa smo jo našli na istem mestu v letu 2004. V vseh teh letih je prihajalo do spremenjenega kvantitativnega in kvalitativnega razmerja med vrstami. V prvih letih so makrofiti naselili predvsem del med čolnarno in pritokom Sopote, medtem ko je bil del ob deponiji neporaščen. V naslednjih letih so se makrofiti razširili tudi v ostala območja litorala, zmanjšala pa se je njihova pojavnost tam, kjer se kaže večji vpliv zaledja – ob nasipu in na območju pešpoti pod vrtičkarskim naseljem »Kinte-Kunte«. Od leta 1996 pa do leta 2004 je število vrst iz 3 naraslo na 9. Medtem ko kažejo vrste *Najas marina*, *Potamogeton filiformis, Potamogeton lucens* in *Potamogeton nodosus* pozitiven trend v razvoju, pa v zadnjih letih opažamo manjše pojavljanje vrst *Chara* sp., *Myriophyllum spicatum*, *Potamogeton crispus* in *Najas minor.*

Le nekaj let je minilo od naselitve prvih makrofitov v jezeru, zato lahko pričakujemo, da bo prišlo v naslednjih letih še do določenih sprememb in sicer tako v vrstni sestavi makrofitov kot tudi v razporeditvi le-teh.

Aknowledgements

Authors thank to Prof. Dr. Alenka Gaberščik and Dr. Mateja Germ for valuable comments on the manuscript.

References

AGAMI M. & Y. WAISEL 2002: Competitive relationships between two water plant species: *Najas marina* L. and *Myriophyllum spicatum* L.. Hydrobiologia **482**: 197-200.

ANDERSSON B. 2001: Macrophyte development and habitat characteristics in Sweden's large lakes. Ambio **30** (8): 503-513.

BEST E. P. H., C. P. BUZZELLI, S. M. BARTELL, R. L. WETZEL, W. A. BOYD, R. D. DOYLE & K. R. CAMPBELL 2001: Modelling submersed macrophyte growth in relation to underwater light climate: modelling approaches and application potential. Hydrobiologia **444**: 43-70.

BLINDOW I. 1992: Long- and short-term dynamics of submerged macrophytes in two shallow eutrophic lakes. Freshwater Biology **28**: 9-14.

BOLDUAN B. R., G. C. VAN EECKHOUT, H. W. QUADE & J. E. GANNON 1994. *Potamogeton crispus* – The Other Invader. Lake and Reserv. Manage. **10** (2): 113-125.

BOWES G. 1987: Aquatic plant photosynthesis: strategies that enhance carbon gain. In: CRAWFORD R. M. M. (ed.): Plant Life in Aquatic and Amphibious Habitats, spec. publ. No. 5. Br. Ecol. Soc., Blackwell Sci. Pub. Oxford, pp. 79-98.

CHAMBERS P. A. & J. KALFF 1985: Depth distribution and biomass of submersed aquatic macrophyte communities in relation to Secchi depth. Can. J. Fish. Aquat. Sci. **42**: 701-709.

CRAWLEY M. J. 1983: Herbivory. The dynamics of animal-plant interactions. Blackwell, Oxford.

FOX A. M. 1992: Macrophytes. In: CALOW P. & E. P. GEOFFREY (eds.): The Rivers Handbook. Volume 1. pp. 216-346.

GERM M. & A. GABERŠČIK 1999: The distribution and abundance of macrophytes of the lowland Ižica river (Slovenia) = Razporeditev in pogostost makrofitov v nižinski reki Ižici. Acta biol. slov. **42** (4): 3-11.

GERM M., M. DOLINŠEK & A. GABERŠČIK 2003: Macrophytes of the river Ižica – comparison of species composition and abundance in the years 1996 and 2000. Arch. Hydrobiol., Suppl. **147** (1-2): 181-193.

HASLAM S. M. 1987: River Plants of Western Europe. The macrophytic vegetation of watercourses of the European Economic Community. Cambridge University Press, Cambridge, New York, New Rochelle, Melbourne, Sydney.

JACOBSEN D. & K. SAND-JENSEN 1992: Herbivory of invertebrates on submerged macrophytes from Danish freshwaters. Freshwater Biology **28**: 301-308.

JEPPESEN E., MA. SONDERGAARD, MO. SONDERGAARD & K. CHRISTOFFERSEN (Eds.) 1998: The structuring role of submerged macrophytes in lakes. Springer Press, New York.

JIAN Y., B. LI, J. WANG & J. CHEN 2003: Control of turion germination in *Potamogeton crispus*. Aquatic Botany **75**: 59-69.

KOHLER A. & G. A. JANAUER 1995: Zur Metodik der Untersuchungen von aquatischen Makrophyten in Flussgewasseren. In: LANDSBERG, H. & H. KLAPPER, (Eds.): Hollandbuch Angewandte Limnologie, Ecomed Verl., Landsberg /Lech, pp. 1-22.

MAZEJ Z. 1998: Macrophytes in the different types of lakes: species composition and species vitality. Master of science thesis, Ljubljana, 104 pp.

MAZEJ Z. & A. GABERŠČIK 1999: Species composition and vitality of macrophytes in different types of lakes. Acta biologica Slovenica **42** (3): 43-52.

MIDDELBOE A. L. & S. MARKAGER 1997: Depth limits and minimum light requirements of freshwater macrophytes. Freshwater Biology **37**: 553-568.

NICHOLS S. A. & B. H SHAW 1986: Ecological life histories of the three aquatic nuisance plants, *Myriophyllum spicatum*, *Potamogeton crispus*, and *Elodea canadensis*. Hydrobiologia **131**: 3-21.

NICHOLS S. A. 1992: Depth, substrate and turbidity relationship of some Wisconsin Lake plants. Wisconsin Academy of Sciences, Arts and Letters 59, 97-118.

OECD 1982: Eutrophication of waters monitoring, assessment and management, Paris.

PALL K. & G. A. JANAUER 1995: Die Makrophytenvegetation von Flußstauen am Beispiel der Donau zwischen Fluß-km 2552.0 und 2511.8 in der Bundesrepublik Deutschland. Arch. Hydrobiol. Suppl. **101** (Large Rivers 9) (2): 91-109.

PALMA-SILVA C., E. F. ALBERTONI & F. A. ESTEVES 2002: The role of Charophytes primary production in a coastal lagoon subjected to human impacts (RJ, Brasil). Acta. Limnol. Bras. **14** (1): 59-69.

PIP E. 1984: Ecogeographical tolerance range variation in aquatic macrophytes. Hydrobiologia **108**: 37-48.

PIP E. 1989: Water temperature and freshwater macrophyte distribution. Aquatic Botany **34**: 367-373.

PRESTON C. D. 1995: Pondweeds of Great Britain and Ireland. Botanical society of the British Isles, London.

SAND-JENSEN K. 1989: Environmental variables and their effect on photosynthesis of aquatic plant communities. Aquatic Botany **34**: 5-25.

REA E. T., D. J. KARAPATAKIS, K. K. GUY, I. J. E PINDER & H. E. MACKEY JR. 1998: The relative effects of water depth, fetch and other physical factors on the development of macrophytes in a small southeastern US pond. Aquatic Botany **61**: 289 – 299.

ROONEY N. & J. KALFF 2000. Inter-annual variation in submerged macrophyte community biomass and distribution: the influence of temperature and lake morphometry. Aquatic Botany 68, 321-335.

TOBIESSEN P. & P. D. SNOW 1984: Temperature and light effects on the growth of *Potamogeton crispus* in Collins Lake, New York State. Can. J. Bot. **62**: 2822-2826.

URBANC-BERČIČ O., A. GABERŠČIK, M. ŠIŠKO & A. BRANCELJ 2002: Aquatic macrophytes of the mountain lake Krnsko jezero, Slovenia. Acta Biologica Slovenica **45** (2): 25-34.

VAN DUIN E. H. S., G. BLOM, F. J. LOS, R. MAFFIONE, R. ZIMMERMAN, C. F. CERCO, M. DORTCH & E. P. H. BEST 2001: Modeling underwater light climate in relation to sedimentation, resuspension, water quality and autotrophic growth. Hydrobiologia **444**: 25-42.

WEISNER S. E. B. 1991: Within-lake pattern in depth penetration of emergent vegetation. Freshwater Biol. **26**: 133-142.

WEISNER S. E. B., J. A. STRAND & H. SANDSTEN 1997: Mechanisms regulating abundance of submerged vegetation in shallow eutrophic lakes. Oecologia **109**: 592-599.

ABS ACTA BIOLOGICA SLOVENICA
LJUBLJANA 2005 Vol. 48, Št. 1: 33-44

Sprejeto (accepted): 2004-11-17

Secular trend in body dimensions in boys from Tuzla region in period 1980 –1996

[1]HADŽIHALILOVIĆ Jasminka, [1]TERZIĆ Rifet, [1]AHMIĆ Adisa, [2]H.HALILOVIĆ Amir, [3]NOŽINOVIĆ Amra, [1]ŠEHIĆ Amela, [1]HAMIDOVIĆ Hajrija, and [4]JUSUPOVIĆ Fatima

[1]Faculty of Natural Science and Mathematics, Department of Biology, University of Tuzla, 75000 Tuzla, Univerzitetska br.4, Bosnia and Herzegovina;
Tel.fax: ++ 387 35 286 454; E-mail: jasnahba@yahoo.com
[2]Gymnasium »M. Selimović« Tuzla, Bosnia and Herzegovina;
[3]School of Phylosophy of Tuzla University, Faculty of Physical Education, Bosnia and Herzegovina;
[4]College of Healt Studies, University of Sarajevo, Bosnia and Herzegovina.

Abstract. Within 16 year-period secular trend in seven measurements of physical growth of male children and youth from Tuzla Region, was researched by corresponding analysis of the sample that involved 1329 researched subjects. Our data were compared with the results of research from 1980 in the sample of 1349 boys. Nine successive generations in 1980 and 1996, from Tuzla region, were involved in this research. Analysis of the data obtained is based primarily on scientific elaboration of the situation registered in 1996 in the tested part of the broader population, after an unnatural and extremely unfavorable period for physical growth of the large majority of that population. The aim of this research was to establish secular trend (negative or positive) for the seven measurements of physical growth of male children and youth comparing our results from 1996 to corresponding research results on growth and development from 1980, the sample of about same population (nearly same number of investigated persons). Although the unfavorable war living conditions negatively affected ontogenesis of the researched subjects, body dimensions of male children and youth were established as harmonious, in the limits of average European standards. However, it seems the unfavorable living conditions caused temporary slowdown in body dimensions, so, for these generations (11 to 19 years old), we could not find any increase of mean values (for certain number) of the researched parameters in comparison with the sample from 1980 year. So, 16 year-acceleration trend for most parameters was established and it is particularly evident in postpubertal period.
Key words: secular trend, boys' growth and development

Introduction

In the last century, nearly in all the world population, gradual acceleration of different increased measures of physical growth was noticed. This occurance was marked as acceleration of growth (sec-

ular trend), and it means faster growth (shortened growth cycle) faster achievement of sexual maturity and increase in body height and weight. Children are »higher« and »heavier« in all ages than the children of the same age 100, 50, even 20 years ago. The term acceleration in broader sense means increase in body dimensions, prolonged reproductive period, longer human lifetime, faster growth of fetus, changes in psychic development, then increased dimensions of certain organs, as well as maturation of their functions (NIKOLIĆ & DOVAT 1978).

Most significant factors causing this occurance are: improvement of general living conditions (among them nutrition is most important), children get sick more rarely, increased possibility of new combination of genes is present due to more frequent migrations (NIKOLIĆ & DOVAT 1978), occurance of increased number of heterozygotes for the genes that prevailing to a certain extent direct growing. There is no direct evidence that it is a causal mechanism, so environmental factors might be main cause of secular changes (SUSANNE 1985, MALINA 1979).

However, secular change of growth is a reversible biological process, so, if socio-economic and growth-hygienic conditions would aggravate to the level they were on a few centuries ago, we could expect come-back of growth type that used to exist in Europe (WIERINGEN 1979). The same author concludes that secular changes may be positive and negative. He relied upon the time of investigation and upon geographic varieties among population (WIERINGEN 1986). This author researched secular changes in Holland, related to the body height, within the period 1850 -1978, and found (1979) strong connection between the secular changes and general socio-economic impacts, nutrition, health conditions, and demographic parametrs. In his opinion there are no indications of certain changes (as a result of selection) in genotype of the Dutch population that could be the cause of that connection. Heterosis did not play a significant role in it, either.

For Belgian population the data for the period 1830-1980 show the average height and weight significantly increased, particularly in the course of the growing period, and to a lower extent, in the adulthood. These data are in accordance to European and North American data on secular trend for the period 1880 –1980, and they are as follows: 1,5 cm/decade in childhood, 2,5 cm/decade in adolescence, and about 1 cm/decade in adulthood. Increase in body dimensions of adults in the recent decades has varied between 0,3 & 3,0 cm/decade (HAUSPIE & AL 1997).

Secular tendency toward a higher growth and earlier maturity are predominantly connected to better nutrition and general health conditions of the population (HISAFUMI & AL 1999).

In the last two or three decades in our country 18-years old boys are every year approximatelly higher for about 0,2 mm, heavier to 0,3 kg (BERBEROVIĆ & HADŽISELIMOVIĆ 1982).

This increase is expected to be followed by improvement of socio-economic conditions and vice versa, and it decreases during crises and wartime periods. Some indications show that this rate has decreased since 1980, or even stopped in these countries (BIELICKI & WALISKO 1991; SANNA, FLORIS & COSSEDU 1993). These indications in industrialized countries show that environmental living conditions enabled reaching their maximal genetic potentials, or their social conditions ceased improving (WEBER & AL 1995). Secular tendency in the achieved growth and growth rate are usually more frequent in children of lower socio-economic status.

Secular trend in growth rate slowdowned in some populations, but in the others it is going on, or it may decrease in future. Lots of researches in the world show that secular tendency in adults' height has been getting slow in most countries during recent 10 to 20 years (HAUSPIE & AL 1996).

The lowest increase in growth of the adults was noticed in Sweden and Norwey, what indicates that adults' growth nearly reached its peak in these countries in recent decades. Western countries like Belgium, England, The Netherlands, show secular tendency between the values of 1,0 – 1,3 cm/decade. The highest increase in the adults' height (3,0 cm/decade was found (in women) in Chech Republic and (in men) in Japan (1950 – 1960; HAUSPIE & AL 1996).

Many countries worldwide have publications with referential data on children and youth's body development, and on secular trend in certain populational measures. In former Yugoslavia secular trend in growth and development was researched by KOVAČ (1973), BRODAR (1961), DOVEČAR

(1978), TOMAZO-RAVNIK (1988, 1999), ŠTEFANČIĆ ET AL. (1996), GAVRILOVIĆ (1972, 1974), PREBEG (1978, 1995, 1997), and IVANOVIĆ (1985).
The tables and centile diagrams (obtained in such researches) are used directly as tables of norms in pediatrics, school medicine, in anthropologic sport institutes (because they deal with normal physical growth of children and youth).
Basic biological factors of secular tendencies are not completely cleared up. However, most of researchers dealing with this phenomenon have been explaining it by genetic factors and factors of external environment.
These data on acceleration of individual physical growth seriously put the question: Will the period required for reaching the boys & girls' sexual maturity keep shortening childhood and will a human being be of a giantic stature in future? However, if the suppositions on heterosis (as a result of increased migrations) and improved socio-economic situation in the world population are the main factors of this acceleration, then it is difficult to believe in such prognoses. In addition to this, possibility of increasing heterezygosity in stimulation of quantitative features (potentially similar genotypes) is naturally limited, the topic that has been discussed about much recently, (HADŽISELIMOVIĆ 1988). HIERNAUX (1975) set a hypothesis that our descendants would probably be higher than we are today, but they should not be giants.

Basic demographic, economic and climate features of the researched Region

Tuzla is a powerful economic, cultural, educational and administrative center, not only in the Region itself, but on all B&H territory. This city is characterized by relatively fast socio-economic development after World War II, what resulted in a hasty increase of inhabitants number, primarily by means of mechanical increase in population (migrations). So, the census from 1971 year established the number of 107 293 inhabitants in Tuzla, out of which 37,08% were immigrants. The biggest increase in inhabitants number was registered between 1955 and 1965. This factor influenced bioanthropologic heterogeneity of the researched sample of the Tuzla's population, as well.
From the time of the researches in Tuzla community (NOVAKOVIĆ 1980) and the researches of ours, number of inhabitants increased for 47 092 more. For the time of 16 years (including the war results, as well), demographic picture of Tuzla changed considerably.In the course of the four year- war (1992-1996), the number of inhabitants in this region increased rapidly due to forced migrations, banishments, so, in 1996 it amounted 154 384; 38.566 (24,98% of expatriates, and 115 818 (75,03%) domiciles (FEDERAL BUREAU OF STATISTICS SARAJEVO 1998). Mixture of the population of different local origin with domiciles before the war, as well as expatriates presence and displaced people during and after the war in the Region of Tuzla affected (and will do it in the future) the changes of genetic structure of the population.
According to the Federal Bureau of Statistics Sarajevo (1998) there were 31 097 (20,14%) employed people. And 16 years ago the number of employed people was 34 885 (32,50%) in 1980 (NOVAKOVIĆ 1980).
According to the same source, an average salary for December 1996 was 253,10 KM or 126,55 Eura. From the data offered it can be concluded that Tuzla's populaton is mainly consisted of workers, what naturally affects socio-economic status of a family and the population in general. The war and migrations changed the national structure of the researched Region.
Speaking about climate, Tuzla has moderate-continental climate. In this Region, summers are warm, winters are cold. The mean year air temperature is relatively high, it ranges from 10,2 to 11,4 ^{0}C, with clearly distinguished seasons. Summer and spring temperatures are changeable (KULENOVIĆ 1980).

Objectives

To analyze 16 year-secular trend in growth dynamic changes by comparison the obtained results with corresponding data from 1980.
To create necessary data – base for research of temporal differences, including four-year aggression on Bosnia and Herzegovina, taking into consideration the fact that all the researched subjects lived one period of physical growth in extremely unfavorable war conditions.

Material and methods

Researches that are a source of data collected in this study, were carried out in Tuzla Region in autumn 1996/97. Anthropological measurements were carried out out in four primary and two secondary schools. Crosss – sectional method was used. The measurement involved 1321 male pupils, age 11 to 19 years.The sample was randomly chosen and stratified according to subjects' decimal age: pupils age 10 years and six months to the 11 years and 5 months made the group of the age 11 years; 16,6 to 12,5 made the group of the age 12 years (Table 1).

Table 1: Age structure of the researched sample

	1980	1996	1996	Total:
Age (years)	N	N Domiciles	N Refugees	
11	113	107	32	139
12	149	117	26	143
13	143	132	33	165
14	188	146	38	184
15	140	115	41	156
16	187	75	58	133
17	138	89	57	146
18	192	86	69	155
19	69	54	46	100
Total:	1349	921	400	1321

Seven anthropometric parameters were used in this research: 1. stature 2. body weight 3. mean chest circumference 4. upper arm circumference; 5. total arm length; 6. biacromial breadth and 7. biiliocristal breadth.
All measurements were carried out according to IBP (International Biologic Program), (HADŽISELIMOVIĆ & LELO 1998).
Descriptive statistics and t-tests were done in the Program Statistics for Windows, Copyright Stat Soft, Inc.1993. Statistical analysis primarily involved evaluation of the central tendency measurements. Statistical significance of differences between the results of our research and the researches from 1980 was evaluted, as well (t-test).

Results and discussion

Stature (body height) is one of the most stable parameters of physical growth, that reflects complex processes in organism. Comparison of the results from 1996 (series II) with those from 1980 (series I) reveals significantly lower mean values of body height in series II than in series I (age 11 to 15 years).This difference in mean values is statistically significant, that is shown in t-test results (Table 2).

Table 2: Stature of the boys of Tuzla Region (1980 & 1996)

Age	N_1 1980.	N_2 1996.	$X_{1\ (cm)}$	$X_{2\ (cm)}$	SD_1	SD_2	Relative Increase (%) 1	2	t-test p<0,05
11	113	139	145,36	142,77	5,98	6,54	-	-	3,28* *
12	149	143	150,07	146,30	7,06	6,55	3,24	2,47	4,74* **
13	143	165	156,02	153,47	8,36	8,40	3,96	4,90	2,66*
14	188	184	163,05	161,13	9,20	8,29	4,50	4,99	2,12*
15	170	156	169,98	168,18	8,03	8,56	4,25	4,37	1,95
16	187	133	170,90	172,44	7,61	6,56	0,54	2,53	1,94
17	138	146	174,07	175,14	6,55	6,99	1,85	1,56	1,33
18	192	155	176,76	177,92	7,16	7,21	1,54	1,59	1,50
19	69	100	176,17	178,87	6,78	6,79	0,99	0,53	2,54*

Boys from series II over 15 year age have higher mean body height than in series I, due to 16 year – accelerating trend. So, for example, 18 years old boy from series II is approximately higher for 1,16 cm than the same boy from series I, so, acceleration trend for that generation is 0,73 cm/decade. Secular trend for the generations from 11 to 15 years, is negative (Fig.1) (due to already mentioned reasons), but for the postadolescent growth (age 16, 17, 18 and 19 years) is evident and amounts 1,01 cm/decade.

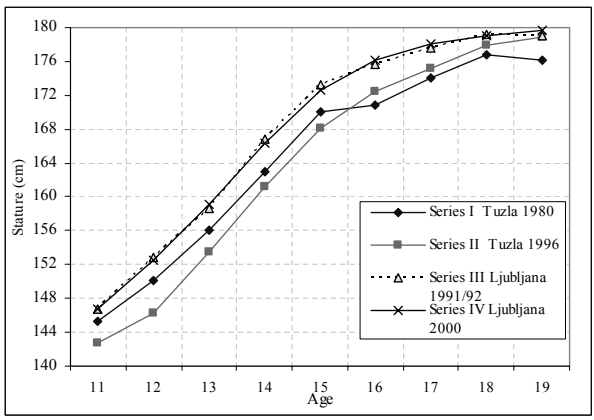


Fig.1. Comparative diagram of stature of Tuzla's male children and youth: 1980 and 1996, and the youth from Ljubljana 1991/92 and 2000

These data correspond to decade diapason of secular trend for Europe and North America, that show secular trend in growth between 1880 and 1980 was 1,5 cm/decade in childhood, 2,5 cm/decade in adolescency, and about 1,0 cm/decade in adulthood (MALINA 1990).

This temporary slowdown in body height increase in boys from series II in prepuberty and puberty is probably due to higher child's sensitivity to negative impact of external factors. Namely, all the external factors that more or less affect physical growth and development, were led to the values that strongly affected children and youth in the mentioned period due to four year-agression.

Results on body height in Ljubljana's boys (Slovenia) from 1991/92, series III (ŠTEFANČIĆ & AL 1996), and from 2000 year – series IV (STREL & AL 2001) show secular trend in that area to stagnate (Fig.1). Figure 1 shows boys from series III and IV approximately »higher« than boys from series II. Those differences in average body height are supposed to be result of genetic potential difference.

Those differences might have been a bit smaller if the boys from series II had not stagnated in growth due to already mentioned reasons.
Examples of negative and stagnating secular trend were found in many countries in a lot of studies including those from wartime and economic crises (TANNER 1968, WALKER & AL 1988, KUH AND WADSWORTH 1989).

Body weight. In addition to body height, body weight is most important parameter in bioanthropology for research and monitoring physical growth and development. Unlike height, weight is labile factor, changable by impact of various endo- and exogenous factors. Body weight increase coincides with body hight increase, so most intensive increase in body weight in series II was registered in age of 14 years (Fig. 2). Negative or stagnating secular trend was registered in series II in age of 11, 12, 13, 17, and 18 years in comparison to series I.
Comparison of results from series II and series I shows boys from series II, aged 11 to13 years have significantly lower average body weight, than in series II (Table 3, Fig. 2).

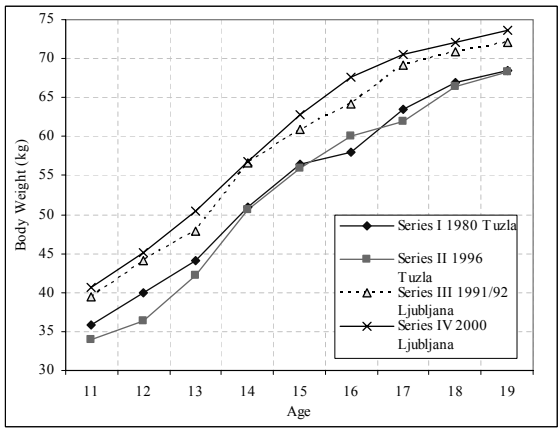


Fig.2. Comparative diagram of body weight of Tuzla's male children and youth: 1980 and 1996, and the youth from Ljubljana 1991/92 and 2000

Temporary stagnation in body weight increase in series II could be explained before all, by incorrect diet (hunger, lack of vitamins and proteins), bad socio-healthy and hygienic conditions our domicile and banished population experinced in the course of the last agression on Bosnia and Herzegovina.
Comparison of the results from series II on average body weight and series III and IV (considering time distance between publishd reseraches in Ljubljana and Tuzla) shows differences that are an outcome of already mentioned differences in average body height (Table 3, Fig. 2).

Table 3. Body weight of the boys of Tuzla Region (1980 & 1996)

Age	N_1 1980.	N_2 1996.	X_1	X_2	SD_1	SD_2	Relative Increase (%) 1	2	t-test p<0,05
11	113	139	35,92	33,92	6,09	6,07	-	-	2,60*
12	149	143	40,02	36,44	8,38	6,57	11,41	7,43	4,07***
13	143	165	44,13	42,28	9,80	10,76	10,26	16,02	1,58
14	188	184	50,98	50,53	0,09	12,30	15,52	19,53	0,5
15	170	156	56,40	55,87	9,46	11,77	10,63	10,56	0,45
16	187	133	58,05	59,98	9,86	9,08	2,92	7,35	1,81
17	138	146	63,50	61,96	8,21	8,79	9,38	3,31	1,53
18	192	155	67,00	66,34	8,72	8,78	5,51	7,06	0,70
19	69	100	68,42	68,35	7,54	9,52	2,11	3,03	0,05

Mean chest circumference. Mean chest circumference represents a few parameters of body dimensions characteristic for growth. Chest circumference is sufficient if it amounts a half of body height, and insufficient if it is under a half of that measure. Comparison of the results from series II with series I, shows acceleration increase of this parameter (1,78 cm/dec) particularly apparent in post-14 years of life (Table 4). Boys from series II have in all age categories higher average values for mean chest circumference than their age boys 16 years ago (series I). At age 15 to 19 years, the established differences between average values are statistically significant (Table 4). Boys from series II have lower value of chest circumference than in series III in all periods of growth (Fig. 3).

Table 4. Mean chest circumference of the boys of Tuzla Region (1980 & 1996)

Age	N_1 1980.	N_2 1996.	$X_{1(cm)}$	$X_{2(cm)}$	SD_1	SD_2	Relative Increase (%) 1	2	t-test p<0,05
11	113	139	67,37	68,66	4,15	4,81	-	-	2,29*
12	149	143	69,64	70,11	5,12	5,05	3,37	2,10	0,80
13	143	165	72,45	73,80	6,19	6,72	4,04	5,27	1,83
14	188	184	78,74	79,14	4,53	8,22	8,68	7,24	0,58
15	170	156	79,70	82,48	5,42	7,98	1,22	4,21	3,65**
16	187	133	79,76	85,79	4,72	5,65	0,08	4,02	10,07***
17	138	146	84,11	86,97	4,57	5,77	5,45	1,37	4,64***
18	192	155	86,03	89,74	4,58	5,29	2,28	3,19	6,90***
19	69	100	87,49	91,05	4,02	5,48	1,70	1,46	4,86***

Upper arm circumference. Increase in upper arm circumference in series II is highest in adolescent shock (age 14 years), but it is most intensive between age 17 and 18 years, as well, (Table 5). The first maximum in relation to intensive general growth and development in puberty, the second one is likely in relation to muscle growth, when maximal sport and physical efforts start. Secular trend in boys from series II is found in puberty, while it stagnates in prepuberty and postpuberty (Fig. 4).

Table 5. Upper arm circumference of the boys of Tuzla Region (1980 & 1996)

Age	N_1 1980.	N_2 1996.	$X_{1(cm)}$	$X_{2(cm)}$	SD_1	SD_2	Relative Increase (%) 1	2	t-test p<0,05
11	113	139	19,23	19,41	1,96	1,99	-	-	0,72
12	149	143	20,07	19,80	2,42	2,02	2,02	4,36	1,04
13	143	165	20,48	20,76	2,36	2,85	4,84	2,04	0,94
14	188	184	21,81	22,39	2,39	2,98	7,86	6,49	2,08*
15	170	156	22,80	23,26	2,21	2,70	3,87	4,53	1,67
16	187	133	23,30	24,27	2,31	2,25	4,32	2,19	3,77**
17	138	146	24,74	24,37	2,11	2,15	0,41	6,18	1,46
18	192	155	25,29	25,39	2,04	2,13	4,18	2,22	0,44
19	69	100	25,92	25,78	2,01	2,24	1,56	2,49	0,43

Total arm length. Results of analysis on the arm length are shown in Tabe 6 and Figure 5. Mean values for the arm length are significantly lower (Tab. 6) in series II in relation to series of all ages, except in 18 years old boys. Negative secular trend is happening here until 19 years age, when mean value in series II came close to values in series I.

Biacromial breadth. Is a good indicator for growth of trunk, skeleton and musculature. Fig.6 shows that shoulder growth in width is happening in the course of puberty. Most intensive growth is in 15 year age, then it slowdowns until 19 year age. The boys from series II of all ages have significantly higher values (Tab. 7) in relation to series I. Tuzla's boys (series II) have around same shoulder width as well as their age boys from Ljubljana (series III) in all ages from 11 to 15 years, and after that period Tuzla's boys have higher values of shoulder width than their age boys from Ljubljana (Fig. 6). Acceleration increase for this parameter is overt in all age categories and it is 1,74 cm/decade.

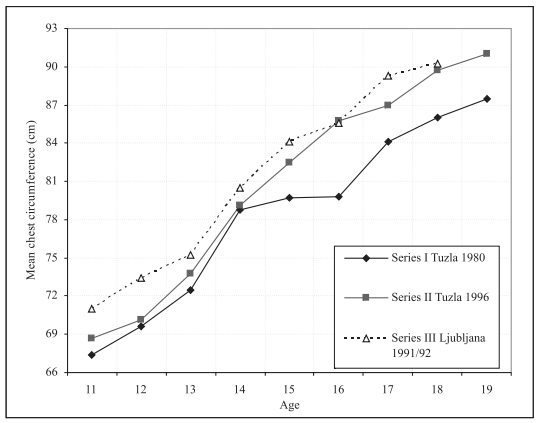


Fig.3. Comparative diagram of mean chest circumference of Tuzla's male children and youth: 1980 and 1996, and the youth from Ljubljana 1991/92

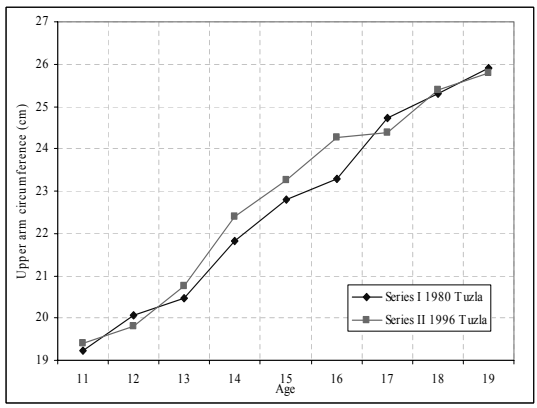


Fig.4. Comparative diagram of upper arm circumference of Tuzla's male children and youth: 1980 and 1996

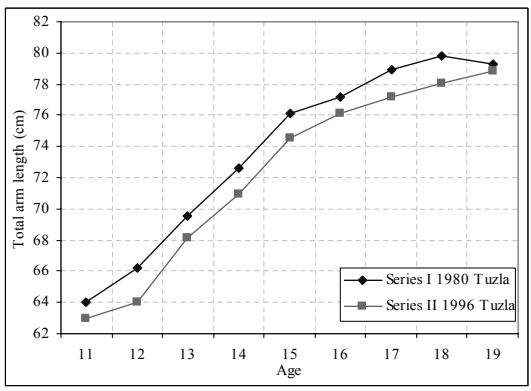


Fig.5. Comparative diagram of total arm length of Tuzla's male children and youth: 1980 and 1996

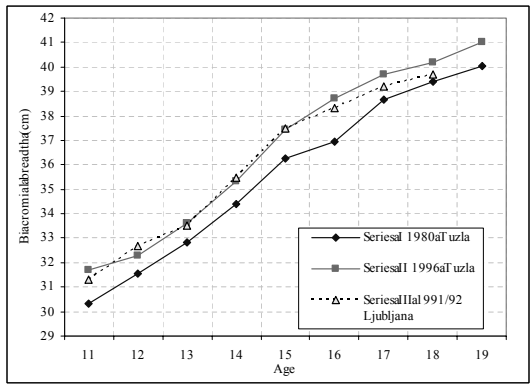


Fig.6. Comparative diagram of biacromial breadth of Tuzla's male children and youth: 1980 and 1996, with the youth from Ljubljana 1991/92

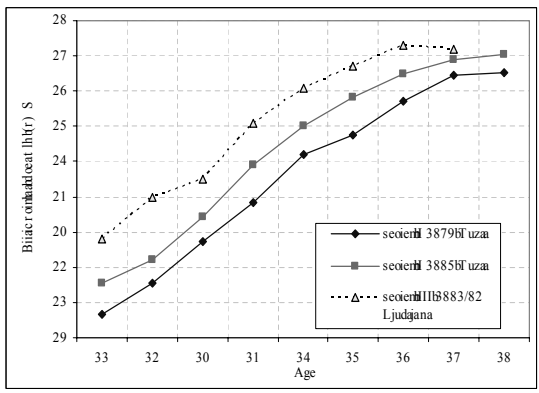


Fig.7. Comparative diagram of biiliocristal breadth of Tuzla's male children and youth: 1980 and 1996, with the youth from Ljubljana 1991/92

Table 6. Total arm length of the boys of Tuzla Region (1980 & 1996)

Age	N_1 1980.	N_2 1996.	$X_{1(cm)}$	$X_{2(cm)}$	SD_1	SD_2	Relative Increase (%) 1	2	t-test p<0,05
11	113	139	64,04	62,93	3,06	3,52	-	-	2,68*
12	149	143	66,25	64,02	3,64	3,29	3,45	1,73	5,49***
13	143	165	69,52	68,13	4,31	4,15	4,93	6,42	2,87*
14	188	184	72,65	70,98	4,88	4,14	4,50	4,19	3,56**
15	170	156	76,15	74,51	4,32	4,23	4,81	4,97	3,46**
16	187	133	77,14	76,08	3,95	3,63	1,30	2,11	2,48*
17	138	146	78,92	77,21	3,65	3,60	2,30	1,48	3,98**
18	192	155	79,79	78,08	3,59	4,17	1,10	1,13	4,04***
19	69	100	79,25	78,81	3,66	3,74	0,70	0,93	0,76

Table 7. Biacromial breadth of the boys of Tuzla Region (1980 & 1996)

Age	N_1 1980.	N_2 1996.	$X_{1(cm)}$	$X_{2(cm)}$	SD_1	SD_2	Relative Increase (%) 1	2	t-test p<0,05
11	113	139	30,31	31,70	1,72	1,78	-	-	9,17***
12	149	143	31,57	32,30	1,68	1,82	4,15	1,60	3,56**
13	143	165	32,82	33,60	2,43	2,29	3,95	4,02	2,89*
14	188	184	34,39	35,32	2,40	2,66	4,78	4,11	3,54**
15	170	156	36,24	37,42	2,54	2,98	5,37	5,95	3,83**
16	187	133	36,97	38,69	2,40	2,28	2,01	3,38	6,52***
17	138	146	38,68	39,67	2,15	2,42	4,49	2,52	3,67**
18	192	155	39,41	40,19	2,19	2,21	2,01	1,31	3,29**
19	69	100	40,05	41,03	1,97	2,19	1,62	2,11	3,04**

Biiliocristal breadth. Yearly relative increase in average width of pelvis is highest between 13 and 14 years age (series II; Tab. 8). Pelvis growth completes in 18 years age. Fig.7 shows presence of growth acceleration for this parameter (after 16 years age) in all ages (1,86 cm/decade). Mean values for the pelvis width are significantly higher in series II in relation to series I (confirmed by t-test; Tab. 8). Boys from series II have significantly narrower pelvis than boys from series III in all ages, except for 18 years age, when those values are about equal in both series.

Table 8. Biiliocristal breadth of the boys of Tuzla Region (1980 & 1996)

Age	N_1 1980.	N_2 1996.	$X_{1(cm)}$	$X_{2(cm)}$	SD_1	SD_2	Relative Increase (%) 1	2	t-test p<0,05
11	113	139	20,65	21,56	1,54	1,91	-	-	4,19***
12	149	143	21,55	22,20	1,63	1,97	4,35	2,96	3,07**
13	143	165	22,74	23,44	1,87	1,74	5,52	5,59	3,42**
14	188	184	23,82	24,90	2,09	2,03	4,74	6,22	5,09***
15	170	156	25,19	26,01	2,01	2,01	5,75	4,45	3,66**
16	187	133	25,76	26,82	1,79	1,74	2,26	3,14	5,30***
17	138	146	26,71	27,48	1,71	1,65	3,68	2,44	3,85**
18	192	155	27,45	27,90	1,74	1,61	2,77	1,52	2,47*
19	69	100	27,53	28,04	1,56	1,64	0,29	0,52	2,05*

Bad living conditions due to the last war did not effect much the width parameters, as they effected body height and circumferences.

Conclusions

Comparison of our results (series II) and corresponding results (from 16 years ago – series I) shows that boys from series II in prepuberty and puberty for some parameters have same or even lower mean values in relation to the boys from series I (negative acceleration trend). This a direct result of negative effect of exogenous factors that caused temporary growth stagnation in all age groups. In postpuberty that stagnation is relatively fast compensated, so a mild secular trend is present for all parameters observed for that growth and developmental period.

All the boys from series II spent one or two intensive periods of heght growth in prewar, wartime and postwar period, so malnutrition, comprehensive indigence and other war misfortunes significantly slowdowned growth and development in both periods.That's why the most intensive growth was prolonged for one year more, as well, in relation to the expected one.

Ten year-secular trend for body height for the boys from series III and IV (Ljubljana) was slowdowned, while 16 year trend for this parameter (series I and II) is evident in postpuberty.
Comparison of our results and those of ŠTEFANČIĆ & AL (1996) and of STREL & AL (2001) revealed larger mean values for almost all tested parameters in Ljubljana's male youth of all tested generations. However, those differences slowly decrease in postadolescent period. We assume those differences occured due to different ecologic factors, living standard, as well as specific genetic features charasteristic for all populations.
We suppose the population of the researched area has not come close to full expression of genetic material for the investiagted features.

Sažetak

Šesnaestogodišnji trend sedam pokazatelja fizičkog rasta i razvoja muške djece i omladine proučavan je odgovarajućom analizom uzorka, koji je 1996. godine obuhvatio 1329 ispitanika. Naši podaci komparirani su odgovarajućim rezultatima istraživanja iz 1980. godine u uzorku od 1349 dječaka. U ovu studiju uključeno je 9 sukcesivnih generacija iz 1980. i 1996. godine sa područja Tuzle. Provedena anliza prikupljenih podataka primarno počiva na naučnoj elaboraciji registriranog stanja iz 1996.godine u proučavanom dijelu šire populacije, nakon jednog neprirodnog i ekstremno nepovoljnog perioda u procesu rasta i razvoja ogromne većine njihovih pripadnika. Cilj ovog rada je bio utvrditi sekularni trend (negativan ili pozitivan) za 7 antropometrijskih pokazatelja rasta i razvoja muške djece i omladine, komparirajući naše rezultate iz 1996. godine sa odgovarajućim podacima rasta i razvoja na uzorku približno iste veličine (iste populacije) iz 1980. godine. Unatoč tome što su nepovoljni (ratni) životni uvjeti negativno djelovali na ontogenezu ispitanika, utvrđeno je da se rast i razvoj muške djece i omladine na proučavanom području odvija skladno te da se nalazi u granicama prosječnih evropskih standarda. Ipak, nepovoljni životni uvjeti izazvali su privremeni zastoj u rastu i razvoju, pa se u pogođenim uzrasnim kategorijama (od 11 do 15 godina života) nije moglo uočiti povećanje srednjih vrijednosti (za jedan broj) izučavanih parametara u poređenju sa uzorkom iz 1980. godine. Šesnaestogodišnji akceleracijski trend za većinu parametara je utvrđen i naročito uočljiv u postpubertetu.

References

BERBEROVIĆ LJ.& R.HADŽISELIMOVIĆ 1982: Medical Antropology. Svjetlost, Sarajevo.

BIELICKI T.& H. WALISZKO 1991: Urbanization-dependent gradients in stature among Polish conscripts in 1976 and 1986. American Yournal of Human Biology, *3*: 419 – 424.

BRODAR V. 1961: Fizički razvoj študentov ljubljanske univerze v času štiriletnega študija. Biol. Vest. **8**: 23 – 30.

DOVEČAR F.& U. ARKO1978: Dinamika razvoja nekaterih telesnih parametrov glede na leto 1939/40. Glasnik ADJ, *15* (15): 61-69.

FEDERAL BUREAU FOR STATISTICS – Sarajevo 1998: N° : 052 – 0212 / 31. 03. 1998.

GAVRILOVIĆ Ž.1972: Body Height and Weight of the First Ten Generations of Medical Technicians from Novi Sad. Medical Survey, 15: 3 – 4.

GAVRILOVIĆ Ž.& V. BOŽIĆ 1974: Some antropological karacteristics of the secondary school children. Zbornik Matice srpske za prirodne nauke, 46: 161 – 172.

HADŽISELIMOVIĆ R. (1988): Introduction into anthropogenesis theory. Svjetlost, Sarajevo: 25 – 53.

HADŽISELIMOVIĆ R.& S. LELO 1998: Bioanthropologic Practical Course. Faculty of Science and Mathematics, Sarajevo University, 35–55.

HAUSPIE R.C., VERCAUTEREN M. & C. SUSANNE 1997: Secular changes in growth and maturation: on update. Acta Pediatr Suppl , *423* : 20 – 7.

HAUSPIE R.C., VERCAUTEREN M. & C. SUSANNE 1996: Secular changes in growth.Horm Res, 45 (Suppl. 2): 8-17.

HIERNAUX J. 1975: Equality & Inequality of Races? Školska Knjiga, Zagreb, 17 – 20.

HISAFUMI M., KOSHI S., SHIGETAKA S.& M. MITSUNORI 1999: Bone Maturation the Secular Trend in Growth. Horm Res, *52*: 125 – 130.

IVANOVIĆ B. 1985: Ontogeny Development and Anthropologic Properties of Monte Negro Youths. CANU, Titograd, (1985): 13–52.

KOVAČ I., EMILI H. & S. URBAN 1973: Body height and body mass of the school children of the island Pag today and before 30 years. Glasnik ADJ, *7*: 123 – 135.

KULENOVIĆ S. 1980: Tuzla Commune Population Structure and its Change Affected by recent economic Development. M. A. Degree Thesis. Faculty of Science and Mathematics, Beograd.

MALINA R.M. 1990: Research on secular trends in auxology. Anthropologische Anzeiger, 48 : 209 – 27.

MALINA R.M. 1979: Secular changes in size and maturity: causes and effects.Monogr. Soc. Res. Child. Dev., 179: 59- 102.

NIKOLIĆ-DOVAT V. 1978: Somatic Growth and Development of a Child Institute of Mother and Child Public Health, Novi Sad: 19 – 25.

NOVAKOVIĆ M. 1980: Growth and Pubertal Develpoment Male Youth From Tuzla's Region. M.A.Degree Thesis. Medical Fakulty University of Tuzla, Tuzla.

PREBEG Ž. 1978: Accelaration growthof the schoolchild and youth from Zagreb.Yugoslav. Akademy of Sciencees and Arts, »Rad« Knjiga 378, Zagreb.

PREBEG Ž., JUREŠA V.& M.KUJUNDŽIĆ 1995: Secular growth changes in Zagreb schoolchildren over four decades, 1951-91. Annals of Human Biology, 22 (2): 99 – 110.

PREBEG Ž. 1997: Variations in Growth Patterns of Schooolchildren in Croatia over the Last Decade. In: Derek F.Roberts, Pavao Rudan, and Škarić-Jurić Tatjana (editors). Growth and Development in the Changing World. Croatian Anthropological Society, Zagreb.

SANNA E., FLORIS G.& COSSEDDU G.G.1993: Secular trend in sardinian consripts drafted from 1979-1883 to 1983-1986. Antropologisher Anzeiger, 51: 225 – 232.

STREL J., KOVAČ M., JURAK G., BRDNARIK J. & B. LESKOŠEK 2001: Comparison of phisical development of school children between 1990 and 2000 on the basis of the data obtained from the sports educational chart. Anthropological Notebooks,VII, N° 1: 11-32.

SUSANNE C. 1985: Living conditions and secular trend. J. Hum. Evol. 14: 357 – 70.

ŠTEFANČIĆ M., ARKO U., BRODAR V., DOVEČAR F., JURIČIĆ M. , MACAROL – HITI M., P. LEBEN – SELJAK & T. TOMAZO – RAVNIK 1996: Ocena telesne rasti in razvoja otrok in mladine v Ljubljani. Zdravstveno varstvo. Letnik 32, supl: 1.

TANNER J. M.1962: Growth at adolescence. Blackwell: Oxford. Scientific Publications. p.313.

TOMAZO – RAVNIK T. 1988: Secular trend in growth of Schoolchildren in Yugoslavia. Coll. Antropol. 12 (1): 121 – 125.

TOMAZO – RAVNIK T. 1999: Body composition of youths in Slovenia. Abstracts. IV th International Anthropological Congress of Aleš Hrdlička.

WIERINGEN J.C. 1986: Secular growth changes. In : FALKNER F, TANNER JM. (eds.): Human growth, (3) 2nd edn. New York: Plenum Press, pp. 307-312.

WIERINGEN J.C. 1979: Secular Growth Changes – An Analysis of Developments in the Netherlands, 1850-1978. Coll. Antropol. 3: 35 – 47.

WEBER G., SEIDLER H., WILFING H. & G. HAUSER 1995: Secular change in height in Austria: an effect of population statification? Annals of Human Biology 22 (4): 277 – 288.

ACTA BIOLOGICA SLOVENICA
LJUBLJANA 2005 Vol. 48, Št. 1: 45-48

Sprejeto (accepted): 2005-09-27

Thinning of 'Golden Delicious' apples with the combination of ethephon and CPPU

Redčenje plodičev jablane sorte Zlati delišes s kombinacijo etefona ter CPPU

Matej STOPAR

Agricultural Institute of Slovenia, Hacquetova 17, 1000 Ljubljana, Slovenia

Abstract. A synthetic cytokinin forchlorfenuron (CPPU) 5 ppm was sprayed at the time of petal fall (PF) on eight years old 'Golden Delicious'/M.9 apple trees with the aim of enlarging the fruit growth. Since CPPU is not recognized as a thinning agent which increases the return bloom, a low dose of ethephon was sprayed on CPPU treated trees to enhance flower bud formation and increase flowering in the next season. Ethephon was applied in successive treatments (alone or in the combination with CPPU) 3 x 30 ppm or 5 x 50 ppm, starting at PF, and continued in week intervals.

The single application of CPPU 5 ppm or the combination of ethephon 3 x 30 ppm with CPPU 5 ppm decreased the fruit set and enhanced the fruit growth to reach the commercial demand. The combination spraying of ethephon 5 x 50 ppm + CPPU 2 x 5 ppm resulted in a too strong thinning response while the yield of bigger size fruit was not enhanced. The alone application of ethephon 3 x 30 ppm had no influence on the fruit thinning or fruit weight while the application of ethephon 5 x 50 ppm reduced the fruit set but, consequently, did not enhance the fruit growth. None of the treatments influenced the return bloom.

Key words: apple thinning, CPPU, ethephon, alternate bearing

Abbreviations: N-(2-chloro-4-pyridyl)-N'-phenylurea (CPPU), 2-chloroethylphosphonic acid (ethephon)

Izvleček. Z namenom povečanja velikosti plodov, smo škropili osem let stare jablane 'Zlati delišes/M.9' s sintetičnim citokininom forklorfenuronom (CPPU), v koncentraciji 5 ppm ob koncu cvetenja (KC) dreves. Ker CPPU ne izboljšuje povratno cvetenje jablan, so CPPU tretiranim drevesom dodali še etefon, kateri naj bi spodbudil nastanek cvetnega brstja ter tako izboljšal cvetenje v naslednjem letu. Etefon je bil nanešen v več zaporednih nanosih (sam ali v kombinaciji s CPPU), v tedenskih intervalih z začetkom ob KC jablan in sicer 3x30 ppm ali 5x50 ppm.

Samostojni nanos CPPU 5 ppm ali pa kombinacija etefona 3x30 ppm s CPPU 5 ppm je zmanjšala rodni nastavek in povečala rast plodov kot zahteva komercial-

ni nivo pridelave. Kombinacija škropljenj etefona 5x50 ppm + CPPU 2x5 ppm je povzročila premočan osip plodičev, tako da se pridelek plodov večjega velikostnega razreda ni izboljšal. Samostojni nanos etefona 3x30 ppm ni imel vpliva niti na redčenje plodičev niti na težo plodov, medtem ko je etefon 5x50 ppm sicer zmanjšal rodni nastavek, kar pa posledično ni povzročilo povečanja teže plodov.

Ključne besede: redčenje plodičev jablane, CPPU, etefon, izmenična rodnost

Okrajšave: N-(2-chloro-4-pyridyl)-N'-phenylurea (CPPU),
2-chloroethylphosphonic acid (etefon)

Introduction

Forchlorfenuron (CPPU), a synthetic cytokinin, has a strong influence on apple fruit growth if applied at petal fall (PF) or a few days later at the concentration below 10 ppm (Greene 1989, Greene 2001). Undesirable side effect, a reduced return bloom, was observed on 'Delicious' and 'McIntosh' apple trees after CPPU 5 ppm applications (Curry & Greene 1993, Greene 1989). By contrast, an old thinner, ethephon, is known to enhance the return bloom of apple trees because its post bloom application mostly results in good flower bud induction (Knight & Browning 1986). The application of ethephon mostly thins apple fruitlets while the growth of the remaining fruit, frequently, does not respond with an enhanced growth rate (Ebert & Bender 1986, Link 2000, Stopar 2000). In the experiment we tried to combine the positive effect of both growth regulators: application of ethephon a few times in a low concentration to enhance the return bloom and not to thin the fruitlets, and the use of CPPU for the enhancement of fruit growth on the high loaded trees.

Material and methods

Eight years old 'Golden Delicious'/M.9 apple trees were selected according to high bloom density and homogeneous growth vigor. In the field trial a standard randomized block design with six replications and a single tree per plot were used.

Successively, ethephon and CPPU were applied at weekly intervals and the first spraying of both agents started at petal fall (PF) time. The treatments were as follows:

1) Control – no thinning
2) Hand thin (done just after the June drop time)
3) CPPU 1x5 ppm = 5 ml Sitofex (SKW, Trostberk, Germany) / L water;
4) Ethephon 3x30 ppm = 0.06 ml Ethrel (Chromos, Zagreb, Croatia) / L water
5) Ethephon 5x50 ppm = 0.10 ml Ethrel / L water
6) Ethephon 3x30 ppm + CPPU 1x5 ppm;
7) Ethephon 5x50 ppm + CPPU 2x5 ppm;

The spraying was done with a hand sprayer to the point of drip. No surfactant was used. When ethephon was combined with CPPU spraying, first, ethephon was applied and an hour later, after the leaves were dry, CPPU was applied. At maturity time the fruit was harvested, counted, weighed and graded into two size classes, < 68 mm and > 68 mm fruit diameter. Phytotoxic effect on trees was estimated a month after spraying with a visual scale (1 = no effect observed, 5 = suppressed shoot growth, bigger and wrinkled leaves with yellow green spots, more transparent crown). The return

bloom was estimated visually next spring at bloom time using the scale 1-10 (1 = no flower clusters present on the trees; 10 = abundant flowering). During the experiment the trees received standard pest and disease management program. Data were subjected to statistical analysis using the statistical program Statgraphics 5.0 (STSC, Rockwille, USA).

Results and discussion

The single PF application of CPPU 5 ppm significantly reduced the final fruit set (Table 1) and increased the fruit growth to satisfy the commercial demand for 'Golden Delicious' apples (Table 2). When GREENE (2001) sprayed CPPU 8 ppm on 'McIntosh' at PF he got no thinning effect while a week later spraying thinned the trees appropriately. STOPAR (1999) did not observe an enhanced fruit drop of small fruited apple cultivars 'Jonathan', 'Elstar' and 'Gala' when CPPU 5 ppm was sprayed a week after PF. It looks that CPPU is not a thinner with a consistent fruitlet abscission response if sprayed either at PF or a week later. In this trial the return bloom after CPPU 5 ppm single spraying was not influenced too.

Table 1: The number of flower clusters at the start of the experiment, the final fruit number and the return bloom of 'Golden Delicious/M.9' apple trees after the application of thinning agents.

Treatment*	Flower clusters (No./tree)	Fruit (No./tree)	Fruit (No./100 clusters)	Return bloom (1-10)**
1) Control	191 a	142 cd	74 b	3,17 ab
2) Hand thin	219 a	101 bcd	47 a	1,83 a
3) CPPU 1x5 ppm	223 a	74 ab	33 a	4,0 ab
4) Ethephon 3x30 ppm	190 a	148 d	83 b	1,93 a
5) Ethephon 5x50 ppm	215 a	96 bc	44 a	4,38 ab
6) Ethephon 3x30 ppm + CPPU 1x5 ppm	221 a	76 ab	34 a	4,67 b
7) Ethephon 5x50 ppm + CPPU 2x5 ppm	196 a	42 a	22 a	4,00 ab

Table 2: Total yield, yield of commercial (> 68 mm) fruit, mean fruit weight and the effect on phytotoxicity after the application of thinning agents on 'Golden Delicious/M.9' apple trees.

Treatment*	Yield (Kg/tree)	Fruit > 68mm (Kg/tree)	Fruit weight (g)	Phytotoxicity (1-5)**
1) Control	16.0 b	5.5 ab	115 a	1,0 a
2) Hand thin	12.7 ab	8.4 ab	125 a	1,0 a
3) CPPU 1x5 ppm	10.9 ab	8.7 ab	161 b	2,2 b
4) Ethephon 3x30 ppm	15.5 b	4.5 a	106 a	1,0 a
5) Ethephon 5x50 ppm	10.8 ab	4.2 a	110 a	1,2 a
6) Ethephon 3x30 ppm + CPPU 1x5 ppm	12.7 ab	11.2 b	174 b	2,0 b
7) Ethephon 5x50 ppm + CPPU 2x5 ppm	7.2 a	6.8 ab	169 b	4,0 b

Application of ethephon 3x30 ppm did not affect the fruit set, yield or fruit growth of 'Golden Delicious', while spraying of ethephon 5x50 ppm reduced the fruit set but did not enhance the fruit growth (Table 1,2). None of the ethephon alone treatments affected the return bloom in this trial significantly. The combination spraying of ethephon 3x30 ppm + CPPU 5 ppm reduced the fruit set and enhanced the fruit growth while the return bloom was not increased, i. e. all similar effects as those which happened after a single CPPU 5 ppm spraying. A stronger combination of both regulators (ethephon 5x50 ppm + CPPU 2x5 ppm) induced the overthinning of 'Golden Delicious' trees. The fruit growth was enlarged but the yield of bigger size fruit was not increased because the fruit was not numerous. A very similar overthinning occurred when CPPU 5 ppm was combined with ethephon 3x70 ppm (PF + weekly intervals) on the cultivar 'Elstar' (STOPAR 1998). In spite of overthinning the return bloom was not increased. This can be speculated as a diminishing effect of CPPU on the return bloom observed by GREENE (1989) and CURRY & GREENE (1993).

A month after spraying the phytotoxic effect of treatments was estimated on trees. A week shoot growth, fewer but bigger leaves with green yellow spots and wrinkled surface were observed on CPPU treated crowns (Table 2). This phytotoxic effect was more pronounced on crowns on which CPPU was applied twice and the effect remaining on trees until harvest.

Spraying of 'Golden Delicious' with CPPU 5 ppm at PF thinned trees in our experiment, however, bearing in mind CPPU trials reported by others no consistent thinning response was observed. If a weak phytotoxic CPPU 5 ppm effect is taken into consideration, no recommendation should be given to apple growers for the use of CPPU. For a more complete understanding of CPPU action the concentration and time response studies should be done on different apple cultivars.

Literature

CURRY E.A. & D.W. GREENE 1993: CPPU Influences Fruit Quality, Fruit Set, Return Bloom and Preharvest Drop of Apples. HortScience **28** (2): 115-119.

EBERT A. & R.J. BANDER 1986. Influence of an Emulsifiable Mineral Oil on the Thinning Effect of NAA, NAAm, Carbaryl and Ethephon in the Apple Cultivar Gala Grown under the Conditions of Southern Brazil. Acta Horticulturae **179**: 667-672.

GREENE D.W. 1989: CPPU Influences 'McIntosh' Apple Crop Load and Fruit Characteristics. Hortscience **24** (1): 94-96.

GREENE D.W. 2001: CPPU Influences Fruit Quality and Fruit Abscission of 'McIntosh' Apples. Hortscience **36** (7): 1292-1295.

KNIGHT J.N. & G. BROWNING 1986. Regulation of Conference Pear Cropping with Gibberelic Acid and Ethephon or Paclobutrazol. Acta Horticulturae **179**: 337-342.

LINK H. 2000: Significance of Flower and Fruit Thinning on Fruit Quality. Plant Growth Regulation **31**: 17-26.

STOPAR M. 1998: Introduction of New Environment Friendly Apple Thinning Compounds. Proceeding of the Conference Agriculture and Environment, Bled, p. 415-420.

STOPAR M. 1999: NAA Thinning of 'Small-Fruited' Apple Cultivars in Combination with CPPU. Phyton **39** (3): 85-90.

STOPAR M., 2000. Comparison of the Most Frequently Used Apple Thinning Compounds for the Thinning of 'Jonagold', 'Elstar' and 'Golden Delicious' Apples. Res. Rep. Biot. fac. UL – Agriculture **75** (1): 89-94.