

**Josip Juraj Strossmayer University of Osijek
and
Ruder Bošković Institute Zagreb**

**University Postgraduate Interdisciplinary Doctoral Study
“ENVIRONMENTAL PROTECTION AND NATURE CONSERVATION”**

Vesna Peršić, M. Sc.

**ALGAL BIOASSAY PROCEDURE FOR ASSESSING
EUTROPHICATION OF WATERS IN EASTERN SLAVONIJA AND
BARANJA**

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Abstract

Excessive algal growth is a major pollution problem and is believed to be caused by large quantities of nutrients loaded into the aquatic environment. Therefore, investigations in this thesis were focused on the relationship between algal growth potential and the distribution of nitrogen and phosphorus in surface freshwaters. To achieve objectives of this thesis a responsive test was designed to assess quantitatively algal growth after nutrient additions in order to obtain a biologically relevant measure of nutrient deficiency. As a result, a bioassay method is introduced for the evaluation of trophic status and limiting nutrients in the investigated waters. Trophic conditions based on algal growth potential throughout the work were inversely related to trophic conditions based on phytoplankton chlorophyll-*a* concentrations. Thus, in defining the trophic state both parameters should be considered as complementary. In the Danube river floodplain system, the most significant factors that influenced nutrient dynamics were the location of floodplain water bodies regarding the river (spatial dimension) and hydrological connectivity between the river and its floodplain (temporal dimension) along with the retention time of floodwaters and nutrient uptake by biota. Based on low nitrogen to phosphorus ratios and the overall high phosphorus concentrations, nitrogen was the expected limiting nutrient for primary productivity in the investigated waters. However, nutrient enrichment results indicated that N/P ratio was not a reliable indicator of nutrient limitation. Watercourses of rural and agricultural catchment (the Biđ-Bosut) were badly damaged or at high risk of eutrophication and their nutrient contents were far above prescribed concentrations. Only moderate signs of distortion resulting from anthropogenic activities were observed in watercourses draining forested catchment (the river Spačva basin), while some watercourses draining agricultural watershed in the Baranja region showed even nutrient deficiency (primarily nitrogen). Nutrient loading from catchments with different land uses exhibited considerable uncertainties making temporal and spatial monitoring of water quality essential for predicting the effects of either nutrient increases or reductions. Moreover, systematic studies involving water quality assessment at catchment scale showed to be necessary for the development of integrated water quality management. Because financial cost and the type of nutrient reduction strategies vary considerably with the targeted nutrient, scientific foundations are needed for management decisions concerning eutrophication control.

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Istraživanja u ovoj disertaciji usmjerena su na odnos između potencijalnog rasta algi i distribucije dušika i fosfora u površinskim kopnenim vodama. Za postizanje ciljeva dizajniran je eksperiment u svrhu kvantitativne procjene rasta algi kulture *Chlorella kessleri* FOT. et NOV. Da bi utvrdili jačinu limitacije, uspoređivan je prirast laboratorijski uzgajane kulture algi sa i bez dodanih hranjivih tvari te je dobivena mjerljiva vrijednost ograničavajućeg djelovanja određene hranjive tvari ($\Delta\mu$, d-1). Također su definirane varijacije u N i P-limitaciji tijekom sušnog i poplavnog razdoblja. Istraživanja su provedena na području istočne Slavonije i Baranje: u poplavnom području rijeke Dunav (Park prirode Kopački rit), te u poljoprivrednom i šumskom području porječja rijeka Biđ i Bosut. Utvrđena je značajna korelacija između potencijala rasta alga i koncentracije nitrata u istraživanim uzorcima vode poplavnog područja Parka prirode Kopački rit. Ograničavajući čimbenik primarne produkcije u tim vodama bio je dušik. Definirane su promjene od nelimitirajućih uvjeta (tijekom sušnog razdoblja) ka N-limitaciji (tijekom razdoblja plavljenja) u Kopačevskom kanalu, kao i stalna N-limitacija na postajama Sakadaškog jezera. Ovisno o fluktuacijama vodostaja kao važnog ekološkog čimbenika u poplavnom području Parka prirode Kopački rit, utvrđeni su hipertrofni uvjeti tijekom sušnog razdoblja i eutrofni uvjeti tijekom razdoblja plavljenja. Dugotrajna plavljenja utječu na koncentracije hranjivih soli, osobito nitrata, te značajno određuju prostornu heterogenost područja. Prema hidrološkom režimu i smanjenju povezanosti između uzorkovanih postaja u poplavnom području i rijeke Dunav, definirani su različiti tipovi vodenih staništa: eupotamal, parapotamal i paleopotamal. Rezultati biotesta pokazali su da nutrijenti u vodama eupotamala podržavaju optimalan prirast *C. kessleri*, dok je potencijalna N-limitacija utvrđena u vodama parapotamala i paleopotamala. Duljim zadržavanjem poplavnih voda utvrđeno je prostorno širenje N-limitacije prema glavnom toku rijeke Dunav. Vode ruralnog i poljoprivrednog područja (porječje Biđ-Bosut) značajno su opterećene nutrijentima. Umjereno opterećenje nutrijentima utvrđeno je u vodama s pretežno šumskim porječjem (Spačvanski bazen), dok je u vodotocima baranjskih slivova povremeno utvrđena i N-limitacija. Dobiveni rezultati omogućit će implementaciju metode biotesta za procjenu stanja trofije kao nužnog preduvjeta definiranja mjera za smanjenje opterećenja voda hranjivim tvarima.

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GENERAL INTRODUCTION

1.1. Introduction

Trophic state is the property of energy availability to the food chain and defines the basis of community integrity and ecosystem function (Dodds, 2007). The

EUTROPHICATION IS A PROCESS OF AN ECOSYSTEM BECOMING MORE PRODUCTIVE BY NUTRIENT ENRICHMENT STIMULATING PRIMARY PRODUCERS

word “trophic” means nourishment, food. Because trophic state is a measure of energy flux to the food web, production of autotrophs and heterotrophs is directly linked to the trophic state. Transparency, biomass of primary producers (chlorophyll *a*) and nutrients are commonly used in the evaluation of trophic state. Aquatic ecosystems can, in fact, be described, referring to their supplies of growth limiting nutrients (Smith et al., 1999), as waters that have relatively large supply of nutrients or eutrophic (well nourished), and those waters that have reduced nutrient supply or oligotrophic (poorly nourished). Eutrophication is, therefore, a process of an ecosystem becoming more productive by nutrient enrichment that stimulates primary producers (Dodds and Whiles, 2010). A variety of definitions of ‘eutrophication’ exists in the literature, some differing fundamentally from others. One difference is in respect of whether eutrophication is only the process of nutrient enrichment, or it should include the problems associated with such enrichment. Eutrophication is a

process, therefore, it is useful to be able to characterise the level at which this process is at any given time in a water body. For this purpose, the ‘trophic state’ is used for the description of the water body. Classification of trophic state of aquatic ecosystems is well accepted and widespread (Vollenweider and Kerekes, 1982; Moss et al., 1994; Dodds et al., 1998; Smith and Bennett, 1999; Wetzel, 2001), and the boundaries that define trophic categories are highly similar, but not universal (Forsberg and Ryding, 1980; Vollenweider and Kerekes, 1982). Trophic classification system has recently been extended to rivers and smaller streams (Dodds, 2006). Still, it is necessary to emphasize that the relationship between the concentration of total phosphorus and suspended chlorophyll-*a* is weaker in lotic than in lentic systems (Wetzel, 2001).

Cultural eutrophication is the Earth's most widespread water quality problem and most scientists agree that it is usually caused by increasing inputs of phosphorus (P) and nitrogen (N), which are abundant in municipal wastewaters and originate from the excrement of livestock and synthetic fertilizers applied to agricultural land. Concerns about the effects of nutrient pollution on aquatic ecosystems have led to a recent surge in information related to the factors that control the trophic state. Although Liebig's Law of the Minimum and relatively low rates of P availability in many freshwaters have led to the notion of phosphorus as the most likely limiting nutrient for phytoplankton growth (Hecky and

Kilham, 1988; Wetzel, 2001), both nitrogen and phosphorus often limit biomass accrual with co-limitation being common (Elser et al., 1990; Jansson et al., 1996). Therefore, there is considerable variability in nutrients limiting algal growth and accumulation in aquatic systems. Lotic systems in the temperate zone appear to be primarily P-limited because of high N/P ratio, N fixation, and sediment retention of P. However, there is recognition of the importance of N in freshwaters (e.g., Elser et al. 1990). Some lakes for instance become N-deficient under summer stratification (Dodds and Priscu, 1990) and with increasing eutrophication (McCauley et al., 1989). Proper understanding of nutrients, which limit phytoplankton growth, is a prerequisite for successful eutrophication control at impacted sites where future changes may alter the existing limitation patterns. In the past, efforts were aimed toward controlling the eutrophication in lakes and reservoirs, but nowadays, these efforts have expanded towards rivers and wetlands. The evaluation of resources limiting algal growth and trophic status of rivers and wetlands, which present hydrological irregularities, must incorporate different approaches in order to provide acceptable understanding of the problem.

The measurements of nutrient concentrations are used to indicate the trophic state, and ratios of their values are used to indicate if a particular nutrient is a limiting factor (Redfield, 1958; Järvinen et al., 1999; Dodds, 2006). The total nitrogen (TN) and total phosphorus (TP) concentrations are useful for determining the trophic state because they represent the total nutrient content available in the algal biomass, or available for incorporation into the biomass. Likewise, the TN to TP ratio is commonly used to indicate nutrient deficiency based on observations of Redfield (1958) that algal cells have 7 to 1 N to P ratio by mass (16 to 1 by moles) under balanced growth. Since then, researchers use N to P ratio to reflect growth limiting nutrients because it correlates well with other measures of nutrient deficiency such as growth based bioassays (Levine and Whalen, 2001; Dodds, 2003; Dzialowski et al., 2005; Peršić

and Horvatić, 2011). Furthermore, laboratory bioassays constitute a practical tool to provide complementary and essential information to infer the strategies of water quality management.

The limnological literature contains descriptions of a large selection of assays that have been used to assess nutrient limitation of phytoplankton growth (Elser and Kimmel, 1986; Ault et al., 2000; O'Farrell et al., 2002; Ojala et al., 2003; Vukić Lušić et al., 2008; Xu et al., 2012). The most widely used methods are algal growth potential (AGP) bioassay and/or nutrient enrichment bioassay, where the biomass yield or growth rate within an isolated water sample is used to determine the capacity of water to support algal production and determine the limiting nutrient. To conduct the algal growth bioassay, surface water samples are collected during the period of maximum algal biomass and placed in the containers (flasks, plates), supplied with one or a group of nutrients, along with a known quantity of the test algae. The cultures are incubated under optimal light and temperature conditions, and their growth is measured over a period of a week or two until growth plateaus are reached. The effects of nutrients on the maximal algal biomass or growth rate signal which nutrient is controlling maximum biomass in the water sample under the test conditions. If the growth rate is high and exponential in the sample that contains certain nutrient, and is reduced in samples that do not contain that particular nutrient, than that nutrient is considered a limiting factor for algal growth.

Algal growth bioassay can be successfully performed in both Erlenmeyer flasks with volumes of 100 ml and microplate wells with volumes of 0.3 ml (Lukavský, 1992; Pavlić et al., 2006). Miniaturised algal bioassay for freshwater samples has become a standardised method used in environmental and ecotoxicology studies (Lukavský, 1992; Horvatić et al., 2006; Pavlić et al. 2006).

1.2. Thesis objective and outline

Excessive algal growth is a major pollution problem and is believed to be caused by large quantities of nutrients loaded into the aquatic environment. Therefore, investigations in this thesis will focus on the relationship between algal growth potential and the distribution of nitrogen and phosphorus in the surface freshwaters.

In the last 15 to 20 years, a prevailing view that rivers are insensitive to nutrient inputs no longer appears to be tenable. Although the majority of freshwater eutrophication research has been focusing on lakes and reservoirs, nutrient enrichment of flowing waters is also of significant concern. The main sources of river pollution are a consequence of anthropogenic activities, which create a greater need to protect the environment, especially those areas that have not yet experienced serious adverse changes.

It is well known that floodplain habitat heterogeneity and hydrological connectivity, driven by flow and flood pulses leads to high levels of productivity and biodiversity (Ward and Stanford, 1995) and any physical impact (e.g., river flow regulation, channelization, levee construction) will cause a decrease in productivity and biodiversity of the main river channel and its accompanying floodplains. Therefore, river floodplains are among the most valuable, but, unfortunately, also the most degraded ecosystems in Europe. Based on the World Wide Fund for Nature (WWF, 1999) for the Danube River Pollution Reduction Program, 80–90% of the original floodplain area in the Danube River basin has been lost. The remaining areas along the Danube are characterized by reduced hydrological exchange between the main river channel and its floodplain area. In today's conditions, the ecology of the Danube and its floodplains has been significantly affected by changed

land-use, by pollution from wastewaters and hydro engineering.

In addition, it is becoming increasingly critical to understand better how the relationship between spatial (location of the water bodies regarding the river) and temporal (hydrological connectivity between the river and its floodplain) dimensions contribute to the integrity of a healthy river-floodplain system. Therefore, this work builds on the knowledge gained from previous PhD theses conducted in the floodplain area of the Danube River in eastern Croatia. Bogut Irella (2005) studied functional structure of macrofauna and meiofauna in macrophyte communities and how changes in complex hydrological variables affect habitat occurrence and development of macrophyte stands in Kopački rit floodplain. Goran Palijan (2010) investigated the effects of hydrology of Kopački Rit floodplain on the bacterioplankton structure and Filip Stević (2011) researched the influence of flooding on the structure and dynamics of phytoplankton in floodplain.

The basic assumptions that are critical in this thesis are:

- a) Algal biomass and net ecosystem productivity can be controlled not only by the type, but also by the magnitude of nutrient limitation.
- b) Spatial heterogeneity of nutrient distribution and phytoplankton chlorophyll-*a* concentration within the floodplain will depend on fluctuations in water level, retention time of floodwaters and nutrient uptake by biota.
- c) Land use characteristics in the watershed will be the most responsible for water quality variations among different catchments.

The main aim of this thesis was, therefore, to provide information on the temporal and spatial variation of resource limitation on algal growth rates, in order to create a scientific basis for evaluating regional strategies of nutrient management in the investigated catchments.

The presented work contains four interrelated chapters – papers already published (Chapters I-IV) in scientific journals.

In **Chapter one**, a responsive experiment was designed to assess quantitatively algal growth after nutrient additions, in order to obtain a biologically relevant measure of the nutrient deficiency that is comparable with other studies. As a result, a bioassay method is introduced for the evaluation of trophic status and limiting nutrients in the Danube floodplain waters (1388-1426 r. km).

The lentic and lotic conditions exchange in river floodplain systems. However, they occur in a temporal dimension (synoptically), rather than simultaneously during different hydrological periods. Wetland scientists and natural resource managers often struggle to predict and quantify ecosystem responses to alternations in hydrological regimes. How does variation in frequency, amplitude, and duration of flooding affect algal growth potential in different floodplain environments? This issue is addressed in **Chapter two**.

It is widely acknowledged that flooding enhances hydrological and ecological connectivity between the main river channel and its floodplain. Therefore, spatial variability is one of the main features of the river-floodplain system and is largely determined by fluctuations in the water level. Much less is known, however, about the spatial patterns of floodplain production and features controlling distribution of these patterns. The focus of **Chapter three** is, therefore, to explain the relevance that hydrological connectivity has on spatial and temporal variability of potential nutrient deficiency in a hydrological dynamic Danube floodplain.

Despite the importance of flooding for the natural ecosystems, many rivers have been subjected to the channelization and are reduced to single-thread channels isolated from their floodplains. Frequencies and magnitude of flooding are predicted to increase due to the climate change. On the other hand, changes in land use may have a greater influence to the ecological status of lotic systems than projected climate change. However, scientists agree that the combined effects of both land use and climate changes will alter the functioning of the ecosystems.

In the agricultural landscape of eastern Croatia rural streams, ditches and melioration canals comprise an extensive drainage system that brings nutrient pollution into receiving watercourses such as large rivers where key issues of eutrophication are associated with nitrogen and phosphorus loading. In order to reverse the effects of eutrophication it is becoming increasingly critical to understand better if, where and how much nitrogen and phosphorus are limiting the production in freshwater ecosystems. **Chapter four** deals with the adequacy of nutrient enrichment bioassays for evaluating the trophic loads and limiting nutrients in watercourses, by providing detailed information needed to mitigate the impacts of eutrophication.

Finally, in the general discussion section, the synthesis of thesis core issues is carried out, focusing on the advantages, limitations, and implications of algal bioassay procedure.

Chapter 1

Bioassay method in evaluation
of trophic conditions and
nutrient limitation in the
Danube wetland waters (1388-
1426 r. km)

Primary Research Paper

Bioassay method in evaluation of trophic conditions and nutrient limitation in the Danube wetland waters (1388–1426 r. km)

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Abstract

The algal growth potential (AGP) in water samples of the Danube wetland waters (1388–1426 r. km) as well as the effect of nitrogen (in final concentration of 0.16 g l^{-1}) and phosphorus enrichment (in final concentration of 0.02 g l^{-1}) on the AGP was investigated by miniaturized bioassay method. Values of the total biomass of *Chlorella kessleri* up to the 14th day of incubation were suitable for the evaluation of trophic conditions according to the classification of AGP. On the basis of the AGP results, trophic conditions in 55% of the samples were oligo-mesotrophic and in 46% of the samples meso-eutrophic. A statistically significant correlation ($r=0.34$) was established between the AGP of *C. kessleri* in original water samples and NO_3 concentrations *in situ*. The TN/TP ratio in the wetland waters indicated a greater limitation due to nitrogen than phosphorous. Significantly lower TSI_{TN} than TSI_{SD} , TSI_{Chla} and TSI_{TP} indicated nitrogen limited conditions. In order to quantify established nutrient limitation by the bioassay method, the effect of added N and P concentrations on the growth rate of *C. kessleri* was expressed as the degree of nutrient limitation ($\Delta r \text{ d}^{-1}$) during 7 days of incubation. In the Danube wetland waters only N limitation was established in June and July; N and P limitation in May and September while in August and October 2003 neither of the tested nutrients were limiting. From May to October 2003 the significantly highest degree of nitrogen ($\Delta r=0.736 \text{ d}^{-1}$) and phosphorus limitation ($\Delta r=0.474 \text{ d}^{-1}$) was determined in Lake Sakadaš.

Introduction

The bioassay method is important for a better understanding of the relation between nutrient concentrations and phytoplankton dynamics in the Danube, its sidearms and wetlands (Horvatić et al., 2003a; Stević et al., 2005; Peršić et al., 2005). The unicellular green alga *Chlorella kessleri* is suitable for the evaluation of the algal growth potential (AGP) in miniaturized algal growth bioassay, since it belongs to a group of ubiquitous species which have a wide tolerance towards environmental conditions (Lukavský, 1992, 1994). The AGP of *C. kessleri* measured in bioassay primarily depends on the nutrient concentration in

the tested waters (Lukavský, 1983). Sladeček (1979), Marvan & Žáková (1981) and Žáková (1986) have defined the criteria for trophic state evaluation on the basis of AGP results for waters of Middle Europe. In all of the mentioned criteria, maximal values of algal biomass were used. According to that, trophic conditions in Nature Park Kopački rit and the Stara Drava (Horvatić & Lukavský, 1997; Horvatić et al. 2003b; Peršić et al., 2005) during the colder part of the year were mainly oligotrophic, and less frequently meso-mesoeutrophic.

Based on the concept of algal nutrient limitation, the algal assay is a responsive test designed to examine algal growth response to nutrient

enrichment (Miller et al., 1978; Downing et al., 1999). Nutrient enrichment bioassays are a useful indicator as to which nutrient has the potential or is most likely to limit phytoplankton growth at a particular time and space (Diaz & Pedrozo, 1996; Ault et al., 2000). Nutrients of primary concern are nitrogen and phosphorus compounds (Verhoeven et al., 2001; Wetzel, 2001). Since the growth rate of phytoplankton in eutrophic waters is usually limited by nitrogen and phosphorus (Olde Venterink et al., 2002), the addition of these nutrients causes a growth response of *C. kessleri* proportional to the magnitude of limitation of the particular nutrient. Accordingly, the interpretation of the degree of algal growth response to nutrient enrichment leads to a sharper definition of the concept of nutrient limitation by providing a quantifiable definition of nutrient limitation (Downing et al., 1999). Algal biomass and overall ecosystem productivity may be controlled by the type and intensity of nutrient limitation (Dodds et al., 2002). Therefore, the magnitude of nutrient limitation has implications for population dynamics, species interactions and ecosystem processes and thus many measures reported in published experiments can be converted to a single biologically meaningful measure of nutrient limitation that is comparable across studies (Downing et al., 1999; Osenberg et al., 1999).

Therefore, the aim of this investigation was to establish the optimal duration of *C. kessleri* cultivation for the evaluation of trophic conditions based on total biomass of *C. kessleri* in wetland water samples. In addition our goal was to establish nutrient limitation by bioassay method and its degree in experimental conditions.

Materials and methods

Study area

The Danubian flood area in northeast Croatia covers approx. 53,000 ha, of which the protected area of the Nature Park Kopački rit includes 23,000 ha. The investigated area was the Danube River (1426 r. km), its flooded area (1426–1388 r. km) and the Stara Drava. The largest, and the most important, is the wetland area of the Nature Park Kopački rit. The deepest, Lake Sakadaš is connected by the Čonakut Channel with Lake

Kopačko, which is connected with the Danube River by the Hulovo Channel. Depending on Danube floodings, the Stara Drava, a side arm of the Drava River, is hydrologically connected with Lake Sakadaš. North of Kopački rit are the Danubian wetlands into which water enters through side arms, of which the largest are Monjoroški Dunavac and Zmajevački Dunavac (Fig. 1).

Monthly sampling was carried out from May 31st to October 23rd 2003 at eight sites (Fig. 1) in the Danube region: Danube r. km 1426 (D); Danube sidearms: Zmajevački Dunavac-Danube r. km 1422 (d₁), Monjoroški Dunavac-Danube r. km 1413 (d₂); Nature Park Kopački rit (1388 rkm Danube): Čonakut Channel (d₅), Lake Sakadaš at dam Sakadaš (LS₁), Lake Sakadaš - central part

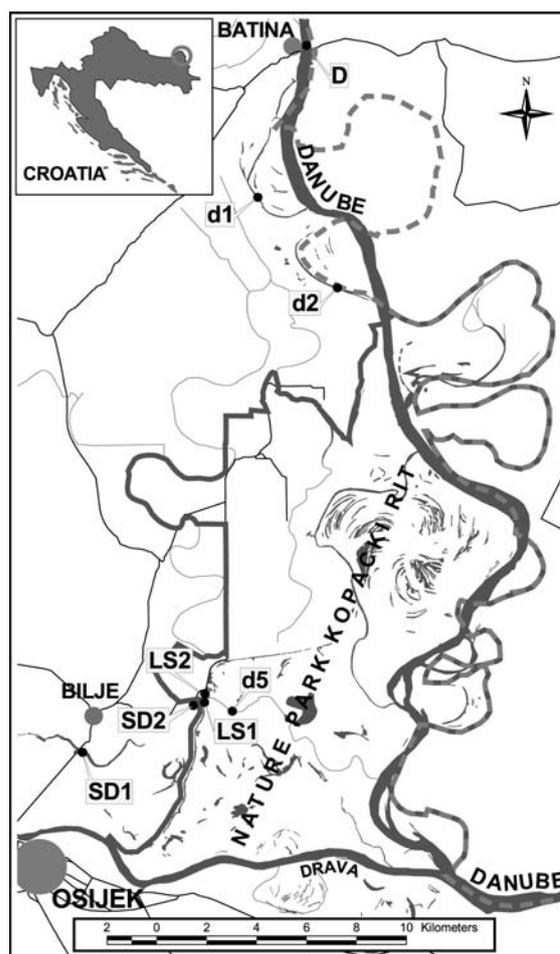


Figure 1. Location of the study site.

(LS₂); side arm of the Drava River (Stara Drava): at dam Bilje (SD₁), and at dam Sakadaš (SD₂).

Analytical methods

Physical parameters such as water temperature (WT), water depth (WD), Secchi-depth (SD) and pH were measured on site. Chemical analyses of water samples in the laboratory included dissolved oxygen (DO) and nutrient concentration such as total nitrogen (TN), ammonium (NH₄), nitrite (NO₂), nitrate (NO₃), total phosphorus (TP) and orthophosphate (PO₄) and were analyzed according to APHA (1985). Phytoplankton chlorophyll concentration (Chl *a*) was calculated according to Komárková (1989). TSI values were calculated for the investigated area using the equations described by Carlson (1977) and Kratzer & Brezonik (1981). The variables used for these equations included SD in meters, Chl *a* and TP in $\mu\text{g l}^{-1}$ and TN in mg l^{-1} .

Bioassay method

The algal growth potential (AGP) in the Danube wetland waters was evaluated in microplates with suspended culture of *Chlorella kessleri* FOTT et NOVAK. strain. LARG/1 by the laboratory miniaturized growth bioassay method according to Lukavský (1992). *C. kessleri* was supplied by Culture Collection Autotrophic Organisms at Třeboň, the Czech Republic. Algae were cultivated at the Department of Biology, J. J. Strossmayer University in Osijek on the BBM solid medium (Bischoff & Bold, 1963), exposed to irradiance with fluorescent tubes (Tungsram 30 W, F 74, daylight, Hungary) by PAR 138 $\mu\text{mol m}^{-2} \text{s}^{-1}$ measured with flat sensor and temperature 25–30 °C. Due to the prior uptake and possible storage of nutrients, it was necessary to starve *C. kessleri* cells before experimental use. Before inoculation the algal cells were washed out with sterile distilled water from the solid medium and subcultured for three days in sterile distilled water. The algal cell density in this solution was determined using a Bürker-Türk counting chamber (Karl Hecht KG, Sondheim, Germany) under a light microscope (Axiovert 25, Carl Zeiss, Inc., Göttingen, Germany). The inoculum solution, used in the experiment, was diluted with sterile

distilled water and the initial cell density of *C. kessleri* was $8 \times 10^5 \text{ cells cm}^{-3}$.

Water samples from the investigated sites were filtered and stored at –20 °C. After melting, the samples were filtered through the Whatman GF/C glass fiber filter to eliminate particles. Bioassay experiments were carried out in polystyrene 96-well microplates (Labsystem, Finland) with $9 \times 13 \text{ cm}$ flat bottom wells of 300 μl . The miniaturized growth bioassays were conducted with six replicates of original water samples (water sample from the investigated sites), six replicates of controls (water sample from the investigated sites diluted with distilled water, 1:1) and six replicates of enriched water samples (water samples with added N or P). Peripheral wells of microplate were filled with distilled water to reduce evaporation. Original water samples were used for evaluation of trophic conditions based on the algal growth potential (AGP) of *C. kessleri*. In nutrient enrichment bioassay the nutrient treatments were added to the water samples as nitrogen in KNO₃ in final concentration of 0.16 g l^{-1} (N) and phosphorus as K₂HPO₄ in final concentration of 0.02 g l^{-1} (P). High concentrations of nutrients ensured that enrichments would not be depleted during the experiment. The uncovered plates were exposed for three hours to UV light for sterilization. Each microplate well was filled with 200 μl of tested water samples and inoculated with 50 μl of algal inoculum solution. The microplates were then closed with lids and exposed in a glass incubation chamber to irradiance with fluorescent tubes (Tungsram 30 W, F 74, daylight, Hungary) by PAR 138 $\mu\text{mol m}^{-2} \text{s}^{-1}$, temperature 25–30 °C and CO₂ ca. 2 % (v/v). The growth of *C. kessleri* was determined by measuring the optical density at 750 nm every day, for 14 days (until the stationary phase of growth), using an automated microplate reader (Multiskan MS, Labsystem, Finland) controlled by GENESIS II software (Windows™ Based Microplate Software). Conversion of optical density at 750 nm to dry weight (mg/l) of *C. kessleri* was described in detail by Lukavský (1992):

$$\text{DW} = 3.31 + 179.45 * \text{Abs}_{750} + 617.45 * \text{Abs}_{750}^2 (\text{mg l}^{-1}).$$

The results were plotted as growth curves (biomass over time) and the total biomass of algae during

the experiment was calculated as the area between the growth curves and the horizontal line (ISO 8692, 1989):

$$B = \frac{B_1 - B_0}{2t_1} + \frac{B_1 + B_2 - 2B_0}{2(t_2 - t_1)} + \dots + \frac{B_{n-1} + B_n - 2B_0}{2(t_n - t_{n-1})}$$

The total biomass of *C. kessleri* was expressed as DW, mg/l (B) with the algal biomass (B_0) at the beginning of the experiment (t_0); algal biomass (B_1) at the first measurement (t_1) and algal biomass (B_n) at the n th measurement (t_n).

The average specific growth rate (μ) during *C. kessleri* exponential growth (up to the 7th day) was calculated according to ISO 8692 (1989): $\mu = \ln(B_n/B_0)/(t_n - t_0)$.

Variance analysis and t-test were used to determine statistically significant effects of added nutrients (N or P) to *C. kessleri* growth rate in comparison to the control growth rate. Linear correlation was based on 48 samples. A significance level of $\alpha = 0.05$ was used for all tests.

Degree of nutrient limitation (Δr) represents the degree of algal response to nutrient enrichment during exponential phase of growth (up to the 7th day), and is expressed as the difference in the growth rate of *C. kessleri* between enriched water samples (μ_E) and control water samples (μ_C) and is calculated according to Downing et al. (1999): $\Delta r = \mu_E - \mu_C$.

Results

Physical and chemical characteristics

The results of the physical and chemical characteristics of the investigated water and phytoplankton Chl *a* are displayed in Table 1. During the investigated period the water level of the Danube River varied from -0.15 m in July to 1.25 m in September 2003, indicating extremely dry conditions in Nature Park Kopački rit (Fig. 2).

The lowest Secchi-depth was marked at site SD₁ and the highest at site d₂. Secchi-depth at the investigated sites was in a statistically significant negative correlation with Chl *a* ($r = -0.6419$, $n = 48$,

$p < 0.05$). At all sites, from May to October 2003, a significant positive correlation was established between pH and ammonium ($r = 0.3628$), pH and Chl *a* ($r = 0.3874$) and pH and DO ($r = 0.4242$). The highest values of PO₄ were determined at sites SD₁ and SD₂ and the lowest at the site d₂. A significant positive correlation was established between PO₄ and Chl *a* ($r = 0.4830$) and a negative correlation between PO₄ and SD ($r = -0.5056$). Throughout the investigated period high NO₃ concentrations were characteristic only for the Danube River. The lowest NO₃ concentrations were measured at the site d₂. A significant positive correlation was established between NH₄ and DO ($r = 0.4372$), NH₄ and Chl *a* ($r = 0.6182$) and NH₄ and TN ($r = 0.3072$), while a significant negative correlation was established between DO and TP ($r = -0.4327$). According to the TN/TP ratio, in June, August and September all sites were nitrogen limited, while in May, July and October 58% of sites were phosphorus and 42% nitrogen limited (Fig. 3).

Values of Chl *a* were minimal at the site d₂, and maximal at the sites SD₁ and SD₂ and at all sites maximal values were established in August. A comparison of TSI values for Danube wetland waters (1388–1426 r. km) is shown in Fig. 4. At all investigated sites TSI_{TN} was significantly lower than TSI_{SD}, TSI_{Chl *a*} and TSI_{TP}. The values of TSI_{SD} varied from 57.8 to 86.4; TSI_{Chl *a*} from 55.6 to 79.7; TSI_{TP} from 79 to 86.9 and TSI_{TN} from 46.6 to 61.5 (Fig. 4).

Bioassay experiments

The algal growth potential in original water samples at investigated sites from May to October 2003 was observed up to the stationary phase of growth of *C. kessleri* which was most frequently established up to the 14th day of incubation. Obtained results of total biomass of *C. kessleri* (Fig. 5) were used for the evaluation of trophic conditions according to classification for AGP values (Table 2).

Values of total biomass of *C. kessleri* varied in 17% of the water samples from 28.19 to 45.12 mg l⁻¹; in 38% from 57.35 to 99.70 mg l⁻¹; in 29% from 108.20 to 198.86 mg l⁻¹ and in 17% from 201.26 to 318.90 mg l⁻¹ (Fig. 5). Based on these values and according to the classification for

Table 1. Mean, minimal and maximal values of physical characteristics of the water and Chl *a* (A) and chemical characteristics of the water (B) at the investigated sites during the period from May to October 2003

(A)	WT (°C)	WD (m)	SD (m)	DO (mg l ⁻¹)	pH	Chl- <i>a</i> (µg l ⁻¹)
Danube (D)	19.58	2.83	0.79	12.03	8.18	82.92
	10.0–24.5	1.15–3.77	0.56–1.15	6.42–20.44	7.85–8.51	6.38–142.26
Danube sidearm (d ₁)	20.0	2.50	0.62	9.66	7.80	81.48
	11.0–24.0	1.70–3.33	0.40–1.00	4.52–19.64	7.40–7.97	14.39–173.40
Danube sidearm (d ₂)	21.33	1.38	1.16	8.61	7.77	12.80
	12.0–25.0	0.80–1.90	0.72–1.57	4.15–17.97	6.79–8.11	10.19–20.42
Čonakut Channel (d ₃)	21.0	1.94	0.46	12.28	8.45	85.89
	9.0–27.0	1.45–2.70	0.31–0.62	6.60–20.42	8.06–8.99	26.00–165.07
Lake Sakadaš (LS ₂)	21.33	4.59	0.76	12.39	7.71	56.60
	10.0–27.0	4.05–5.90	0.44–1.42	7.90–19.66	7.03–8.74	13.42–103.53
Lake Sakadaš (LS ₁)	22.50	2.49	0.52	12.27	8.02	106.09
	10.0–28.0	1.78–3.61	0.26–0.93	4.26–23.15	7.58–8.45	36.89–142.47
Stara Drava (SD ₂)	22.50	0.86	0.35	11.51	8.34	149.18
	11.0–27.0	0.40–1.50	0.16–0.67	4.92–22.30	7.17–9.38	75.98–229.18
Stara Drava (SD ₁)	23.33	0.27	0.16	11.30	8.30	115.82
	12.0–28.0	0.20–0.30	0.10–0.30	6.47–17.08	7.85–8.54	28.60–187.69
(B)	NH ₄ (mg N l ⁻¹)	NO ₂ (mg N l ⁻¹)	NO ₃ (mg N l ⁻¹)	TN (mg N l ⁻¹)	TP (mg l ⁻¹)	PO ₄ (mg P l ⁻¹)
Danube (D)	0.21	0.02	1.46	1.63	0.26	0.07
	0.04–0.40	0.02–0.03	1.02–2.24	0.96–2.50	0.05–0.66	0.02–0.10
Danube sidearm (d ₁)	0.53	0.03	0.89	0.93	0.22	0.07
	0.06–1.47	0.00–0.06	0.39–1.66	0.41–1.55	0.08–0.59	0.03–0.15
Danube sidearm (d ₂)	0.24	0.02	0.52	0.58	0.18	0.03
	0.01–0.34	0.00–0.06	0.30–0.94	0.31–0.85	0.07–0.49	0.01–0.08
Čonakut Channel (d ₃)	0.80	0.02	0.92	0.78	0.30	0.13
	0.02–1.46	0.01–0.04	0.75–1.62	0.34–1.48	0.06–0.87	0.05–0.29
Lake Sakadaš (LS ₂)	0.63	0.02	0.86	0.85	0.31	0.07
	0.02–1.40	0.01–0.06	0.61–1.64	0.34–1.62	0.08–0.64	0.04–0.11
Lake Sakadaš (LS ₁)	0.61	0.03	0.79	0.94	0.24	0.10
	0.06–1.19	0.01–0.05	0.09–1.44	0.29–1.53	0.06–0.56	0.03–0.16
Stara Drava (SD ₂)	1.05	0.03	1.11	0.98	0.22	0.15
	0.41–2.41	0.01–0.05	0.80–1.34	0.37–1.75	0.05–0.48	0.04–0.28
Stara Drava (SD ₁)	0.70	0.02	0.98	1.07	0.22	0.10
	0.32–1.36	0.01–0.04	0.72–1.26	0.53–1.78	0.03–0.51	0.05–0.16

waters of Middle Europe (Marvan & Žáková, 1981; Sládeček, 1979; Žáková, 1986), Table 2, the trophic conditions in Danube wetland waters during the investigated period were determined in 55% of the water samples to be oligo-oligomesotrophic (17% oligotrophic, 38% oligomesotrophic) and in 46% of the water samples meso-mesoeutrophic (29% mesotrophic and 17% mesoeutrophic).

Comparing the results obtained by the chemical analysis of water *in situ* (Table 1) and the total biomass of *C. kessleri* up to 14th day of incubation,

in all investigated water samples a statistically significant correlation ($r = 0.34$) was established only between the NO₃ concentration and total biomass of *C. kessleri*.

Results of nutrient enrichment bioassay in the Danube wetland waters from May to October 2003 were summarized in Figures 6 and 7. The growth of *C. kessleri* was estimated as the average daily specific growth rate during the exponential phase of growth which lasted up to the 7th day. Through enrichment of investigated water samples

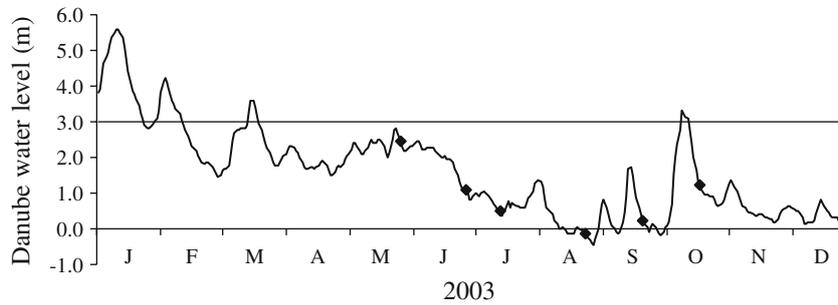


Figure 2. The Danube water level at Apatin, 1401 r. km. The horizontal line represents the effect of the borderline water level of the Danube River on the inflow and outflow of water in the Nature Park Kopački rit, and the points represent sampling time.

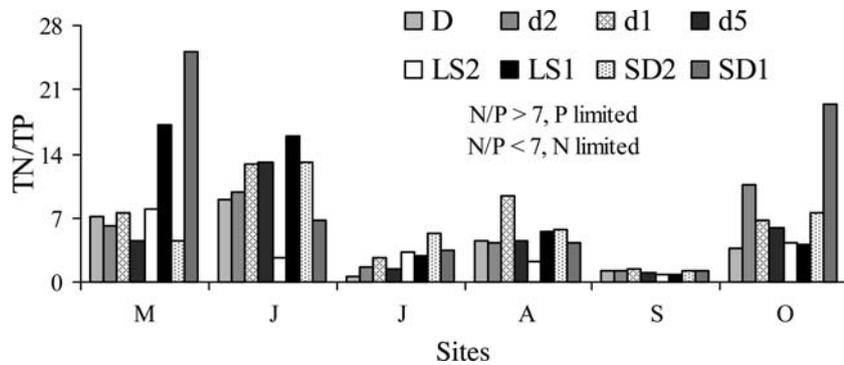


Figure 3. TN/TP ratio for Danube wetland waters (1388–1426 r. km) during the period from May to October 2003.

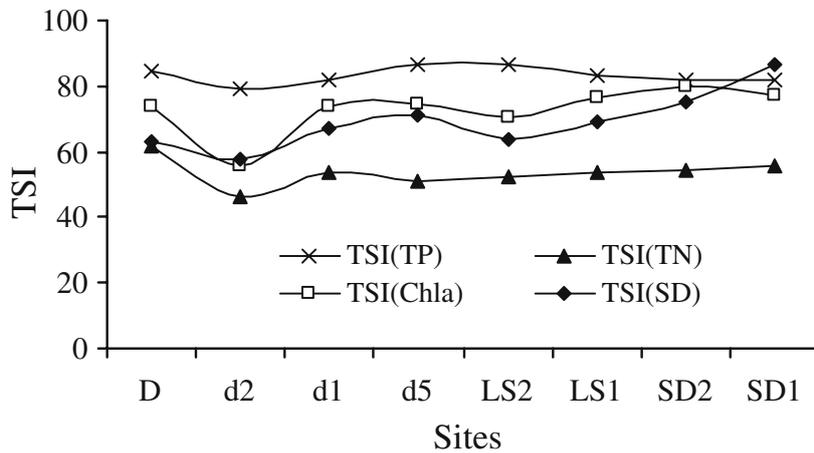


Figure 4. Comparison of TSI values (TSI_{SD} , TSI_{Chla} and TSI_{TP}) according to Carlson (1977) and TSI_{TN} according to Kratzer & Brezonik (1981) for Danube wetland waters (1388–1426 r. km) plotted as median values from May to October 2003.

with N, a significant difference was determined between the control samples and samples enriched with N at sites d_1 , SD_2 , LS_1 and LS_2 . A significant difference between the control samples and sam-

ples enriched with P was determined only at sites of Lake Sakadaš (LS_1 and LS_2), with the highest ($\Delta r = 0.474 d^{-1}$) degree of P limitation (Figs. 6a and 7a).

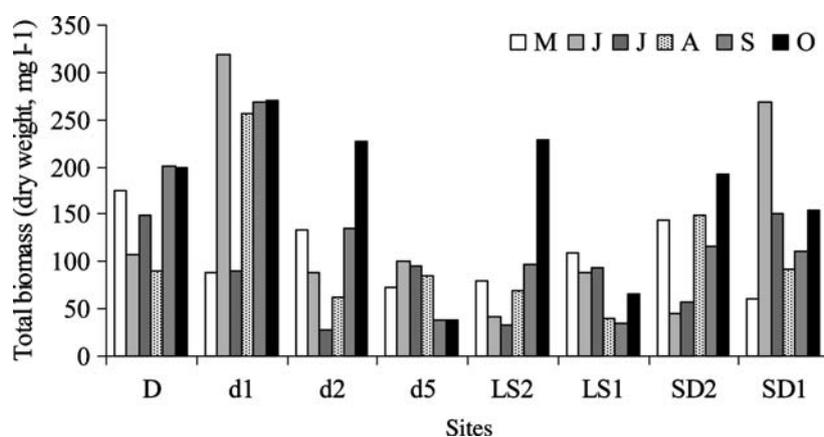


Figure 5. Values of total biomass of *C. kessleri* (dry weight, mg l^{-1}) up to the 14th day of incubation in original water samples at the investigated sites from May to October 2003.

Table 2. A comparison of Czech proposals of classification for AGP values (dry weight of *C. kessleri*, mg l^{-1})

	Ultraoligotrophy	Oligotrophy	Oligomesotrophy	Mesotrophy	Mesoeutrophy	Eutrophy	Polytropy	Hyper-trophy
S	25	<50		<100	<500	<3000		
M	<5	5–50		50–200	200–1000	500–1000	<1000	
Ž	<5	<50	<100	<200	<350	<500	<1000	<1000

S – Sládeček (1979); M – Marvan & Žáková (1981); Ž – Žáková (1986)

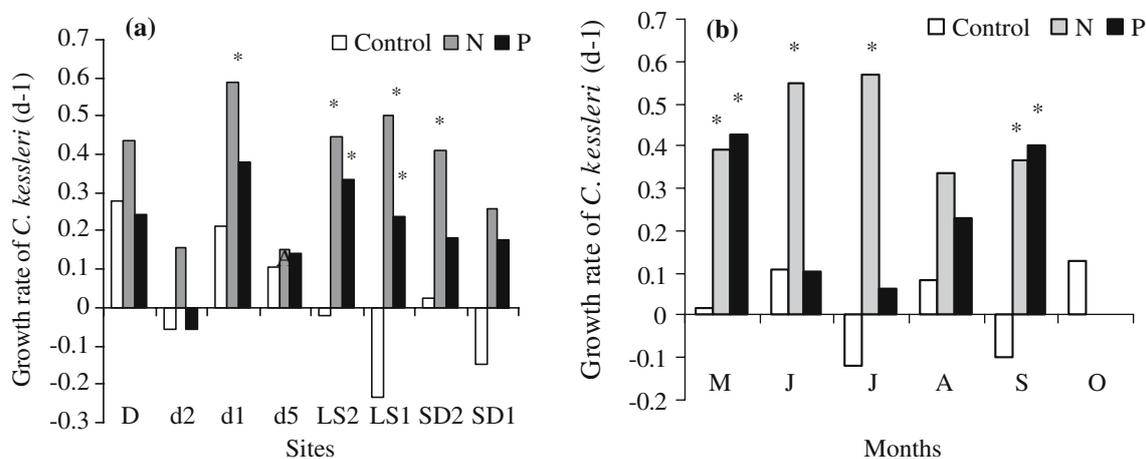


Figure 6. Average specific growth rate of *C. kessleri* (day^{-1}) during 7 days of incubation in the control water samples as well as samples enriched with N ($0.16 \text{ g l}^{-1} \text{ KNO}_3$) and P ($0.02 \text{ g l}^{-1} \text{ K}_2\text{HPO}_4$) at the investigated sites of Danube wetland waters (a), from May to October 2003 (b). * Statistically significant difference between average specific growth rate of *C. kessleri* in the control sample and samples enriched with N and P.

The most significant magnitude of N limitation was established at site LS_1 ($\Delta r = 0.736 \text{ d}^{-1}$), and the lowest at site d_1 ($\Delta r = 0.374 \text{ d}^{-1}$), Figure 7a. In June and July, only enrichment with N had a sig-

nificant effect on the growth rate of *C. kessleri* in the Danube wetland waters, with the degree of N limitation from 0.440 d^{-1} to 0.686 d^{-1} . In May and September both, N and P had a significant effect on

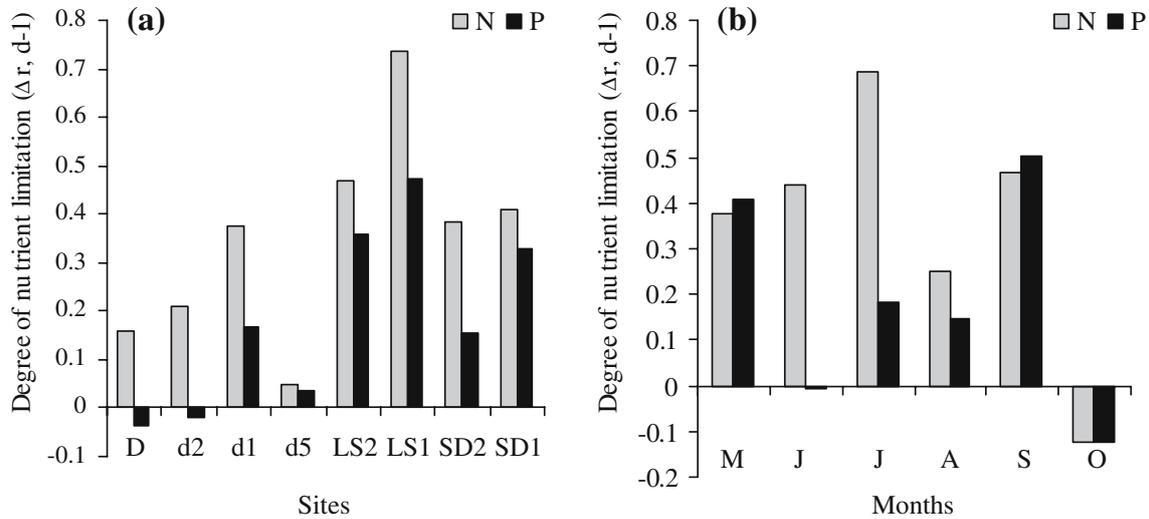


Figure 7. The effect of added nitrate and phosphate concentrations on the growth rate of *C. kessleri*, expressed as the degree of nutrient limitation ($\Delta r, d^{-1}$) during 7 days of incubation; (a) at the investigated sites and (b) during the investigated period.

the change in the *C. kessleri* growth rate, with a similar degree of limitation ($\Delta r_N = 0.375 d^{-1}$, $\Delta r_P = 0.409 d^{-1}$). In contrast, in August and October not one of the added nutrients had a significant effect on the growth rate of *C. kessleri* (Figs. 6b and 7b).

Discussion

The hydrology of natural nets of channels and lakes in Kopački rit depends on the flood dynamics of the Danube River, while the Drava regime influences it indirectly. When the water level of the Danube River near Apatin exceeds 3 m, the Danube water enters Kopački Rit (Mihaljević et al., 1999). A higher water level of the Danube River is characteristic of the spring season and the beginning of summer, while the beginning of autumn (September and October) as well as the entire winter is characterized by lower water levels. Because of the extremely low water level of the Danube River (Fig. 2) during the investigated period in 2003, there was no periodical flooding of the wetland area in Nature Park Kopački rit. The values of TSI_{SD} , TSI_{Chla} and TSI_{TP} in the Danube wetland waters ranged from eutrophic to hyper-eutrophic states (Carlson, 1977), and the values of TSI_{TN} indicated a mesotrophic state, except on site

D (Kratzer & Brezonik, 1981). Because all nutrients in the water are not in the form that can be used by algae (there may be substances that inhibit algal growth), bioassay provides a better indication of trophic conditions than can be determined by chemical measurement of nutrient concentration *in situ*. Therefore, the total biomass of *C. kessleri* in the waters of the investigated sites determined the maximum amount of algal growth that the nutrients in water samples can support during the 14 day period of the experiment. The trophic conditions based on the values of total biomass of *C. kessleri* (Fig. 5) and according to the classification for waters of Middle Europe (Table 2), in 55% of sites in Danube wetland waters were oligo-oligomesotrophic and in 46% of sites meso-mesoeutrophic.

A significantly lower TSI_{TN} index than TSI_{SD} , TSI_{Chla} and TSI_{TP} (Fig. 4), the total biomass of *C. kessleri* in the original water samples (Fig. 5) as well as the positive correlation between the NO_3 concentration *in situ* and the total biomass of *C. kessleri* indicated nitrogen limited conditions in the investigated waters. The trophic conditions based on the AGP results are determined by the limiting nutrient. Phytoplankton Chl_a is a response to the algal biomass *in situ* and the total biomass of *C. kessleri* in bioassay represents an algal response to the nutrient

concentrations of the investigated water samples in laboratory conditions. The results have shown that the total biomass of *C. kessleri* is a good parameter for potential algal growth in surface waters. It is not directly related to phytoplankton Chl_a , on the contrary, trophic conditions based on the algal growth potential were inversely related to $\text{TSI}_{\text{Chl}_a}$ ($r = -0.3812$). Therefore, both parameters should be considered as supplementary. To precisely determine which nutrient was limiting for the *C. kessleri* growth in the waters of the investigated area, water samples were enriched with nitrates and phosphates in excess. According to Downing et al. (1999), the definition of nutrient limitation in nutrient enrichment bioassay assumes that nutrients were added in excess. Enrichment with low concentrations of N and P individually did not achieve significant growth of *C. kessleri* (Horvatić et al., 2000). The primary production of fresh water systems is mainly phosphorous limited, while nitrogen is less often reported as a limiting nutrient (Scheffer, 2001). However, Wetzel (2001) suggested that nitrogen, rather than phosphorus, is more likely to be a limiting nutrient for phytoplankton growth in fresh water systems. In nutrient enrichment bioassay the response of the growth rate of *C. kessleri* to added N and P indicated N limitation at 25% of the sites, 13% of the sites were limited with P, and 62% of the sites were limited neither by N nor P. In May and September, the growth of *C. kessleri* biomass after the addition of N and P was significant and similar (Fig. 6b), meaning that both N and P limited growth of *C. kessleri* and the determined similar magnitude of N and P limitation (Fig. 7b) suggested possible co-limitation. An increase in N limitation from June to July could be explained with abundant development in phytoplankton communities (Horvatić, 1989; Horvatić & Gucunski, 1997; Horvatić et al., 2003a). In August, nutrient enrichment indicated that N and P did not limit the growth of *C. kessleri* during bioassay experiments. However, the TN/TP ratio indicated N limitation at majority of the sites. The high trophic state according to the values of TSI_{SD} , $\text{TSI}_{\text{Chl}_a}$ and TSI_{TP} (Carlson, 1977) indicated that algae dominate light attenuation and that the investigated area is probably phosphorus limited. This is in contrast to the high PO_4

concentrations determined *in situ* (Table 1). Stanley (2003) explains high phosphorus concentrations in forest wetlands as a consequence of leaf and wood debris, where light, together with nitrogen limits algal growth. Established alkaline values of pH and low values of DO in shallow wetland waters resulted in high NH_3 concentrations as well as in the internal loading of phosphorus (Kisand & Nõges, 2003; Kinnear & Garnett, 1999; Søndergaard et al., 2003). Since algae do not tend to assimilate other inorganic sources of nitrogen in conditions of ammonium availability, significant nitrate utilization can be expected to occur only if the ammonium becomes depleted (Von Ruckert & Giani, 2004). According to Ault et al. (2000), ammonium is the preferred nitrogen source for many species and uptake of nitrate is suppressed once the concentration of ammonium exceeds $1\text{--}2 \mu\text{M NH}_4$. Accordingly, high concentrations of ammonium could be responsible for the lower degree of nitrogen limitation in August 2003. The positive correlation determined between NH_3 concentration and phytoplankton Chl_a *in situ* ($r = 0.6182$, $n = 48$, $p < 0.05$) indicated that there were sufficient nutrients present to support algal growth and none of the analyzed nutrients was limiting growth. In September, the TN/TP ratio indicated nitrogen limitation at all sites, and the nutrient enrichment bioassay indicated N and P limitation of similar magnitude suggesting possible co-limitation. Nutrient enrichment of water samples in October showed that none of the nutrients were limiting the growth of *C. kessleri*, suggesting that sufficient nutrients were present to support algal growth at all sites. This is confirmed by the highest values of the trophic level in algal growth potential bioassay (Fig. 5). According to Olde Venternik et al. (2002), nitrate concentration decreases when the water inflow in the wetland area is interrupted. Thus, we assume that the abundance of nutrients in the water in October was the consequence of the new flooding of Nature Park Kopački rit (Fig. 2). The indirect influence of flooding was the resuspension of wetland sediment, due to which the internal loading of phosphorous increased (Kisand & Nõges, 2003). Since the nutrient concentration in the Danube wetland waters in great part depends on flood dynamics, further investigations should

be aimed at the comparison of trophic conditions and nutrient limitation during the usual flooding dynamics. These results would be the basis for trophic valorization of Danube wetland waters (1388–1426 r. km) and other similar wetland areas.

Conclusions

According to the results of the AGP of *C. kessleri* in Danube wetland waters optimal duration of *C. kessleri* cultivation for the evaluation of trophic conditions was up to the 14th day. The trophic conditions based on the total biomass of *C. kessleri* were at 55% of the investigated sites oligo-mesotrophic and meso-eutrophic at 46%, which corresponds to the trophic condition based on TSI_{TN}. The correlation between the AGP of *C. kessleri* and the NO₃ concentration *in situ* indicated possible N limitation. The TN/TP ratio and lower TSI_{TN} than TSI_{SD}, TSI_{Chla} and TSI_{TP} also indicated N limitation. Based on the results of nutrient enrichment, the growth of *C. kessleri* in the Danube wetland waters was limited in June and July only with N, in May and September with both N and P and in August and October 2003 with neither N nor P. The highest magnitude of N and P limitation was established at the Lake Sakadaš. According to all results, the bioassay method is suitable for the evaluation of trophic conditions and nutrient limitation.

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Chapter 2

Changes in N and P limitation
induced by water level
fluctuations in Nature Park
Kopački Rit (Croatia): nutrient
enrichment bioassay

Changes in N and P limitation induced by water level fluctuations in Nature Park Kopački Rit (Croatia): nutrient enrichment bioassay

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Abstract The importance of nutrients as limiting factors might vary in environments with different limnological characteristics. In this article we evaluate the effect of frequency and duration of flooding on nutrient limitation in a riparian floodplain. Variations in N and P limitations were studied in the period of low (2003) and high (2004) water level in two different floodplain habitat types in the Nature Park Kopački Rit (Croatia), a floodplain area of the Danube River. In 2003 and 2004, the limnological characteristics of floodplain lake (Lake Sakadaš) and the channel (Stara Drava) differed due to their hydrological regimes. Potential for nutrient limitation was determined by DIN:TP and TN:TP ratios, while the actual nutrient limitation was assessed by nutrient enrichment bioassay. A change from non-limited to N-limited conditions in the channel, and consistent actual N limitation in the lake was determined by the nutrient enrichment experiment. Of the two ratios, DIN:TP matched better with the bioassay data. Phosphorus limitation was only occasionally evident. Changes in trophic conditions from hypereutrophy to eutrophy (between low and high water levels)

reflected the importance of the hydrologic regime as a factor which can modify the trophic state of Lake Sakadaš. Compared with 2003, the increase of total nitrogen concentrations in 2004 calls attention to the importance of nitrogen inputs from the Danube to the system with excessive phosphorus concentrations.

Keywords *Chlorella kessleri* · Chlorophyll *a* · Danube water level · Lake Sakadaš · Stara Drava Channel · N limitation

Introduction

Hydrologic fluctuations coupled with periodicity, water chemistry and community structure are the key elements affecting habitat and species diversity in river–floodplain systems (Schemel et al. 2004), as the floodplains of Nature Park Kopački Rit, Croatia. The typology of floodplain water bodies is primarily based on the hydrological regime. Depending on the Danube water level, the hydrological regime within Nature Park Kopački Rit switches between two phases: the limnophase, when the floodplain remains isolated from the river and lentic conditions prevail, and the potamophase, a flood period characterised by more lotic conditions (Mihaljević et al. 1999; Palijan and Fuks 2006). The flood-pulse concept proposed by Junk et al. (1989) emphasises the importance of an alteration between periods of flooding and drought in the aquatic floodplain ecosystem. The fluctuating

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hydrological connectivity of the Danube and its floodplain induced changes in water chemistry and phytoplankton chlorophyll *a* (Chl *a*) of the Danube wetland waters (Horvatić et al. 2006). Dokulil and Mayer (1996) reported that nutrients are never limiting factors for phytoplankton Chl *a* concentrations of the Danube in Austria. This finding can also be applied to an even more nutrient rich stretch of the Danube in Hungary (Kiss et al. 1996).

In freshwaters, phosphorus (P) has been regarded as the most probable limiting nutrient for phytoplankton growth (Hecky and Kilham 1988; Wetzel 2001) but co-limitation by P and nitrogen (N) is not uncommon (Elser et al. 1990; Jansson et al. 1996). The identification of limiting nutrients requires multiple assessment methods including the bioassay approach (Ojala et al. 2003; Camacho et al. 2003). Although nutrient limitation of phytoplankton is common in aquatic ecosystems, it is difficult to quantify it. Evaluating the growth response of algae after nutrient additions in laboratory conditions makes quantification of nutrient limitation possible. The addition of a limiting nutrient causes an immediate growth response of the tested algae proportional to the intensity of limitation of that particular nutrient. These results reflect algal growth responses to increased nutrient availability under specific experimental conditions. They are comparable with in situ nutrient limitation of algal growth in the absence of other limiting factors (Elser and Kimmel 1986). Therefore, the interpretation of the degree of algal growth response provides a quantifiable measure of nutrient limitation that is comparable across studies (Downing et al. 1999).

Although the presence of a periodic flood pulse is a key factor in maintaining a healthy river ecosystem, flooding has a destabilising effect on nutrient dynamics and trophic interactions in floodplain wetlands (Junk and Wantzen 2004). In previous studies, an increase in transparency and a decrease in nutrient concentrations were reported along a transversal axis from the main stream of the Danube towards the floodplain (Peršić et al. 2005; Horvatić et al. 2006). The main objective of this investigation was to obtain a better understanding of how water level fluctuations affect nutrient limitation among different floodplain habitat types. We hypothesised that the relative importance of nutrients as limiting factors might vary in environments with different limnological

characteristics. To attain the objective we assessed nutrient limitation by determining nutrient conditions in situ and by miniaturised bioassay method applied in laboratory conditions. Also, variation in N and P limitation was investigated in different hydrological conditions (high versus low water periods).

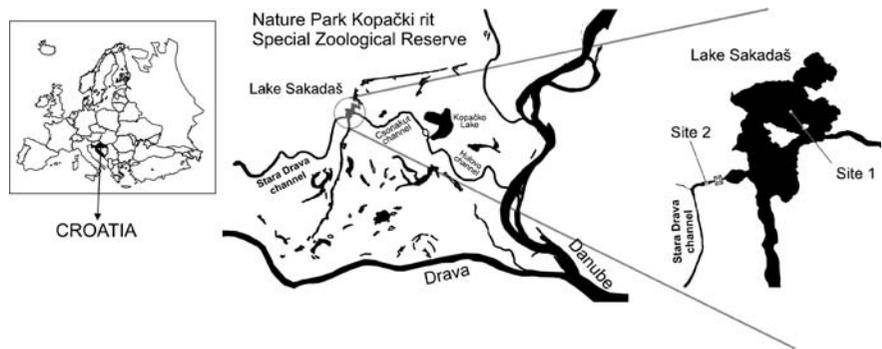
Materials and methods

Study area

Study sites are located in the Nature Park Kopački Rit (Special Zoological Reserve), a floodplain area of the Danube (between 1,410 and 1,383 r. km, with a surface of ca. 100 km², Fig. 1). Kopački Rit is situated in eastern Croatia at the confluence of the Danube and Drava rivers. The complex structure of the Kopački Rit floodplains consists of big depressions permanently under water (lakes), occasionally flooded areas (sloughs) and recesses with flowing water (backwater channels). The hydrology of its natural networks of channels and lakes directly depends on the water level fluctuations of the River Danube, while the Drava regime has an indirect influence. According to hydrological records, the high water period is usually in spring and at the beginning of summer, while the low water period begins by late summer, reaching minimum levels in autumn (September–October) and winter (Mihaljević et al. 1999).

Both investigated sites are located in the marginal part of the Kopački Rit floodplain (some 10 km away from the main Danube flow) and bordered by the Drava–Danube flood-dyke from the west (Fig. 1). The first site is Lake Sakadaš (Site 1), the deepest of the lakes (mean depth, 5 m; maximum depth, 7 m; surface area: 0.12 km²) with relatively steep slopes. The lake was formed during the flooding of the Danube in 1926, which changed the configuration of the Kopački Rit floodplain. The second site is the Stara Drava channel (Site 2), a natural backwater channel with abundant littoral macrophyte vegetation and a very slow surface water velocity with a longitudinal flow that may cease during dry seasons. During the study period, the mean water depth (WD) of the Stara Drava channel at the sampling site was 1.5 m and the maximum depth was 3 m. The water level of the Stara Drava channel during the drought period is under a greater influence of precipitation

Fig. 1 Location of the study site in Nature Park Kopački Rit (Site 1—Lake Sakadaš, Site 2—Stara Drava channel)



and evapotranspiration than of fluctuations in the water level of the Danube and Drava rivers. This is because the channel is situated on the right side of the Drava–Danube flood-dyke and is hydrologically connected with Lake Sakadaš through the dam. We presume that both investigated sites represent different floodplain habitat types.

Analytical methods

Water temperature (WT), WD, Secchi-depth (SD) and pH measurements and sampling for chemical and phytoplankton Chl *a* analyses were performed at monthly intervals from May to October, 2003 and 2004. Surface water samples were collected from the centre of the lake, and near the dam at the Stara Drava channel, also from the centre. Dissolved oxygen (DO) was determined by the Winkler method. Samples for chemical analysis and Chl *a* were collected in plastic bottles previously rinsed with sampling water, stored in containers with ice and analysed the same day. Chemical analysis of nutrient concentrations ammonium (NH₄), nitrate (NO₃), total nitrogen (TN), orthophosphate (PO₄) and total phosphorus (TP) followed standard methods (APHA 1985). To determine Chl *a* concentration, water samples were filtered through Whatman GF/C filters, extracted with acetone and measured spectrophotometrically. Phytoplankton Chl *a* concentration was calculated according to Komárková (1989).

Trophic State Indices of transparency (TSI_{SD}), total phosphorus (TSI_{TP}) and phytoplankton chlorophyll *a* (TSI_{Chl_a}) were calculated using the equations described by Carlson (1977). TSI values of total nitrogen (TSI_{TN}) were calculated according to Kratzer and Brezonik (1981).

Relationships among physical and chemical parameters of investigated sites were determined by correlation analysis. To discern the possible interaction between year (water regime) and site (habitat), based on physical and chemical parameters, the two-way analysis of variance (two-way ANOVA) was applied. Tukey's honestly significant difference test was used for post hoc comparisons. All data except pH were log transformed.

Bioassay method

The algal growth potential (AGP) in the Danube wetland waters was evaluated in microplates with suspended culture of *Chlorella kessleri* FOTT et NOVAK, strain LARG/1 by the laboratory miniaturised growth bioassay method according to Lukavský (1992) and Horvatić et al. (2006). *Chlorella kessleri* was supplied by Culture Collection Autotrophic Organisms at Třeboň, the Czech Republic. Algae were cultivated at the Department of Biology, J. J. Strossmayer University, Osijek, Croatia in the Bolds basal medium (BBM) (Bischoff and Bold 1963). Cultures were incubated in a 25°C controlled temperature room and illuminated by fluorescent tubes (Tungsram, Hungary) that provided continuous white light (PAR, 400–700 nm) at approximately 138 μmol photon m⁻² s⁻¹. Due to the prior uptake and possible storage of nutrients, it was necessary to starve *C. kessleri* cells before experimental use. Before inoculation, the algal cells were washed using the medium with sterile distilled water in which they were subcultured for 3–5 days. The algal cell density in this solution was determined using a Bürker-Türk counting chamber (Karl Hecht KG, Sondheim, Germany) under a light microscope (Axiovert 25,

Carl Zeiss, Inc., Göttingen, Germany). The inoculum solution, used in the experiment, was diluted with sterile distilled water and the initial cell density of *C. kessleri* was 8×10^5 cells ml^{-1} .

Water samples from the investigated sites were filtered and stored at -20°C . After melting, the samples were filtered through the Whatman GF/C glass fibre filter to eliminate particles. Bioassay experiments were carried out in polystyrene 96-well microplates (TPP, Switzerland) with 9×13 cm flat bottom wells of $300 \mu\text{l}$. In nutrient enrichment bioassay, the nutrient treatments were added to the water samples: N as KNO_3 in final concentration of 0.16 g l^{-1} (N) and P as K_2HPO_4 in final concentration of 0.02 g l^{-1} (P). High concentrations of nutrients ensured that enrichments would not be depleted during the experiment (Horvatić et al. 2006). In addition, the growth rate definition of limitation assumes that nutrients should be added in great excess, above saturating levels, since nutrient enrichment experiments measure how far phytoplankton is from nutrient-saturated growth (Downing et al. 1999). Each microplate well was filled with $200 \mu\text{l}$ of tested water samples. The uncovered plates were exposed for three hours to UV light for sterilisation and inoculated with $50 \mu\text{l}$ of algal inoculum solution. The microplates were then closed with lids and exposed in a glass incubation chamber to the cultivation conditions.

The growth of *C. kessleri* was determined by measuring the optical density at 750 nm every day, for 14 days (until the stationary phase of growth), using an automated microplate reader (Multiskan MS, Labsystem, Finland) controlled by GENESIS II software (WindowsTM Based Microplate Software). Conversion of optical density at 750 nm to dry weight (mg l^{-1}) of *C. kessleri* was described in detail by Lukavský (1992) and Horvatić et al. (2006). The results were plotted as growth curves (biomass over time), and during the experiment, the total biomass of algae was calculated as the area between the growth curves and the horizontal line. The total biomass of *C. kessleri* was used for evaluation of trophic conditions according to the classification for waters of Middle Europe (Žakova 1986).

The average specific growth rate ($\mu \text{ day}^{-1}$) for *C. kessleri* during its exponential growth period (for 7 days) was calculated according to ISO 8692 (1989): $\mu = \ln(A_n/A_0)/(t_n - t_0)$, where A_0 and A_n were

absorbencies at 750 nm at the start of the test (t_0) and after n days (t_n). The difference in the growth rate of *C. kessleri* in the enriched (μ_E) and control water samples (μ_C) is expressed as the degree of algal response to nutrient enrichment ($\Delta\mu$) and is calculated according to Osenberg et al. (1999) and Downing et al. (1999): $\Delta\mu = \mu_E - \mu_C$.

Variance analysis and Dunnett test were used to determine the statistically significant effects of nutrient enrichment by N or P on the *C. kessleri* growth rate. If the growth rate from a single nutrient addition was found to be statistically greater than the growth rate for the control, the results were taken to indicate limitation by that particular nutrient. A significance level of $\alpha = 0.05$ was used for all tests.

Results

Physical and chemical characteristics

During the period of investigation, the Danube water level at 1404.1 r. km varied from -0.46 m in September 2003 to 3.3 m in October 2003 (mean 1.18 m , $\text{CV} = 81.67\%$), and from 0.39 m in September 2004 to 4.88 m in July 2004 (mean 2.41 m , $\text{CV} = 48.55\%$, Fig. 2). The Danube water level was significantly higher in 2004 than in 2003 (t test: $t = -6.92$, $\text{df} = 728$, $P < 0.0001$). The inflow/outflow boundary level of the Danube waters into the Nature Park Kopački Rit was 2.5 m . Two hydrological phases were distinguished according to this inflow/outflow boundary level. From June to September 2003, Lake Sakadaš and the Stara Drava channel were completely isolated from the Danube. Flood pulses in 2003 occurred (maximal Danube water level $> 2.5 \text{ m}$) in May (duration of 4 days, amplitude 0.3 m) and in October (duration of 6 days, amplitude 0.7 m). In our study, amplitude presented the difference between the maximal Danube water level and the specified threshold level when flooding occurs. During the investigated period in 2004, a flood phase was established in May, June, July and in the beginning of August (duration of 90 days, amplitude 2.4 m) while a short flood pulse occurred at the end of September (duration of 3 days, amplitude 0.1 m).

Significant difference (two-way ANOVA; $F_{12,9} = 9.84$, $P = 0.0009$) was found for: Chl *a* concentration (Tukey HSD; $P = 0.04$), WD (Tukey

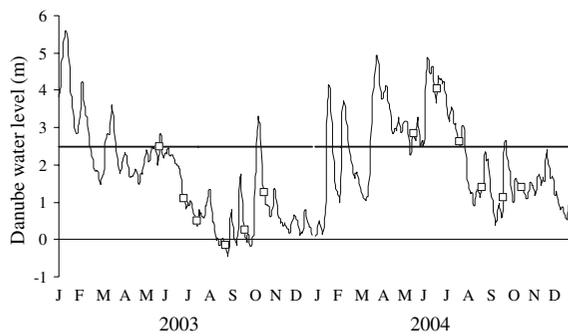


Fig. 2 Danube water level (1404.1 r. km) during 2003 and 2004. Horizontal line represents the inflow/outflow boundary level of the Danube waters into the Nature Park Kopački Rit, and the points represent sampling time

HSD; $P = 0.0002$), SD (Tukey HSD; $P = 0.03$) and PO_4 concentration (Tukey HSD; $P = 0.01$) between two investigated sites (Table 1). Significant changes (two-way ANOVA; $F_{12,9} = 3.92$, $P = 0.02$) between the years were determined in WD (Tukey HSD; $P = 0.0005$), SD (Tukey HSD; $P = 0.02$), DO (Tukey HSD; $P = 0.04$), NH_4 (Tukey HSD; $P = 0.003$) and TN (Tukey HSD; $P = 0.02$) concentrations and TN:TP ratio (Tukey HSD; $P = 0.007$). The interaction between the investigated sites and years was not significant.

At both sites, the concentration of phytoplankton Chl *a* was negatively correlated with WD (for the

Stara Drava channel $r = -0.71$ ($P = 0.009$) and for Lake Sakadaš $r = -0.59$, $P = 0.04$) and SD (for the Stara Drava channel $r = -0.76$ ($P = 0.004$) and for Lake Sakadaš $r = -0.85$, $P = 0.001$). At the Stara Drava channel, Chl *a* was positively correlated with NH_4 ($r = 0.72$, $P = 0.009$). Higher values of PO_4 from May to August 2003 (low water level), than in the same period in 2004 (high water level) at Lake Sakadaš, coincided with higher values of Chl *a* concentrations (Table 1). Moreover, a positive correlation between phytoplankton Chl *a* and PO_4 concentrations ($r = 0.78$, $P = 0.003$) was observed. A significant positive correlation was found between the Danube water level and the TN:TP ratio ($r = 0.706$, $P = 0.01$) in Lake Sakadaš.

The difference between the years in TSI indices was found for TSI_{SD} (two-way ANOVA, $F_{1,20} = 6.46$, $P = 0.02$) and TSI_{TN} (two-way ANOVA, $F_{1,20} = 6.85$, $P = 0.02$), and between sites only in TSI_{SD} (two-way ANOVA, $F_{1,20} = 5.39$, $P = 0.03$) (Fig. 3).

Bioassay experiments

The AGP in original water samples from the Stara Drava channel and Lake Sakadaš was investigated up to the stationary phase of *C. kessleri* growth, which

Table 1 Physical and chemical characteristics of water at the investigated sites from May to October 2003 and in the same period 2004

	Lake Sakadaš		Stara Drava channel	
	2003	2004	2003	2004
Water temperature (°C)	22.5 ± 6.7	19.8 ± 2.4	22.5 ± 6.2	20.3 ± 3.5
Water depth (m)	4.6 ± 0.7	5.6 ± 0.8	0.9 ± 0.5	2.2 ± 0.6
Secchi depth (m)	0.8 ± 0.4	1.1 ± 0.8	0.4 ± 0.2	0.8 ± 0.4
Oxygen saturation (%)	145.2 ± 60.6	103 ± 52.3	122.6 ± 73.6	66 ± 36.8
pH	7.9 ± 0.6	7.6 ± 0.4	8.3 ± 0.6	7.4 ± 0.6
NH_4 ($\mu\text{g l}^{-1}$)	497 ± 340	203 ± 364	936 ± 735	229 ± 375
NO_3 ($\mu\text{g l}^{-1}$)	885 ± 376	815 ± 445	1,114 ± 221	651 ± 548
PO_4 ($\mu\text{g l}^{-1}$)	75 ± 26	91 ± 148	143 ± 94	310 ± 302
TN ($\mu\text{g l}^{-1}$)	829 ± 438	1,200 ± 240	967 ± 569	1,291 ± 166
TP ($\mu\text{g l}^{-1}$)	340 ± 241	132 ± 28	206 ± 154	201 ± 96
TN/TP	3.6 ± 2.4	9.5 ± 2.8	6.3 ± 3.9	7.4 ± 2.7
DIN/TP	7.6 ± 8.2	7.2 ± 3.9	15.3 ± 11.3	4.6 ± 2.6
Chl <i>a</i> ($\mu\text{g l}^{-1}$)	57 ± 33.4	70.3 ± 61	149.2 ± 60	83.3 ± 57.8

Values are the mean ± standard deviation, $n = 6$ for each year and each site

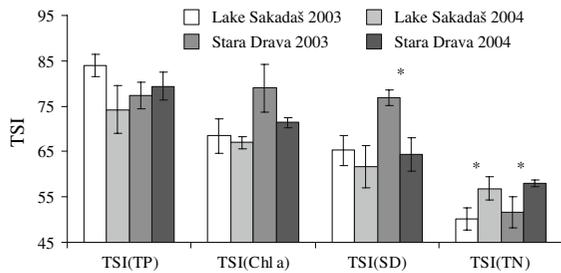


Fig. 3 Mean values of trophic state indices for transparency (TSI_{SD}), total phosphorus (TSI_{TP}), phytoplankton chlorophyll *a* ($TSI_{Chl a}$) and total nitrogen (TSI_{TN}) at the Stara Drava channel and Lake Sakadaš in 2003 and 2004. Error bars represent standard error of the mean. *Statistically significant differences between TSI indices

was usually established by the 14th day of incubation. The total biomass of *C. kessleri* in experiments using the Stara Drava channel water ranged from 63.6 to 192.9 mg l⁻¹ in 2003 (mean 121.9 mg l⁻¹), while in 2004 it ranged from 43.3 to 361.8 mg l⁻¹ (mean 204 mg l⁻¹). In experiments using Lake Sakadaš water, the total biomass of *C. kessleri* ranged from 30.7 to 228.1 mg l⁻¹ in 2003 (mean 91.2 mg l⁻¹), while in 2004 it ranged from 73.7 to 445.9 mg l⁻¹ (mean 226.2 mg l⁻¹). For both sites, the mean values of the total biomass were higher in 2004 (one-way ANOVA, $F_{1,22} = 5.06$, $P = 0.03$).

In the water samples from Lake Sakadaš, a significant response of the *C. kessleri* growth rate to N enrichment was determined during both years, with the exception of May and October 2003 (Fig. 4a). The maximal degree of N limitation (Fig. 5a) was determined in September 2003 ($\Delta\mu = 0.39$ d⁻¹) and in June 2004 ($\Delta\mu = 0.64$ d⁻¹). A significant response of the *C. kessleri* growth rate to P enrichment was determined only in August in both 2003 and 2004, with a higher degree of P limitation ($\Delta\mu = 0.72$ d⁻¹) in 2003 than in 2004 ($\Delta\mu = 0.19$ d⁻¹). During the investigated period of 2003, addition of N to the samples of the Stara Drava channel caused a significant increase in the *C. kessleri* growth rate compared to the *C. kessleri* growth rate in the original water samples only in June (Fig. 4b), with the degree of N limitation $\Delta\mu = 0.36$ d⁻¹ (Fig. 5b). However, in 2004 there was a significant increase in the *C. kessleri* growth rate in N enriched samples of the Stara Drava from June to October, with the maximal degree of N limitation ($\Delta\mu = 0.59$ d⁻¹) in June.

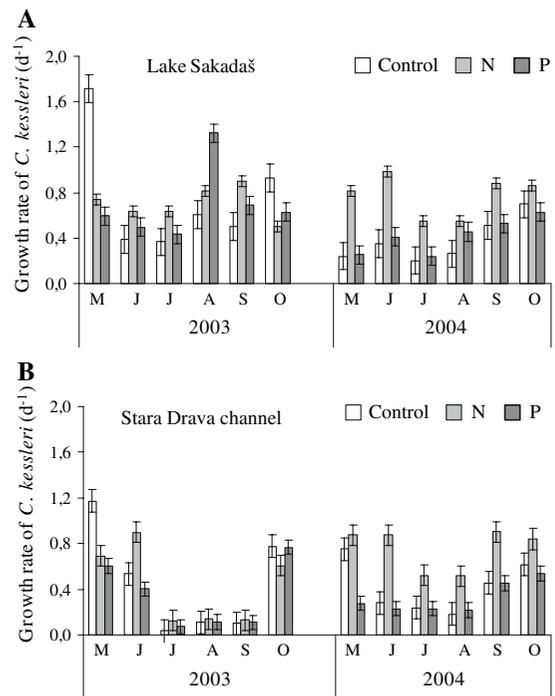


Fig. 4 The average specific growth rate of *C. kessleri* (day⁻¹) in the control water samples (water samples without added nutrients) as well as samples enriched with N and P from Lake Sakadaš (a) and Stara Drava channel (b). Error bars represent standard error of the mean

Discussion

As the hydrological regime within the Nature Park Kopački Rit depends on the Danube water level, we compared variations in N and P limitation during the two study periods with contrasting hydrological characteristics. The first period was from May to October 2003, characterised by low waters (more lentic conditions) and the second was the same period in 2004, characterised by high waters (more lotic conditions). According to Mihaljević et al. (1999), when the water level of the Danube near Apatin exceeds +3 m, the Danube waters enter the Kopački Rit floodplain. We have compared our results with a lower threshold, according to which flooding starts when the Danube water level gauge at Apatin exceeds +2.5 m, as suggested by Palijan and Fuks (2006). Fluctuations of the Danube water level significantly influenced the physical and chemical characteristics of the investigated floodplain lake and channel waters. As the water discharge level increased, dilution of water led to a decrease in

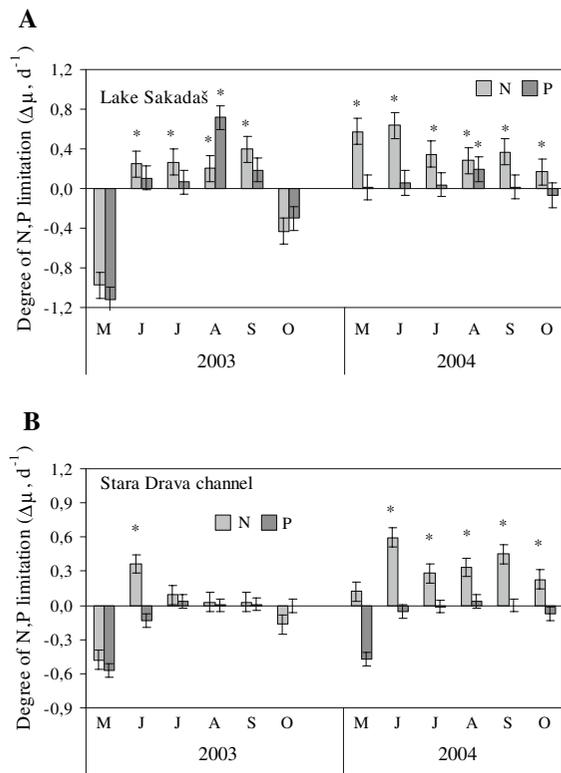


Fig. 5 Algal response to added N and P concentrations expressed as the degree of nutrient limitation ($\Delta\mu, d^{-1}$) during 7 days of incubation in water samples from investigated sites Lake Sakadaš (a) and Stara Drava channel (b) during the period of investigation. Error bars represent standard error of the mean. *Statistically significant effect of N or P addition

several dissolved nutrient concentrations, particularly those of NH_4 and NO_3 . This was likely due to low concentrations of the same nutrients in the source waters as well as physical processes such as short water residence time. Therefore, higher flushing rates in the Stara Drava channel associated with flooding and short water residence time were considered responsible for low phytoplankton biomass in 2004. According to Schemel et al. (2004), the inundation period in a large floodplain of the Sacramento River, USA, reflected on the low concentrations of several dissolved nutrients and phytoplankton biomass. In Lake Sakadaš, the phytoplankton biomass was lower in the year with less hydrologic variation. Nutrient enrichment and an increase in phytoplankton biomass during flooding were also reported in other floodplains and shallow lakes (Tockner et al. 1999; Zalocar de Domitrovic 2003; Hein et al. 2004; Rennella and Quiros 2006).

The N:P ratios can be used to estimate the relative importance of the most frequent limiting nutrients. According to Dzialowski et al. (2005), TN:TP ratios reflect only the potential for nutrient limitation, while actual limitation should be determined by concentrations of available dissolved inorganic N and P. However, the ratios of inorganic N to P are not reliable indicators of nutrient availability (Dodds 2003). Therefore, according to Dodds (2006), when possible, total N:total P should be used to characterise stoichiometry of nutrient loading; alternatively, more reliable estimate of nutrient limitation can be derived from the dissolved inorganic N:total P ratios. A correlation found between the Danube water level and the TN:TP ratio in Lake Sakadaš implies that water level fluctuations are most likely the largest single disturbance affecting nutrient dynamics in the Danube wetland waters. Also, the two-way analysis of variance revealed that changes in N:P ratios resulted from an independent effect of inter-annual variability in the water level. In 2003, when water level was low in Lake Sakadaš, the TN:TP ratio suggested an N rather than P limitation of phytoplankton. According to Jeppesen et al. (1997) and Søndergaard et al. (2003), summer TP concentrations in shallow lakes depend on internal nutrient recycling. The higher TN:TP ratio was probably caused by a decrease in TP concentrations (due to dilution) and an increase in TN concentrations. This increase in TN concentrations during high water levels could be an indirect influence of flooding (Olde Venternik et al. 2002). The ratio of DIN:TP showed that phytoplankton growth in Lake Sakadaš should have been N limited during both years. According to Hein et al. (2004), in river–floodplain systems, the mean duration of connectivity was significantly related to geochemical conditions, nutrients and particle concentrations in the floodplain.

Values of the TN:TP ratio at the Stara Drava channel, in both years, suggested that phytoplankton growth should have been N-limited. During the low water period, mean values of the DIN:TP ratio were close to the Redfield ratio, which is the ideal ratio for algal growth. High concentrations of phytoplankton Chl *a* were observed during this period. In 2004 (high water period), the DIN:TP ratio significantly decreased, which was also observed for phytoplankton Chl *a* concentrations. The highest NH_4 concentrations occurred during Chl *a* peaks at the Stara Drava channel. This emphasises the importance of NH_4 as a primary N

source for phytoplankton in shallow wetland waters (Kinnear and Garnett 1999; Horvatić et al. 2006; Palijan and Fuks 2006). In addition, Schemel et al. (2004) determined that after the inundation period in a river–floodplain system other sources and processes, such as mineralisation of organic matter indicated by high concentrations of NH_4 , induced an increase in phytoplankton abundance.

Based on the nutrients and Chl *a* concentrations, during the whole investigated period, the Stara Drava channel was classified as hypereutrophic by Carlson (1977), and eutrophic by Kratzer and Brezonik (1981). According to Carlson (1977), trophic conditions changed in Lake Sakadaš from hypertrophy (in low water period) to eutrophy (in high water period) and reflected the importance of the hydrologic regime as a factor which can modify the trophic status of Lake Sakadaš. On the other hand, an increase in the TSI_{TN} values in 2004 in comparison with 2003 calls attention to the importance of nutrient inputs, especially of N from the Danube.

The concentration of Chl *a* phytoplankton is an indication of the algal biomass at in situ conditions and the total biomass of *C. kessleri* is the algal response to the total nutrient concentrations in the investigated samples in bioassay laboratory conditions (Horvatić et al. 2006). The trophic conditions based on the values of *C. kessleri* total biomass in water samples from both sites and according to the classification for waters of Middle Europe (Žakova 1986) were mesoeutrophic in 2003 and eutrophic in 2004. Increased biomass indicates increased nutritional conditions and production potential, whereas decreased biomass indicates the opposite.

Taking into consideration that the algal biomass and overall productivity can be controlled by the type and the intensity of nutrient limitation (Dodds et al. 2002), many bioassay experiments provide no information with respect to magnitude or the intensity of nutrient limitation (Ojala et al. 2003; Dzialowski et al. 2005; Levine and Whalen 2001). By estimating the difference between the growth rate of *C. kessleri* in the original and in the enriched water samples we can quantify nutrient limitation. In nutrient enrichment bioassay, N limitation in Lake Sakadaš was determined during both years with the exception of May and October 2003. Both, N and P limitation was determined only in August in 2003 and 2004. Consistent actual N limitation in Lake Sakadaš

determined by nutrient enrichment bioassay corresponds with the DIN:TP ratios observed in lake water. In the experiment in which the water samples of the Stara Drava were enriched, the intensity of limitation decreased during the low water period, and a significant limitation was established only for N in June 2003. In 2004 (high water period), N limitation was found in all samples with the exception of May 2004. These results are in agreement with the conditions established by N:P ratios observed in the water of the Stara Drava channel at both years. Therefore, N limitation predominates in these riparian floodplains, with greater degrees of N limitation observed during flooding conditions. According to Forshay and Stanley (2005), denitrification is often assumed to be the dominant process driving N reduction during floodplain inundation, as these environments provide an opportunity for extensive contact between N-rich flood water from the river and often oxygen depleted organic matter-rich soils.

Nutrient ratios do not always indicate the real limiting nutrient. For example, ambient N:P ratios in Australian reservoirs correctly indicated if N or P is limiting in 33% of bioassay experiments (Kobayashi and Church 2003). Similarly, in the Great Salt Lake, USA, TN:TP ratios normally suggest P limitation, but bioassay experiments routinely demonstrate N limitation of phytoplankton (Wurtsbaugh 1988). Several problems have been identified in the interpretation of nutrient enrichment bioassay results. The first was the lack of N + P combo treatments. Also, using a single species bioassay may or may not be useful for determining nutrient limitation of in situ phytoplankton since different phytoplankton communities have different nutrient requirements. Bioassays are performed over a number of days under controlled conditions. Therefore, factors other than nutrient limitation may be minimised or magnified (Holland et al. 2004). Other factors such as temperature, light and grazing also need to be considered (Elser and Kimmel 1986; Beardall et al. 2001). Nevertheless, nutrient addition bioassays indicate only limitation under predefined conditions.

Conclusions

The characteristic ecology of floodplain lakes and channels is greatly related to variations in frequency

and duration of inundation. The inter-annual variability in the hydrological regime (exchanges of flooding and drought periods) provokes changes in nutrient limitation in different floodplain habitat types. The longer isolation time of the floodplains from the main river (more lentic conditions) results in a higher eutrophication level. On the other hand, periodical flooding can slow down the eutrophication process of the riparian floodplains (lower eutrophication level in lotic conditions). In floodplain systems of Kopački Rit, N limitation predominates, with greater degrees of N limitation observed during flooding conditions. Nevertheless, continued high nutrient input during flooding may lead to further eutrophication of these riparian floodplains. Of the two ratios used to assess nutrient limitation in situ, the DIN:TP ratio matched better with the bioassay data. An identification of limiting nutrients is necessary for the selection of appropriate nutrient control measures to reduce nutrients as well as to slow down eutrophication process in aquatic systems. Combining the information, from both water quality parameters in situ and nutrient enrichment bioassay in laboratory could provide an integrative approach that would enable extrapolation of laboratory results to the real environment conditions.

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Chapter 3

Spatial distribution of nutrient limitation in the Danube River floodplain in relation to hydrological connectivity



Spatial Distribution of Nutrient Limitation in the Danube River Floodplain in Relation to Hydrological Connectivity

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Abstract In this paper, we describe the influence of flooding on nutrient conditions and phytoplankton Chl-*a* concentrations in a hydrologically dynamic Danube floodplain in North–Eastern Croatia. Based on hydrological typology, the connectivity gradient extends from the main river stream (Eupotamal) to the backwater channels and side-arms (Parapotamal) to the edge of the floodplain (Paleopotamal). A spatial gradient of phytoplankton Chl-*a* occurred during low water levels, while uniformity of Chl-*a* across wetland habitats was observed during high water levels. Nutrient enrichment assays indicated that inorganic nutrients in Eupotamal waters (N/P ratio 16–22) could support a higher growth rate of the assay alga *Chlorella kessleri*, while potential N limitation was found in Parapotamal and Paleopotamal waters (both with N/P ratios < 14). Spatial expansion of N limitation toward the main river was observed as inundation continued. Lateral flood pulse connectivity along with the duration of inundation period affects the input and the retention of nutrients, especially of nitrogen. Understanding of the relationship between spatial (location of the floodplain waterbodies regarding the river) and temporal (hydrological connectivity between the river and its floodplain) dimensions contribute to the importance of healthy floodplains for the maintenance of water quality and environmental conditions in the Danube River.

Keywords *Chlorella kessleri* · Chlorophyll-*a* concentrations · N limitation · Nutrient enrichment assay · Spatial heterogeneity

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Introduction

Algal biomass and overall ecosystem productivity may be controlled by both nutrient composition and nutrient availability. In freshwaters, phosphorus (P) has been regarded as the most probable limiting nutrient for phytoplankton growth (Wetzel 2001), but co-limitation by P and nitrogen (N) is not uncommon (Elser et al. 1990; Jansson et al. 1996). Nitrate is the most common dissolved nitrogen form in rivers and agricultural runoff and it is often used to assess water quality and ecosystem ecological integrity (Smith et al. 2007). For example, in the Danube floodplain restoration project in Austria, nitrate is used as a kind of “hydrological tracer” (Tockner et al. 1998). Although nutrient limitation of algal growth is common in aquatic ecosystems, it is difficult to carry out its quantification. Evaluating the growth response of algae after nutrient additions in laboratory conditions makes quantification of nutrient limitation possible and comparable across studies (Downing et al. 1999; Horvatić et al. 2006; Peršić et al. 2009). The use of green algae *Chlorella kessleri* in bioassay approaches is suitable since it belongs to a group of ubiquitous species. It is a unicellular alga whose growth can be monitored accurately and rapidly and is amenable to laboratory cultivation (Lukavský 1992). Also, the algal growth potential of *C. kessleri* varies widely for various freshwater environments (Peršić et al. 2005; Horvatić et al. 2006; Peršić et al. 2009).

Floodplain-river ecosystems are natural fragmented systems with periodic hydrological connections (Thoms et al. 2005). The overall value of floodplain production is difficult to assess and is likely to vary greatly depending on the variations of river flow and flood pulses (Schemel et al. 2004). Physical and chemical conditions within the floodplain are affected by the size and position of floodplain

waterbodies and their hydrological connectivity with the parent river (Junk and Furch 1993; Junk and Wantzen 2004). Hydrological connectivity greatly contributes to the exchange of nutrients and organic matter between the river and its floodplain (Tockner et al. 1999) and plays a crucial role for species that require different habitat conditions during their life-cycle (Amoros and Bornette 2002). Spatial heterogeneity of water chemistry and phytoplankton biomass appears with decreasing water levels and is accelerated by the subsequent surface separation of water bodies (Pithart et al. 2007). Therefore, spatial variability of limnological characteristics is one of the main features of river-floodplain system and is largely determined by the fluctuations in water level. However, considerably less is known about spatial patterns of floodplain productivity and what controls these patterns (Ahearn et al. 2006).

Therefore, riparian floodplains are among the most valuable, but unfortunately also the most degraded ecosystems in Europe. Based on the World Wide Fund for Nature (WWF 1999) for the Danube River Pollution Reduction Program, 80–90% of the original floodplain area in the Danube basin has been lost. The remaining areas along the Danube River are characterized by reduced hydrological exchange between the main river channel and its floodplain area. In fact, one of the last and the largest transboundary floodplains in the Middle Danube consists of a natural complex of more than 70,000 ha and embraces three protected areas: the Kopački Rit Nature Park in Croatia, the Danube-Drava National Park in Hungary, and the Gornje Podunavlje Special Nature Reserve in Serbia.

The main objective of this study was to describe and explain the effect of hydrological connectivity with the parent river on algal growth (chlorophyll-a concentrations) and nutrient concentrations in a river-floodplain system. We intended to identify spatial variability of potential nutrient limitation in the Danube floodplain in relation to hydrological connectivity. To determine the limiting nutrients of algal growth in different floodplain waterbodies, we used nutrient enrichment assays in laboratory conditions. The importance of measuring algal growth potential in nutrient enrichment assays is the differentiation between nutrients determined by chemical analysis and nutrients that are actually available for algal growth.

Methods

Study Area and Hydrological Classification of the Floodplain Waters

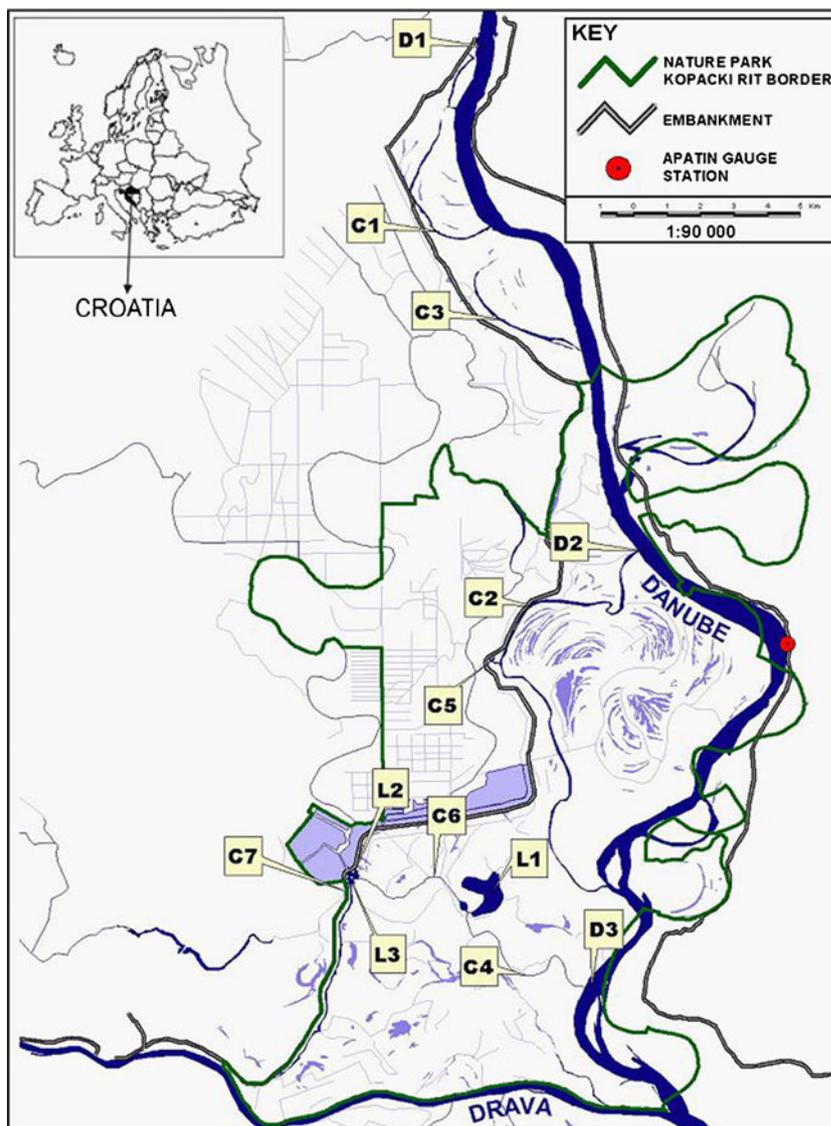
The Danube River is the second longest river in Europe with a length of 2,778 km and watershed that forms the largest international river basin (817,000 km²). It rises in

Germany and flows through ten countries (Germany, Austria, the Slovak Republic, Hungary, Croatia, Serbia, Bulgaria, Romania, Moldova and Ukraine) to its mouth on the Black Sea. The present study was conducted in a 37 km long river-floodplain section of the Danube River in North–Eastern Croatia (r. km 1,426–1,389). In the investigated section of the Danube River the altitude ranges from 78.08 m (Aljmaš) to 80.64 m above sea level (Batina/Bezdan). Its width here is about 250–880 m, the depth of the bed when water is in the middle is 3–6 m and the oscillation of water level over last 50 years is from 78.70 to 87.08 m asl with the range of 8.38 m (Tadić et al. 2003, Maričić 2005). Land configuration and low slope (0.055‰) are typical characteristics of lowland fluvial rivers and provide adequate conditions for meandering. The average current velocity in this river section is about 0.4 ms⁻¹. The Danube discharge is mainly influenced by alpine flow conditions and peaks in spring and early summer due to snowmelt. The average discharge at Batina/Bezdan gauge is 2268 m³ s⁻¹ (the mean flow of 30 years course), with the mean minimum and mean maximum values varying between 742 and 8360 m³ s⁻¹ (Maričić 2005). Hydrological measurements for this study were conducted on a daily basis at the Apatin gauging station (r. km 1404) and were provided by Croatian Water Management Authority.

The Danube floodplain area in North–East Croatia covers approx. 53,000 ha, of which the protected area of the Nature Park Kopački Rit includes 23,000 ha and is a worldwide known wetland in the region of Slavonia and Baranja, Croatia. The Nature Park Kopački Rit is situated at the confluence of the Drava and Danube Rivers (45° 37' 51" N, 18° 53' 31" E), which forms the inland delta where the rivers meet. An inland delta is not known among other European rivers and it makes this area globally important (Schwarz 2005). The aquatic environments of the Kopački Rit floodplain include floodplain lakes, occasionally flooded areas and recesses with flowing water, as well as relict oxbow lakes and backwater channels (Schwarz 2005). The Nature Park Kopački Rit has been selected as the Ramsar Area protected by the Ramsar Convention (Convention on internationally important wetland areas) and included in the Important Bird Area (IBA) list. Nevertheless, the Nature Park Kopački Rit has recently been subjected to some degradation and therefore included on the 'Montreux record' - the list of designated Ramsar sites where substantial changes in ecological character have occurred. Also, a recent war left behind nearly 3,000 ha of floodplain contaminated with mines and unexploded ordnance.

In our investigation 13 sampling sites were established. Three sites are located along the main river channel and 10 sites are located in the lateral dimension from the main river stream towards the floodplain (Fig. 1, Table 1). Along the

Fig. 1 The map of the investigated area. The study sites are shown, coding as in Table 1



main river channel three sampling sites (within the same water body) were chosen along the main transport ways of Danube floodwater into and out of the investigated floodplain. Based on hydrological connectivity among the chosen sites and the river, we have distinguished five types of aquatic habitats along the flooding gradient (Table 1). The typology was made according to existing approaches of Ward and Tockner (2001), Ward et al. (2002) and Schwarz (2005). Similar approaches are regularly used in Danube River catchments (Šporka 1998; Illyová and Némethová 2005; Chovanec et al. 2006; Schwarz 2006; Ot'ahel'ová et al. 2007). The sites within the floodplain were chosen based on the connectivity patterns of a river-floodplain system. The water flow in the Kopački Rit floodplain is directed from the north to the south towards the lower part of the floodplain. To the north of the present park's boundaries lay important, but still unprotected parts

of the Danube floodplain (Fig. 1) that extend to the Danube-Drava National Park in Hungary (Schwarz 2005).

Analytical Methods

Water samples were collected monthly from March to July 2004. Water temperature (WT, °C), water depth (WD, m), Secchi depth (SD, m), pH, and conductivity (EC, $\mu\text{S cm}^{-1}$) were measured at each site. Dissolved oxygen (DO, mg l^{-1}) was determined using the Winkler method (APHA 1995). Samples for chemical and phytoplankton pigment analysis were collected in plastic bottles previously rinsed with sampling water, stored in containers with ice and analyzed the same day. The following nutrients were assayed: ammonium (NH_4 , mg NI^{-1}); nitrate (NO_3 , mg NI^{-1}); nitrite (NO_2 , mg NI^{-1}); Kjeldahl nitrogen (mg NI^{-1}); total nitrogen (TN, mg NI^{-1}); orthophosphate (o-PO_4 , mg P l^{-1});

Table 1 The locality and description of sampling sites assigned to specific aquatic habitat types

Site	Locality	Potamal type (aquatic habitat type)	Description
D1	Danube River (1426 r. km) - shore	Eupotamal A	Site located north of the Danube floodplain.
D2	Danube River (1407 r. km) - central part	Eupotamal A	Located at the confluence of the Vemelj Channel to the Danube River.
D3	Danube River (1389 r. km) - central part	Eupotamal A	Located at the confluence of the Hulovo Channel to the Danube River.
C1	Zmajevac Channel - shore	Eupotamal B	Side-arm with an active flow connected with the Danube River upstream at 1422 r. km and downstream at 1418.2 r. km.
C2	Vemelj Channel - central part	Eupotamal B	Active side channel (upstream connected with the Danube River at 1407 r. km, and downstream at 1392 r. km), transports Danube waters in and out of the Nature Park Kopački Rit.
C3	Monjoroš Channel - shore	Parapotamal A	Backwater channel permanently connected with the Danube River only at the downstream end (1413.2 r. km).
C4	Hulovo Channel - central part	Parapotamal A	Side-arm only downstream permanently connected with the Danube River (1389 r. km), transports Danube waters in and out of the Nature Park Kopački Rit.
C5	Site near the pump station Tikveš - central part	Parapotamal B	Backwater prone to inundation and located further from the main channel. Receives flood waters from the Vemelj Channel and represents entry point of meliorated waters from the surrounding agricultural fields into the floodplain.
C6	Csonakut Channel - central part	Parapotamal B	Permanent water link within the wetland which receives water from connected higher side arm system (Hulovo Channel).
L1	Kopačko Lake - central part	Paleopotamal	Large shallow floodplain lake connected with the Danube River with Hulovo Channel.
L2	Sakadaš Lake - central part	Paleopotamal	The deepest floodplain lake located in the marginal part of floodplain (some 10 km away from the main Danube flow).
L3	Sakadaš Lake - near the dam	Paleopotamal	Site situated near dam of the Drava-Danube flood-dyke.
C7	Kopačevo canal - central part	Paleopotamal	Artificial canal hydrologically connected with Sakadaš Lake through the dam, situated on the right side of Drava-Danube flood-dyke, represents entry points of waters from the surrounding agricultural fields into the floodplain.

and total phosphorus (TP, mg P l⁻¹), using standard methods (APHA 1995). Water samples (0.5–1 l) for phytoplankton pigment analyses were filtered through Whatman GF/C filters, extracted with acetone and were kept for 2–4 h in darkness in a refrigerator at 4°C (Komárková 1989). Chlorophyll concentrations (Chl-*a*, Chl-*b* and Chl-*c*) of the prepared samples were determined spectrophotometrically and calculated according to equations of Strickland and Parsons (1968). To reveal how habitat types respond to physical disturbance (flooding), spatial heterogeneity was indicated by site-specific coefficients of variation (CV) of phytoplankton Chl-*a* and N-NO₃ concentrations based on mean monthly level. Variance rather than mean environmental value is a valuable parameter for understanding how patterns and processes are linked in floodplain systems (Palmer et al. 1997; Tockner et al. 2000).

Homogeneity of variances was tested using Levene's test and data, apart from pH values, were log transformed, if necessary, to reduce heterogeneity of variances. Significant differences in water quality parameters among the investigated sites were calculated by one-way analysis of

variance (ANOVA) and Tukey Unequal N HSD tests. Relationships among physical and chemical parameters were determined by Pearson correlation. Regression analysis was used to model Danube water level through time (separately for each month). A significance level of $p < 0.05$ was used for all tests. Statistical analysis of data was performed using Statistica for Windows 7.0 (StatSoft, Inc. 2005).

Algal Bioassays

Algal growth potential was evaluated in microplates with a suspended culture of *Chlorella kessleri* Fott et Nov. strain LARG/1 by the laboratory miniaturized growth bioassay method according to Lukavský (1992) and Horvatić et al. (2006). Cultures were incubated in the Bold's basal medium (BBM) in a 25°C controlled temperature room and illuminated by fluorescent tubes (Tungsram, Hungary) that provided continuous daylight (PAR, 400–700 nm) at approximately 140 μmol photon m⁻² s⁻¹. Due to prior uptake and possible storage of nutrients, it was necessary to starve *C. kessleri* cells before experimental use (Lukavský

1992; Horvatić et al. 2006). If algae are cultivated in dilute media, the amount of growth from nutrient carry-over in subsequent test waters is negligible (Shoaf 1978). The algal culture in its exponential phase was washed from the nutrient medium with sterile distilled water and cultivated for 3–5 days in sterile distilled water. The algal cell density in this solution was determined using Bürker-Türk counting chamber (Karl Hecht KG, Sondheim, Germany) under a light microscope (Axiovert 25, Carl Zeiss, Inc., Göttingen, Germany). The initial cell density in the inoculum solution was 8×10^5 cells ml^{-1} . Water samples from the investigated sites were filtered through Whatman GF/C glass fibre filters to eliminate particles. Bioassays were carried out in polystyrene 96-well microplates (TPP, Switzerland) with well volume of 300 μl . The miniaturized growth bioassays were conducted with six pseudo replicates of control samples (250 μl tested water sample from each site and month+50 μl algal inoculums in distilled H_2O), N enriched samples (250 μl tested water sample+50 μl algal inoculums with added nutrient N as KNO_3 in final concentration of 0.16 g l^{-1}), and P enriched samples (250 μl tested water sample+50 μl algal inoculums with added P as K_2HPO_4 in final concentration of 0.02 g l^{-1}). High concentrations of nutrients ensured that enrichments would not be depleted during the experiment (Horvatić et al. 2006). Prior to adding algal inoculum solution, the uncovered microplates with water samples from the investigated sites were exposed to UV light for sterilization. The microplates were covered with lids, placed in a glass incubation chamber and exposed to cultivation conditions (temperature of 25°C , continuous daylight (PAR, 400–700 nm) at approximately $140 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$, and CO_2 ca. 2% v/v). The growth of *C. kessleri* was determined by measuring the optical density at 750 nm every day, for approximately 14 days (until the stationary phase of growth), using an automated microplate reader (Multiskan MS, Labsystem, Finland) controlled by GENESIS II software (Windows™ Based Microplate Software). The results were plotted as growth curves. The average specific growth rate for exponentially growing culture of *C. kessleri* ($\mu \text{ day}^{-1}$) was quantitatively determined from the measured absorbance using the exponential model $y=e^{a+\mu x}$ (where y = the population estimate, x = time, a = integration constant and μ = slope or growth rate) during its exponential growth period (up to the 7th day). Growth rate was calculated from six parallels, with respect to time (for each day of exponential phase). The difference in the average specific growth rate of *C. kessleri* in the enriched (μ_E) and control water samples (μ_C) is expressed as the degree of algal response to nutrient enrichment ($\Delta\mu$) was calculated using the method in Downing et al. (1999). The bioassay experiments were used to determine whether nutrient addition stimulates *C. kessleri* growth relative to the control

and thereby ascertain the limiting nutrient in the original water sample. The differences between treatments and the control growth with respect to time were tested with ANOVA. Dunnett's test was used as a *post hoc* test to identify treatment growth rates that were significantly different from the control growth rate. If no significant difference in the *C. kessleri* growth rate was determined between the control and the treatments - no limitation was inferred. An increase in the growth rate of *C. kessleri* following the addition of N or P alone was inferred as N or P limitation. To test statistically significant differences between habitat types, a one-way ANOVA design was used with Tukey's HSD *post hoc* test.

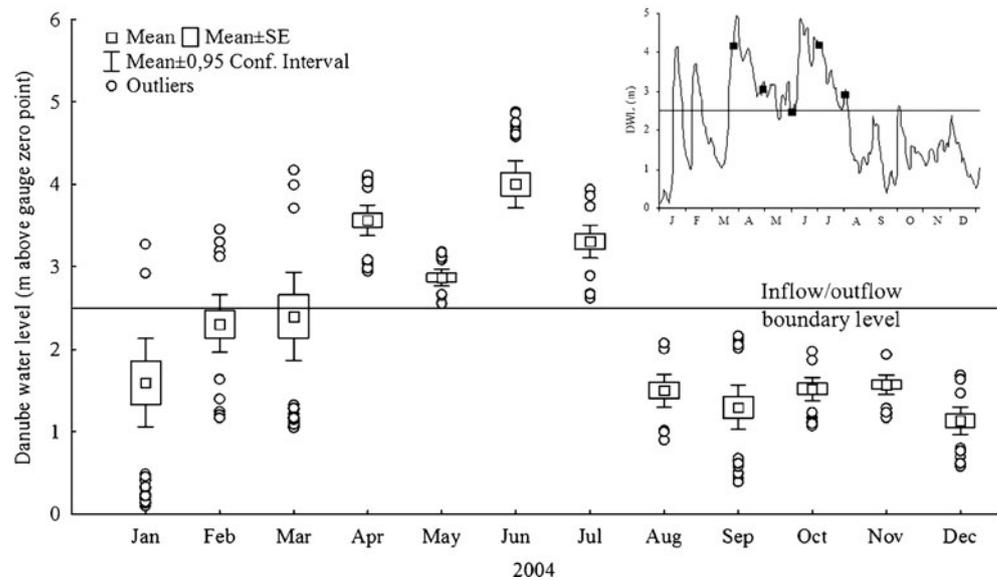
Results

Hydrology

Spring and early summer flooding of the Danube floodplain started at the end of March and lasted until the beginning of August 2004. The flooding had two flood pulses. The first flood pulse started at the end of March and the second one started in June, both with amplitude of 2.4 m, and total duration of 55 and 74 days (Fig. 2). When the Danube water level (Dwl) reaches 2.5 m above the Apatin gauge zero point (1404 r. km), floodwaters enter channels that fill the lakes of the floodplain. Furthermore, when the Danube water level reaches 4 m, water in the floodplain channels starts to overflow into surrounding lowland areas.

An overbank flooding of the Danube floodplain in March was caused by the rapid increase in the Danube water level when Dwl ranged from 1.05 to 4.94 m above gauge zero point (mean 2.40 m, with coefficient of variation 60%). Linear regression analysis also indicated ascending water level of the Danube in March ($R^2=0.84$, $F=70.2$, $p<0.001$). On the other hand, a slow decrease in the water level resulted in the receding of floodwaters to channels and lakes by the end of April (descending water level with $R^2=0.87$, $F=88.6$, $p<0.001$) and Dwl ranged from 2.85 to 4.58 m above gauge zero point, mean 3.56 m with CV=14%. During May, water levels stagnated ($R^2=0.28$, $F=2.4$, $p>0.05$) ranging from 2.28 to 3.28 m above gauge zero point, with mean water level 2.86 m and CV=10%, while another rapid increase in water level (from 2.50 to 4.88 m above gauge zero point, mean 4.0 m, CV=19%) caused an overbank flooding in June (ascending water level, $R^2=0.53$, $F=11.1$, $p=0.002$). Finally, by the end of July floodwaters receded to floodplain channels and lakes and the main river channel (descending water level ($R^2=0.91$, $F=147.3$, $p<0.001$) ranging from 2.5 to 4.22 m above gauge zero point, mean 3.3 m and CV=17%).

Fig. 2 Mean monthly water levels with average daily course in 2004 of the Danube River, 1404 r. km (in m above Apatin station gauge zero point). Horizontal line represents the threshold level of the Danube floodwaters flow into/out of the floodplains of Nature Park Kopački Rit. The hydrograph data were provided by Croatian Water Management Authority



Physical-Chemical Characteristics and Phytoplankton Chl-a Concentrations

Table 2 summarizes the mean values and ranges of physical and chemical parameters as well as of phytoplankton Chl-a at five investigated habitat types across the river-floodplain

transect. Nitrogen concentrations (N-NO₃, TN) were significantly higher (Tukey HSD, $p < 0.05$) in Eupotamal waters (the Danube River and its side-arms) than in the Parapotamal A, Parapotamal B, and Paleopotamal waters (floodplain). Habitat types significantly differentiated also in WD, SD, DO, EC, Kjeldahl nitrogen, and N/P ratios

Table 2 Physical and chemical parameters and phytoplankton chlorophyll concentrations at five investigated floodplain habitat types during 5 month long inundation period (n is the number of sampling sites and dates; mean values with minimal and maximal values in parentheses)

	Eupotamal A ($n=15$)	Eupotamal B ($n=10$)	Parapotamal A ($n=10$)	Parapotamal B ($n=10$)	Paleopotamal ($n=20$)
WD (m)	4.8 ^a (1.0–8.6)	3.0 ^{ab} (0.5–4.7)	2.3 ^b (1.4–3.1)	2.8 ^{ab} (1.2–4.3)	4.0 ^{ab} (1.6–6.9)
SD (m)	0.8 ^{ab} (0.6–2.2)	0.6 ^a (0.4–0.8)	1.0 ^b (0.5–2.1)	1.0 ^b (0.5–2.0)	1.1 ^b (0.4–2.5)
WT (°C)	16.8 ^a (7–21)	16.3 ^a (6–21)	17.3 ^{ab} (7.5–22)	17.2 ^{ab} (7–22)	17.6 ^b (7–23)
DO (mg O ₂ l ⁻¹)	10.3 ^a (1.3–17.2)	10.1 ^a (1.3–16.9)	7.7 ^{ab} (1.3–13.5)	8.3 ^{ab} (1.3–17.7)	6.2 ^b (1.4–13.5)
pH	8.0 ^a (6.6–8.8)	8.0 ^a (7.1–8.8)	7.9 ^a (6.8–8.4)	7.7 ^a (6.3–8.8)	7.6 ^a (6.6–8.4)
EC (μS cm ⁻¹)	410 ^a (330–637)	369 ^a (326–462)	390 ^a (338–492)	455 ^{ab} (323–736)	505 ^b (362–780)
o-PO ₄ (μg l ⁻¹)	65.3 ^a (35.4–117.7)	49.7 ^a (9.8–84.4)	57.1 ^a (8.2–91.5)	36.2 ^a (10.2–69.2)	49.3 ^a (6.9–198.4)
TP (μg l ⁻¹)	117.3 ^a (35.2–243.5)	134.9 ^a (68.3–351.3)	188.2 ^a (57.3–724.0)	103.7 ^a (45.4–184.0)	118.4 ^a (58.4–246.0)
N-NH ₄ ⁺ (μg l ⁻¹)	38.9 ^a (1.9–84.8)	34.6 ^a (2.1–53.8)	35.9 ^a (10.9–114.2)	63.6 ^a (21.7–190.7)	46.9 ^a (10.7–143.6)
N-NO ₃ ⁻ (μg l ⁻¹)	1493.2 ^a (183.5–2596.4)	1391.7 ^a (212.5–2503.6)	903.8 ^b (199.1–1787.9)	852.2 ^b (217.7–1575.4)	804.9 ^b (237.9–1400.0)
N-NO ₂ ⁻ (μg l ⁻¹)	25.6 ^a (5.6–73.1)	21.6 ^a (6.2–38.3)	17.3 ^a (6.0–30.4)	21.5 ^a (6.3–38.7)	13.8 ^a (1.5–36.9)
Kjeldahl N (μg l ⁻¹)	297.3 ^a (172.7–87.0)	281.4 ^{ab} (99.2–467.4)	267.8 ^{ab} (107.8–481.3)	324.3 ^{ab} (117.7–501.3)	374.1 ^b (62.6–544.9)
TN (μg l ⁻¹)	1713.0 ^a (376.0–2915.7)	1575.2 ^a (420.4–2729.2)	1113.9 ^b (404.9–2026.6)	1102.7 ^b (339.4–1822.3)	1057.1 ^b (452.3–1666.2)
TN/TP	22 ^a (3–77)	16 ^{ab} (4–38)	9 ^b (3–25)	12 ^b (3–20)	11 ^b (3–25)
DIN/TP	20 ^a (2–73)	14 ^{ab} (2–36)	8 ^b (2–24)	10 ^b (3–18)	9 ^b (2–22)
Chl-a (μg l ⁻¹)	55.4 ^a (15.6–130.2)	64.3 ^a (14.8–151.0)	39.3 ^a (23.1–86.3)	44.4 ^a (5.3–92.9)	46.8 ^a (4.9–130.8)
Chl-b (μg l ⁻¹)	1.5 ^a (0.01–9.4)	2.1 ^a (0.1–9.4)	3.2 ^a (0.1–12.8)	3.5 ^a (0.3–13.6)	4.3 ^a (0.01–18.5)
Chl-c (μg l ⁻¹)	11.1 ^a (0.7–25.3)	12.9 ^a (0.2–28.0)	9.8 ^a (0.6–28.8)	10.4 ^a (1.6–32.2)	9.2 ^a (0.1–45.2)

Letters in superscript following values indicate statistical significance at $p < 0.05$ (ANOVA and Tukey Unequal n HSD test). Different letters indicate significant differences among different habitat types

(Table 2). Although phosphate concentrations did not differ significantly among different floodplain habitat types, they reflected the same decreasing spatial trend.

A wide range of parameter values were found at all sites during the investigation period. For example, minimal values of DO concentration (from 1.3 to 1.4 mg O₂ l⁻¹) and WT (from 6 to 7.5°C) were measured in March at all sites. Dissolved oxygen concentration increased rapidly after the first flood pulse in April and stayed high during May when maximal values were determined at all habitat types (from 13.5 to 17.7 mg O₂ l⁻¹). A similar pattern was found for the N-NO₃ and TN concentrations.

A strong positive correlation occurred between Danube water level (Dwl) and N-NO₃ concentrations ($r=0.82$, $p<0.001$), as well as between Dwl and TN ($r=0.82$, $p<0.001$). Also, a significant positive correlation occurred between Dwl and EC ($r=0.38$, $p=0.008$), as well as between Dwl and DO ($r=0.50$, $p<0.001$). On the other hand, a significant negative correlation was determined between Dwl and phytoplankton Chl-*a* concentrations ($r=-0.57$, $p<0.001$).

To reveal how habitat type responded to physical disturbance (flooding), habitat heterogeneity was measured as the coefficients of variation (CV) of phytoplankton Chl-*a* and N-NO₃ concentrations (Fig. 3a and b). The CV of phytoplankton Chl-*a* (CV=87%) peaked at stagnating water level in May (Fig. 3a). In May, maximal values of phytoplankton Chl-*a* concentration were found in the Eupotamal waters (Danube River and its side-arms), while a decrease of phytoplankton Chl-*a* concentration was observed in lateral dimension from the main stream (Eupotamal) towards the floodplain (Parapotamal and Paleopotamal, Fig. 4a). On the other hand, uniformity of river-floodplain habitat types with low CV was observed during ascending water level in March (CV=24%), and in June (CV=22%). In March, an increase of phytoplankton Chl-*a* concentration was determined in the lateral dimension from the main stream of the Danube (Eupotamal A) towards the marginal part of the floodplain (Paleopotamal) (Fig. 4a). In addition, high concentrations of phytoplankton Chl-*b* and Chl-*c* were determined in March (at the beginning of an early spring inundation period) at all investigated habitat types (from 9.4 to 18.5 μg l⁻¹ Chl-*b* and from 25.3 to 45.2 μg l⁻¹ Chl-*c*, Table 2).

Unlike phytoplankton Chl-*a*, spatial heterogeneity of N-NO₃ concentrations increased significantly with increasing water level (Fig. 3b). At the beginning of the flood period in March 2004 (mean Danube water level 2.4 m), nitrate concentrations were uniformly low in all habitat types (CV=12%). A concentration gradient (CV=36%) first formed after the first flood pulse in March (Figs. 3b and 4b). By the end of April, as floodwaters receded to floodplain channels and lakes, mean nitrate

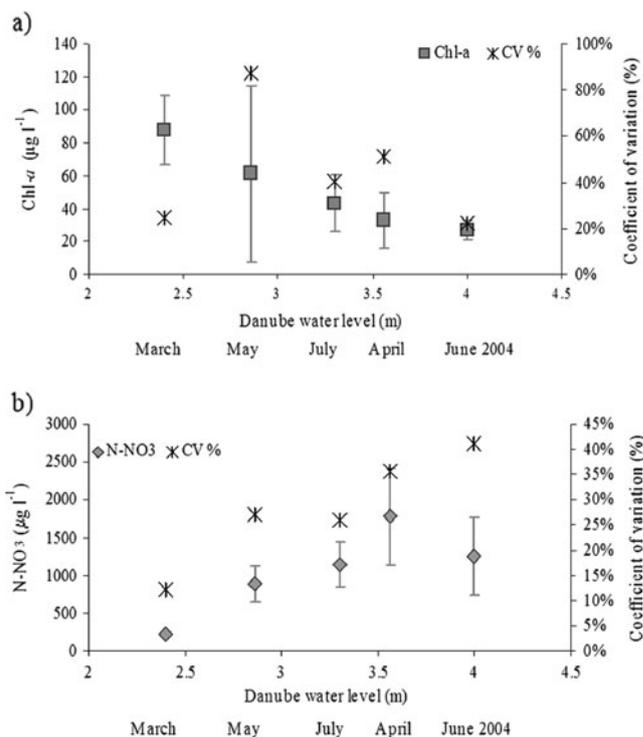


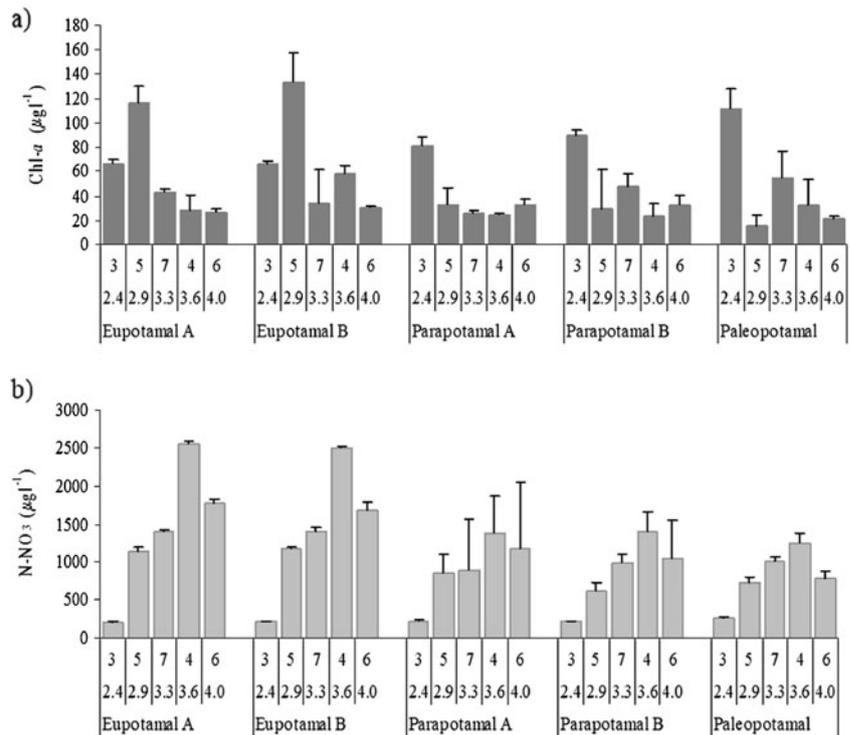
Fig. 3 a Phytoplankton Chl-*a* concentrations (mean values±standard deviations) and habitat heterogeneity measured as the coefficient of variation of Chl-*a* concentrations; b N-NO₃ concentrations and habitat heterogeneity measured as the coefficient of variation of N-NO₃ concentrations across different mean monthly water level of the Danube River during the study (March–July 2004), number of replicates per sampling date: 13

concentrations differed by a factor of 2 between Eupotamal A (2549.1 μg N l⁻¹) and Paleopotamal waters (1256.7 μg N l⁻¹). The highest spatial heterogeneity (CV=41%) for nitrates was determined during the highest mean monthly water level of the River Danube in June (Fig. 3b), with mean nitrate concentrations in Eupotamal A waters of 1764.4 μg N l⁻¹ and in Paleopotamal waters of 789.3 μg N l⁻¹ (Fig. 4b).

Nutrient Enrichment Bioassay

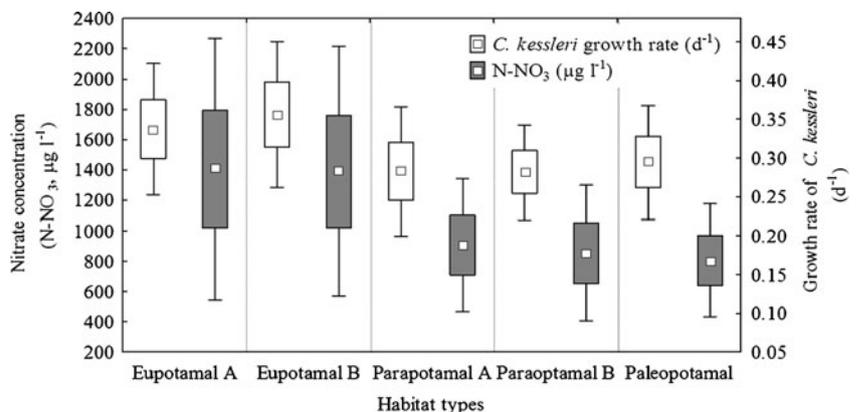
The algal growth potential in original water samples from the investigated sites of the river-floodplain system was investigated up to the stationary phase of *C. kessleri* growth, which was usually established by the 14th day of incubation. The average specific growth rate of *C. kessleri* was the highest in the control water samples of Eupotamal waters ($\mu=0.337$ d⁻¹ in Eupotamal A, $\mu=0.356$ d⁻¹ in Eupotamal B) (Fig. 5). A decreasing trend in the growth rate of *C. kessleri* was observed from the main river channel towards the floodplain. This pattern was similar to that of nitrate concentrations and the N/P ratio (Fig. 5, Table 2).

Fig. 4 Mean values \pm standard deviations of phytoplankton Chl-*a* concentrations (a) and mean values \pm standard deviations of N-NO₃ concentrations (b) during the inundation period (horizontal axis represents months indicated by numbers 3,5,7,4,6 for March, May, July, April, June, respectively, and mean monthly water level for each month) at five investigated river-floodplain habitat types



A quantitative index of nutrient limitation was calculated from the difference in the growth rate of the control samples and the growth rate of treatment samples (N, P) and was expressed as the degree of nutrient limitation (Fig. 6). A significant degree of N limitation (Tukey HSD, $p < 0.01$) occurred in water samples from Parapotamal A ($\Delta N = 0.180 \text{ d}^{-1}$), Parapotamal B ($\Delta N = 0.206 \text{ d}^{-1}$), and Paleopotamal ($\Delta N = 0.213 \text{ d}^{-1}$) habitat types. A significant degree of P limitation ($\Delta P = 0.116 \text{ d}^{-1}$) was only determined in water samples from Parapotamal A habitat type (Fig. 6). Table 3 summarizes the main findings of the nutrient enrichment assay. A significant N limitation was determined at all sites of the Paleopotamal habitat type over the entire 5 month inundation period.

Fig. 5 Mean, mean \pm standard error (box) and mean \pm standard deviation (whisker) values of the growth rate of *C. kessleri* and nitrate concentrations in the water samples from each habitat type (number of samples per habitat type: 10–20)

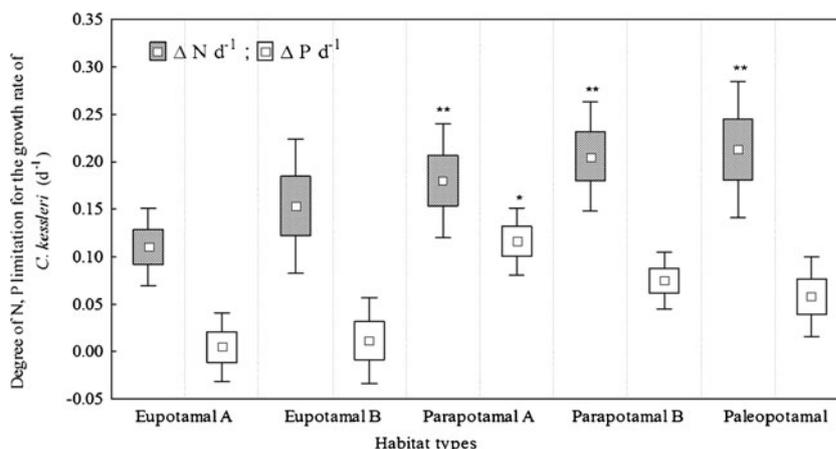


Discussion

The Effect of Hydrological Connectivity on Nutrients and Phytoplankton Chl-*a* Concentrations

The characteristic ecology of floodplain channels and lakes in the investigated river-floodplain area strongly relates to variations in frequency and duration of inundation. Based on hydrological records, high water levels in the Danube floodplain of Kopački Rit are characteristic for spring (March/April) and the beginning of summer (June/July) (Mihaljević et al. 1999). Spring and early summer flooding started at the end of March and lasted until August 2004. Without these natural flood disturbances, the floodplains of

Fig. 6 Mean, mean ± standard error (box) and mean ± standard deviation (whisker) values of the degree of nutrient limitation ($\Delta N d^{-1}$, $\Delta P d^{-1}$) for the growth rate of *C. kessleri* in the water samples from each habitat type (number of samples per habitat type: 10–20). “*” and “**” represents statistically significant nutrient limitation at $p < 0.05$ and $p < 0.01$, respectively



the Danube River have a tendency towards geographical and temporal uniformity with a resulting reduction in biodiversity (Tockner et al. 1998; Ward and Tockner 2001). Based on hydrological typology, the connectivity gradient extends from the main river stream (Eupotamal A) to the edge of the floodplain (Paleopotamal). Among the investigated sites, a gradient was observed in physical and chemical conditions from the main channel (Eupotamal A) towards the marginal part of the floodplain (Paleopotamal).

At the beginning of the inundation period when phytoplankton Chl-*a* concentrations were high, the average oxygen saturation indicated under-saturation. The possible continuous input and decomposition of allochthonous organic matter could have had an influence on decreased oxygen concentrations, even in periods of autotrophy. These results emphasize the general heterotrophic conditions that prevailed at the investigated habitat types. Moreover, Palijan and Fuks (2006) found a higher number of oligotrophic and eutrophic bacteria in the surface waters of the Csonakut Channel and Lake Sakadaš (in this paper classified as Parapotamal and Paleopotamal waters) during March 2004, thus indicating heterotrophic conditions. Therefore, the low nitrogen concentrations occurring in

March could be the consequence of nutrient uptake by phytoplankton (Hein et al. 2003) or bacterioplankton (Palijan and Fuks 2006).

Chlorophyll-*a* concentration was inversely related to the mean monthly water level. March, which included both the pre-flood low flow and the rising limb of the hydrograph, had the lowest mean flow during the study and also had the highest mean Chl-*a* concentration. The high March Chl-*a* concentration was consistent with an annual spring phytoplankton bloom (Vidaković et al. 2008). The March bloom was dominated by diatoms (Mihaljević et al. 2009), which is consistent with the high Chl-*a*, Chl-*b*, and Chl-*c* concentrations. The highest mean monthly flow during the study occurred in June, when Chl-*a* concentration was lowest. The June sampling occurred 3 weeks after the start of flooding and, because the floodplain retention capacity was already filled, the June flood pulse had less effect on connectivity and water exchange. That could explain why habitat heterogeneity concerning nitrogen (high CV) was highest during that time. The March bloom and the low June Chl-*a* concentrations were consistent across sites, as indicated by the accompanying low Chl-*a* coefficients of variation (low CV).

Table 3 Summary of the results of nutrient enrichment bioassay conducted in water samples from the investigated habitat types in river floodplain ecosystem. Categories of nutrient status include nitrogen

limitation (N), nitrogen and phosphorus limitation (N, P) and no limitation ($p > 0.05$), a significant type and degree of nutrient limitation is indicated by p values (one-way ANOVA and Tukey HSD test)

Sampling time (Month and water level)	Eupotamal A (n=3×6)	Eupotamal B (n=2×6)	Parapotamal A (n=2×6)	Parapotamal B (n=2×6)	Paleopotamal (n=4×6)
March 2004 ascending water level	no limitation (p>0.05)	no limitation (p>0.05)	no limitation (p>0.05)	no limitation (p>0.05)	N (p=0.008)
April 2004 descending water level	no limitation (p>0.05)	no limitation (p>0.05)	no limitation (p>0.05)	N (p<0.001) P (p=0.008)	N (p<0.001)
May 2004 stagnating water level	N (p=0.002)	no limitation (p>0.05)	N (p<0.001) P (p=0.02)	N (p<0.001)	N (p<0.001)
June 2004 ascending water level	no limitation (p>0.05)	N (p<0.001)	N (p=0.02)	N (p<0.001) P (p=0.04)	N (p<0.001)
July 2004 descending water level	N (p<0.001)	N (p=0.002)	N (p=0.002)	N (p<0.001)	N (p<0.001)

The stagnating water flow occurred in May and is consistent with high spatial heterogeneity in phytoplankton Chl-*a* (high CV). At that time, the clear water phase occurred in Paleopotamal waters (Mihaljević et al. 2009), in contrast to the high planktonic production phase in Eupotamal waters. The maximal development of phytoplankton in floodplain waters occurred after floods (Mihaljević et al. 2009). Therefore, phytoplankton responded slower to the reduction in water flow than water chemistry. According to Tockner et al. (2000), the relationship between habitat heterogeneity and the river stage, which has a major influence on biodiversity patterns, is a functional characteristic of a river-floodplain system.

Throughout the study nitrate concentrations decreased with the distance from the main river channel. This gradual decrease towards the floodplain could have been a result of N uptake by macrophytes, algae, or bacteria (Hamilton and Lewis 1987; Knowlton and Jones 1997; Unrein 2002; Olde Venterink et al. 2003). Since the highest N-NO₃ concentrations occurred in Eupotamal waters (River Danube and its active side-arms), it seems that the floodwaters of the Danube River are the source of nutrients, especially nitrogen. High amounts of nitrogen brought by the over-bank flooding waters or sediment resuspension related to the inflow of flooding waters, could have supported the abundant macrophyte development recorded during the study period (Vidaković and Bogut 2007; Čerba et al. 2009; Bogut et al. 2010).

Spatial Distribution of Nutrient Limitation

Understanding the nutrients essential for phytoplankton growth is important in the successful control of eutrophication (Smith et al. 1999). Such control efforts must be directed toward the limiting nutrient(s), which promote algal growth. The limiting nutrient can be determined using different methods, for example, by measuring ambient nutrient concentration and nutrient ratios based on intracellular nutrient concentrations (Redfield 1958; Järvinen et al. 1999) or by enrichment assays (Levine and Whalen 2001; Dzialowski et al. 2005; Horvatić et al. 2006; Peršić et al. 2009). Using a single-species assay may or may not reflect nutrient limitation of the entire phytoplankton community, because a single species may not always be representative of algae in the investigated waters and may respond to nutrients differently from the complete community. For example, freshwater cyanobacteria often dominate at N/P ratios from 5 to 10, while a high N/P ratio (> 29) stimulates growth of green algae (Schindler 1977; Smith 1982). In our study, the growth rate of green algae *C. kessleri* increased when the N/P ratio was greater than 14 (mass ratio) observed in Eupotamal waters.

In our enrichment assays, the highest specific growth rate of *C. kessleri* in water from Eupotamal habitats and the gradual decrease in the growth rate of *C. kessleri* in waters from the main river channel towards the floodplain are consistent with physical and chemical features of the river-floodplain system. Inorganic nutrients present in Eupotamal waters were sufficient to support the higher growth rate of *C. kessleri*. Potential N limitation of the growth rate of *C. kessleri* was determined in Parapotamal and Paleopotamal waters that had N/P ratio < 14. Furthermore, a spatial expansion of N limitation was observed as inundation continued. For example, in March, N limitation was determined only in Paleopotamal waters, while at the end of the inundation period N limitation was determined along the lateral dimension from the main stream of the Danube River (Eupotamal A) towards the edge of floodplain (Paleopotamal). As explained earlier, the water level during sampling in June was high but the hydrological action was probably low. Therefore, Danube water was not entering the floodplain. This could have enabled biota to deplete nitrate imported during the flooding making the floodplain N limited.

The abiotic and biotic characteristics (e.g., N-NO₃ concentrations, lower transparency, higher Chl-*a* concentrations) of the Danube River and its active side-arms (Eupotamal waters) suggest that the light limitation is a more probable growth regulator than nutrient limitation. As reported by Hamilton and Lewis (1990) and Knowlton and Jones (1997), light limitation of algal growth prevails in floodplain waters with high connectivity. On the other hand, higher transparency, lower depth, and lower N-NO₃ concentrations in Parapotamal and Paleopotamal waters are consistent with potential N limitation of algal growth. Therefore, the long inundation periods along with the pulsing connectivity controls the input of nutrients in particular nitrates and greatly influences spatial heterogeneity (Amoros and Bornette 2002). Distribution of nutrients in different habitat types of the Danube River floodplain strongly depends on the retention time of the floodwaters and nutrient uptake by phytoplankton, bacterioplankton, and macrophytes. Therefore, this approach to the typology of Danube River floodplain habitat types should be further developed using other biological, geomorphologic, and hydrodynamic parameters, in addition to hydrological connectivity.

In conclusion, nutrient availability decreased with the hydrological connectivity gradient. Furthermore, a spatial gradient of phytoplankton Chl-*a* was observed during stagnating water flow, while uniformity of Chl-*a* among habitat types followed the increase of water level. Consequently, the increase in the degree of N limitation was observed from the Eupotamal waters towards the edge of floodplain and N limitation expanded spatially as inunda-

tion continued. According to Thoms et al. (2005), for nutrient dynamics in river-floodplain systems the most important factors are the location of the floodplain waterbodies regarding the river (spatial dimension) and its hydrological connectivity (temporal dimension).

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Chapter 4

Water quality and algal growth
potential of watercourses
draining agricultural and
forested catchments in eastern
Croatia (Middle Danube Basin)



Water quality and algal growth potential of watercourses draining agricultural and forested catchments in eastern Croatia (Middle Danube Basin)

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With 7 figures and 3 tables

Abstract: The water quality of watercourses in eastern Croatia was examined in terms of the influence of land use and sampling time in the agricultural and forested catchments. Based on small nitrogen to phosphorus ratios (N/P) and the overall high phosphorus concentrations, nitrogen was expected to limit primary production in the spring sampling. The results indicate that N/P ratio was not a reliable indicator of nutrient limitation since ambient concentrations were sufficient to preclude nutrient limitation. The study, therefore, examined nutrient enrichment experiments and found that watercourses of the rural and agricultural catchment (the Bid-Bosut) are severely damaged or at high risk of eutrophication and their nutrient contents are far above prescribed concentrations. Only moderate signs of distortion resulting from anthropogenic activities were observed in watercourses draining the forested catchment (the river Spačva basin) while some watercourses draining the agricultural watershed in the Danube-Drava catchment showed nutrient deficiency (primarily nitrogen).

Key words: agriculture, flowing waters, phosphorus, N/P ratio, nutrient enrichment experiment, trophic conditions.

Introduction

Flowing waters are important links in global biogeochemical cycles. They transport organic matter from terrestrial sources, produce organic matter within the aquatic environment, and degrade organic matter downstream (Hedges et al. 2000). Over the past years, a prevailing view that rivers are insensitive to nutrient inputs no longer appears to be tenable. Although the majority of freshwater eutrophication research has been focusing on lakes and reservoirs, the nutrient enrichment of flowing waters is also of considerable concern (Smith et al. 1999, Dodds 2006, Jones et al. 2008). The main sources of river pollution are a consequence of anthropogenic activities, which result in a greater need to protect the environment, particularly those areas that have not experienced significant

adverse changes. The problem of water pollution in lowland forest ecosystems stands out in particular because forests are usually located in near-pristine environments.

Rural streams, ditches, and canals constitute an extensive drainage system in the agricultural landscape, which can transfer nutrient pollution into receiving watercourses, such as large rivers, where major issues of eutrophication are associated with nitrogen and phosphorus loading (Withers et al. 2000, Jarvie et al. 2006, Neal et al. 2010). Diffuse pollution with high amounts of nutrients is one of the most important environmental problems (Dodds 2006, Munn et al. 2010, Tu 2011). However, Alexander & Smith (2006) documented declines in nutrient concentrations, and improvements in trophic conditions in streams and rivers of the U.S.; also, Merseburger et al. (2011) re-

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ported that, in the U.S., the loading of nutrients from agriculture has decreased in recent years. According to Istvánovics & Honti (2012), the efficiency of nutrient emission control in the Middle Danube Basin is reduced in small and medium sized systems while higher-order rivers are less polluted than medium ones. This implies that the selection of appropriate protection measures to reduce river nutrient loads must be based on local circumstances.

Flowing waters are considered saturated with nutrients because the penetration of light and water flow limits or prevents the response of algae to nutrient enrichment. On the other hand, the limitation of algal growth by nutrients in rivers is not uncommon (Smith et al. 1999, Francoeur 2001, Dodds 2006). Phosphorus is an essential factor for the classification of trophic status because it is a nutrient that often limits primary production in freshwaters (Carpenter et al. 1998, Neal & Robson 2000, Wade et al. 2001). Weak or no correlation between nutrients and suspended biomass of algae, a high turbidity and water flow represent a problem for the trophic classification of lotic systems. For example, suspended particles limit the light penetration through the water column and potentially limit the growth and development of benthic and suspended algae in rivers. Estimates of nutrient limitation that are more accurate are obtained when nutrient ratios are evaluated parallel with controlled bioassays, such as nutrient enrichment experiment. The nutrient enrichment bioassay is a reproducible standard procedure that provides the possibility to determine the potential of various waters to support, accelerate, or inhibit algal growth. Previous studies showed that algal growth potential is useful in projecting maximum potential biomass in standing or slow moving waters (Horvatić et al. 2006, Horvatić et al. 2009).

The objective of this paper is to compare the quality of waters draining agricultural and forested watersheds and changes in water quality during the growing season. The goal was to identify the key limiting nutrient as an important consideration in developing appropriate eutrophication control measures in the watershed. The efficiency of such measures depends

largely on our ability to control nutrients driving the eutrophication process. In addition to the monitoring of physical and chemical parameters in running waters, we applied ecophysiological laboratory studies (bioassays) to determine algal growth potential and the availability of nutrients for the growth of *Chlorella kessleri* in waters draining agricultural and forested watersheds. The results of ecological and physiological studies will enable the implementation of bioassay methods for the evaluation of trophic state as a necessary precondition for defining the measures to reduce nutrients in the water.

Material and methods

Environmental setting

The area under investigation comprises a channel system in eastern Croatia. It is fertile agricultural and forested lowland between 80 and 100 m a.s.l., located in the Pannonian Plain bordered by the Danube, Drava and Sava rivers. Out of the 53 sites chosen to investigate water quality and eutrophication risk of water flows, 27 were located in the most intensive agricultural area at the confluence of the Danube and Drava rivers (Danube-Drava catchment area). The remaining sites (26 of them) were located in the Biđ-Bosut catchment area and the river Spačva sub catchment (Table 1, Fig. 1). Table 1 presents estimated percentages of land use in the study catchments. According to Köppen's classification the investigated area has a moderate continental climate in which precipitation is uniformly distributed over the year. The average annual temperature in the year 2008 was 12.7 °C, and the average annual rainfall was 640.9 mm. The warmest month was August and the coldest January, with average temperatures of 22.6 and 1.8 °C (raw unpublished DHMZ data).

In terms of water management, the Danube-Drava catchment in the Baranja region consists of three hydrographical units or sub catchments – Danube, Drava and Karašica, with a total area of 105,025 hectares. It is predominantly arable land with intensive agricultural production in a low-lying region with a very young relief (the highest peak has 243 m). Holocene and Pleistocene sediments (loess and loess like sediments, sands, etc.) dominate in the composition and structure of soil. Very moist alluvial plain (especially in Kopački Rit Nature Park) cover 12 % of the investigated area and belongs to the Danube watershed. Floods pose a problem in this area, which is being resolved for a century and a half. So far, nearly 100,000 ha has been improved. Around 135 km of embankments, and over a

Table 1. Total areas and percentages of land use types for study catchments.

Catchment	Area, ha	Land use, %			
		Urban	Agriculture	Forest	Wetlands
Danube-Drava (DD)	105 025	2.1	63.4	23.2	11.3
Biđ-Bosut (BB)	172 076	3.1	73.0	23.8	0.1
River Spačva (SP)	85 149	2.4	46.8	50.5	0.2

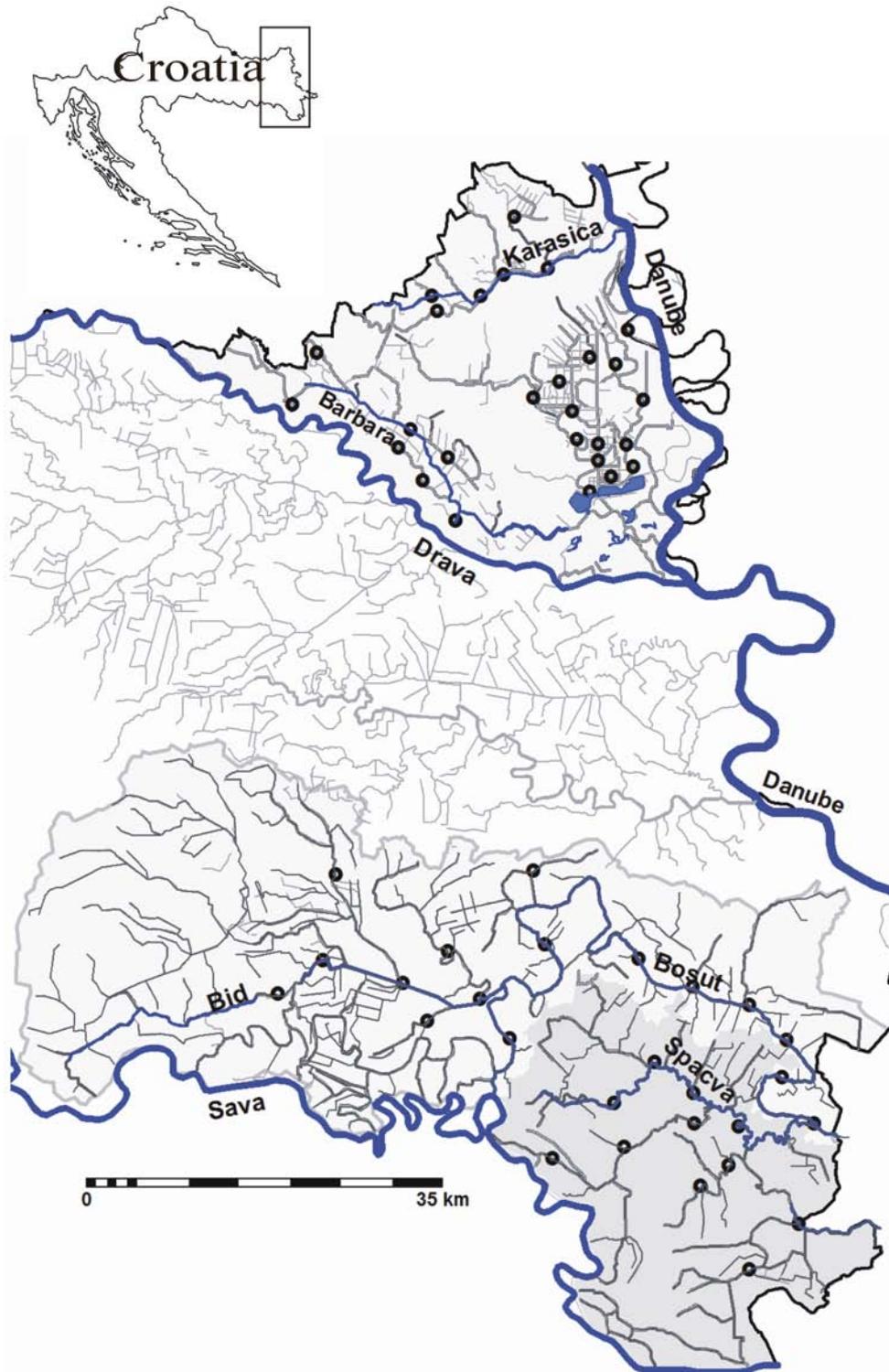


Fig. 1. Study area representing the Danube-Drava catchment to the north in the Baranja region (agricultural land use) and the Bid-Bosut River Basin (a mosaic of agricultural land and forests) with the river Spačva sub catchment (mainly forested land use).

1000 km of canals have been built. The total length of the improvement drainage system is about 887 km, of which 248 km are main canal network (first and second order canals) and 639 km are third and fourth order canals. The main water chan-

nel in the northernmost area is the river Karašica. This is “the most natural stream of Baranja region in the broader sense” and a right tributary to the Danube. The river Karašica is a recipient of waste water treatment water (it has a mechanical puri-

fier that usually does not work) which covers all industry and most of the population of the regions central city (10,549). The main water flow in the southern area is the Barbara Channel that runs almost parallel with the river Drava from the Croatian-Hungarian border. It is divided into the Old Barbara, which is 26.7 km long, and the Barbara Canal 7 km long. Numerous improvement canals and semi natural streams are its tributaries. This watercourse is a recipient of agricultural drainage waters where various mineral fertilizers and pesticides are used. The water quality in the Barbara channel influences the water quality of the river Drava and the nearby floodplain of the Nature Park Kopački Rit. Also, in the surroundings of the Nature Park Kopački Rit there are more than 20,000 ha of arable land with intensive agricultural production and a developed improvement system that drains surface waters towards the Kopački Rit and further towards the river Danube. This network of canals forms the third sub catchment area in the Baranja region.

The Biđ-Bosut Basin with a catchment area of 257,225 ha is characterized by a patchwork of meadows, pastures, fields and forests. The main drainage recipient of this catchment is the river Sava. The composition and structure is dominated by amphigleyic and Holocene clay soils, which are largely under grassland and forest vegetation. The whole valley of the river Bosut is an old, fossil river bed of the river Sava. This area is also known as a region with the highest evaporation in Croatia (Prpić et al. 1997). The river Bosut is known for meandering and extremely slow flow. It has an unusually small inclination in its basin – less than 10 m from its source until its mouth. In its lower course, the Bosut flows through a forested area in the region of the Spačva Basin where it receives the water of the river Spačva as a right tributary. The Spačva sub catchment area includes part of the river Bosut Holocene alluvial plains. Oak forests occupy the valley landscape: plains and peripheral terraces. The whole area is rich in groundwater, and with a significant inflow of surface waters. The central and the longest watercourse (length 40 km) is the river Spačva. Forests of the river Spačva catchment area represent the largest complex of near-natural pedunculate oak forests in this part of Europe. The “Spačva” forest basin covers approximately 43,000 ha in Croatia and about 18,000 ha in Serbia and makes one fifth of all pedunculate oak forests in Croatia. In today’s terms, the vitality of trees is significantly compromised due to changes in the hydrological regime (absence of periodic flooding and the appearance of consecutive dry periods) and the pollution of air and water flows.

Sample collection and chemical analysis

We sampled each of the 53 stations (Fig. 1) twice during the growing season, once during spring and once during summer conditions to characterize the seasonal influence. The sampling occurred between May and August 2008. Water depth (WD, m) and Secchi depth (SD, m) were measured at each site. Water temperature (WT, °C), pH and conductivity (EC, $\mu\text{S cm}^{-1}$) were measured with a portable multi-meter (Multi 340 i, WTW, Germany). Sampling was conducted from a boat or the bank of the channel. Dissolved oxygen (DO, mg l^{-1}) was determined using the Winkler method (APHA 1995). The organic matter content in surface waters was measured according to EN ISO 8467 (1995) as the chemical oxygen demand by decomposition of organic carbon with KMnO_4 (COD_{Mn} , $\text{mg O}_2 \text{ l}^{-1}$). Samples for chemical analysis and suspended phytoplankton analysis were collected in plastic bottles that were previously rinsed with

sampling water, stored in containers with ice, and analysed the same day. The following nutrients were assayed: ammonium (NH_4 , mg N l^{-1}), nitrate (NO_3 , mg N l^{-1}), nitrite (NO_2 , mg N l^{-1}), Kjeldahl nitrogen (KN, mg N l^{-1}), total nitrogen (TN, mg N l^{-1}), orthophosphate (o- PO_4 , mg P l^{-1}), total phosphorus (TP, mg P l^{-1}), chloride (Cl^- , mg l^{-1}), and sulphate (SO_4 , mg l^{-1}) using standard methods (APHA 1995). Water samples for phytoplankton chlorophyll-*a* analysis (0.5–1 l) were filtered through Whatman GF/C filters extracted with acetone for 2–4 h in darkness at 4 °C (Komárková 1989). Chlorophyll-*a* concentrations (Chl-*a*) of the prepared samples were determined spectrophotometrically and calculated according to the equations of Strickland & Parsons (1968). The assessment of water quality of the investigated channels was carried out according to the National regulations (Regulations on quality standards for waters 2010), the European Union Water Framework Directive (WFD) criteria (European Union 2000), and Dodds et al. (1998).

Shapiro-Wilks tests showed that water quality variables were not normally distributed among catchments. Also, because of skewed distributions, small sample size and different standard deviations, nonparametric tests were used for statistical data analysis. To address the problem of non-normal and moderately heteroscedastic data, mean, median, inter-quartile range (IQR) and robust coefficient of variation (CVR, %) were used as a descriptive statistics (Reimann et al. 2009). The comparison between different catchments were performed using Kruskal Wallis ANOVA and between sampling dates using Wilcoxon test. Kendall’s τ was used to identify significant correlations. Saturation of dissolved oxygen (DOSAT) was used in statistical analysis since it removes the temperature dependency of DO due to solubility variations at different temperature. A significance level $p < 0.05$ was used for all tests. Statistical analysis of data was performed using Statistica 7.1 (StatSoft, Inc. 2005).

Algal bioassays

Algal growth potential was evaluated in microplates with a suspended culture of *Chlorella kessleri* Fott et Nov. strain LARG/1 by the laboratory miniaturized growth bioassay method according to Lukavský (1992) and modified by Horvatić et al. (2006, 2009). Cultures were incubated in the Bold’s basal medium (BBM) in a 25 °C controlled temperature room and illuminated by fluorescent tubes (Tungsram, Hungary) that provided continuous daylight (PAR, 400–700 nm) at approximately 140 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, and about 2 % v/v CO_2 . It was necessary to starve *C. kessleri* cells before experimental use due to prior uptake and possible storage of nutrients (Lukavský 1992, Horvatić et al. 2006). If algae are cultivated in dilute media, the amount of growth from nutrient carry-over in subsequent test waters is small (Shoaf 1978). The algal culture in its exponential phase was washed from the nutrient medium with sterile distilled water and cultivated for three days in sterile distilled water. The algal cell density in this solution was determined using Bürker-Türk counting chamber (Karl Hecht KG, Sondheim, Germany) under a light microscope (Axiovert 25, Carl Zeiss, Inc., Göttingen, Germany). The initial cell density in the inoculum suspension was 5×10^5 cells ml^{-1} . Water samples from the investigated sites were filtered to remove particles through Whatman GF/C glass fibre filters. Bioassays were carried out in polystyrene 96-well microplates (TPP, Switzerland) with well volume of 300 μl . The miniaturized growth bioassays were conducted with six pseudo replicates of control samples (240 μl water sample from each site and month +10 μl algal inoculum

in distilled H₂O), N enriched samples (240 µl water sample +10 µl algal inoculum +1.5 mg N l⁻¹ KNO₃ final concentration), P enriched samples (240 µl water sample +10 µl algal inoculum+K₂HPO₄ and KH₂PO₄ with a final concentration of 0.4 mg P l⁻¹), and their combination (N+P treatment). High concentrations of nutrients ensured that they would not be depleted during the experiment (Horvatić et al. 2006). Prior to adding the algal inoculum, the uncovered microplates with water samples from the investigated sites were exposed to UV light for sterilization. The microplates were covered with lids, placed in a glass incubation chamber and exposed to cultivation conditions. The growth of *C. kessleri* was determined by measuring the optical density at 750 nm every day, for approximately 14 days (until the stationary phase of growth), using an automated microplate reader (Multiskan MS, Labsystem, Finland) controlled by GENESIS II software (Windows™ Based Microplate Software). The results were plotted as growth curves (biomass over time). Optical density at 750 nm was converted to algal dry weight (mg l⁻¹) as described by Lukavský (1992). The average growth rate for exponentially growing culture of *C. kessleri* (µ day⁻¹) was quantitatively determined from the measured biomass using an exponential model, $y = e^{a+\mu x}$ (where y is the population biomass estimate, x is time, a is the integration constant and μ is the slope or growth rate), during the exponential growth phase (up to the 7th day). Growth rate was calculated from six parallels, with respect to time (for each day of exponential phase). Treatment effects were tested by comparing slopes of growth curves using ANCOVA (Zar, 2010).

During the experiment the biomass integral (B) of algae was calculated as the area under the growth curve (Horvatić et al. 2006). The idea of calculating the biomass integral is to extract as much as information as possible. This approach will result in estimates that are dependent of the test duration and the absolute maximum of the growth rate. The total biomass of *C. kessleri* in the water samples unamended with nutrients was used for evaluation of trophic conditions (Žáková 1986). Determination of the trophic potential of waters using total biomass rather than a fluctuating growth rate can be considered to yield a more stable result. Therefore, to determine whether the nutrient addition is stimulating *C. kessleri* growth, relative to that in the control water sample, and thereby ascertain the limiting nutrient in the surface water sample we have used a nutrient deficiency index (based on the integrated biomass) instead of the degree of nutrient limitation (based on growth rate). Since different components of phytoplankton community can be limited by different nutrients, and since individual cells can be simultaneously limited by more than one nutrient even under very homogeneous conditions (Egli 1991), we calculated the index of N or P deficiency for *C. kessleri* using the following relationship:

$$\begin{aligned} \text{N deficiency} &= (B_{NP} - B_P) \times B_C^{-1}, \text{ and} \\ \text{P deficiency} &= (B_{NP} - B_N) \times B_C^{-1}, \end{aligned}$$

where B_{NP} is the integrated biomass in the N+P treatment, B_N and B_P are the integrated biomasses in the N and P treatments, and B_C is the integrated biomass in the control treatment. A high value means a high degree of nutrient deficiency and low value means low degree of nutrient deficiency. This measure reflects N deficiency without the confounding effects of P deficiency, and assumes that even secondary limitation by N can alter biomass. This also applies to P deficiency (Dodds et al. 2004).

Significant differences in *C. kessleri* growth, and in nutrient deficiency of water samples among catchments were tested

with Kruskal Wallis ANOVA; between spring and summer sampling by Wilcoxon test. Kendall's τ was used to identify significant correlations between *C. kessleri* growth and ambient nutrient concentrations in the surface water samples.

Results

Water quality

Water quality variable data for the investigated channel system in eastern Croatia is summarized in Tables 2 and 3.

The Cl⁻ concentrations ranged from 12.76 mg l⁻¹ (improvement canal) up to 94.3 mg l⁻¹ (site which is a recipient of wastewaters). Higher concentrations occurred near sewage and other waste outlets, or irrigation drains. The highest SO₄ concentrations were measured in the rural catchment area during spring sampling (i.e. 101.3 and 102.66 mg l⁻¹ at sites located in improvement canals) and most probably came from the intensive use of fertilizers.

The chlorophyll-*a* concentration showed a wide variation within catchment areas (Table 2). Overall, in 27 % of samples Chl-*a* concentrations were lower than 10 µg l⁻¹ (oligotrophic – mesotrophic limit) and in 33 % of samples Chl-*a* concentrations were greater than 30 µg l⁻¹ (mesotrophic – eutrophic limit, Dodds et al. 1998). Chl-*a* was not significantly related to any nutrient forms. A significant correlation existed only between Chl-*a* and WD (Kendal τ =0.24), SD (-0.14), DOSAT (0.16), pH (0.16), KMnO₄ (0.15), as well as EC (-0.15).

There were significant differences in water quality variables among catchments and between sampling occasions. The results of Kruskal Wallis test, χ^2 (2, 106), indicated that there is a significant difference in medians of water quality parameters among catchments in COD_{Mn} ($\chi^2 = 13.05$, $p = 0.002$), NH₄-N ($\chi^2 = 15.87$, $p = 0.0004$), NO₃-N ($\chi^2 = 10.97$, $p = 0.004$), NO₂-N ($\chi^2 = 6.99$, $p = 0.03$), TP ($\chi^2 = 28.12$, $p < 0.0001$), o-PO₄ ($\chi^2 = 44.83$, $p < 0.0001$) and TN/TP ratio ($\chi^2 = 12.89$, $p = 0.002$). Pairwise comparison among the three groups revealed significantly higher concentrations of NH₄-N and P in the Biđ-Bosut catchment in comparison to the Danube-Drava catchment (for NH₄-N, $p < 0.0001$, TP, $p < 0.0001$, o-PO₄, $p < 0.0001$) as well as in comparison to the river Spačva catchment (for NH₄-N, $p = 0.001$, TP, $p = 0.01$, o-PO₄, $p = 0.0005$).

Significant pair-wise differences between means of the main factors in the Danube-Drava catchment (agricultural land use) showed a higher values in SD, SO₄, EC and COD_{Mn} in the spring (Wilcoxon test, $p = 0.002$,

Table 2. Descriptive statistics of selected water quality variables (water depth – WD, Secchi depth – SD, saturation of dissolved oxygen – DOSAT, phytoplankton chlorophyll-*a* concentration – Chl-*a*, pH, conductivity – EC, chloride concentration – Cl⁻, sulphate concentration – SO₄²⁻, and COD_{Mn}) in watercourses of the Danube-Drava and the Bid-Bosut catchment areas (with significant agriculture land use), and of the river Spačva catchment (with significant forest land use).

	WD (m)	SD (m)	DOSAT (%)	Chl- <i>a</i> (µg l ⁻¹)	pH	EC (µS cm ⁻¹)	Cl ⁻ (mg l ⁻¹)	SO ₄ ²⁻ (mg l ⁻¹)	KMnO ₄ (mg O ₂ l ⁻¹)
Danube Drava catchment (n = 54)									
Mean	1.1	0.8	88	31.0	8.1	688	33.62	47.11	9.43
Median	1.1	0.7	80	18.5	8.1	696	28.01	43.57	9.50
Min	0.3	0.3	16	4.2	7.3	345	12.76	9.52	1.00
Max	2.0	1.5	281	210.8	9.1	1143	94.30	102.66	16.00
IQR	0.5	0.3	54	20.8	0.5	200	16.00	36.11	4.75
CVR, %	27	20	51	80	5	20	43	52	39
Bid Bosut catchment (n = 28)									
Mean	1.7	0.9	60	37.4	7.7	732	34.42	27.24	12.04
Median	2.0	0.9	56	26.2	7.7	706	32.61	28.09	11.65
Min	0.4	0.4	11	3.0	6.7	589	21.63	3.22	8.00
Max	2.6	1.8	150	238.9	8.4	984	58.49	48.44	21.62
IQR	1.0	0.4	58	29.1	0.5	108	7.62	15.32	2.79
CVR, %	8	8	78	89	5	12	16	44	21
River Spačva catchment (n = 24)									
Mean	1.3	0.8	88	30.0	7.9	716	39.99	34.51	13.36
Median	1.3	0.8	75	16.4	8.0	679	35.98	28.96	13.00
Min	0.4	0.3	18	2.4	6.9	518	24.82	5.70	9.00
Max	2.5	1.3	206	165.2	8.6	1241	81.18	76.67	17.29
IQR	0.7	0.4	90	35.7	0.7	146	14.18	22.71	4.04
CVR, %	12	5	83	104	7	12	29	74	23

Table 3. Ammonium (NH₄-N), nitrate (NO₃-N), nitrite (NO₂-N), Kjeldahl nitrogen (TKN), total nitrogen (TN), total phosphorus (TP) and orthophosphate (o-PO₄) concentrations in watercourses of Danube-Drava and Bid-Bosut catchment areas (with significant agriculture land use) and of the river Spačva catchment (with significant forest land use).

	NH ₄ -N (mg N l ⁻¹)	NO ₃ -N (mg N l ⁻¹)	NO ₂ -N (mg N l ⁻¹)	TKN (mg N l ⁻¹)	TN (mg N l ⁻¹)	TP (mg P l ⁻¹)	o-PO ₄ (mg P l ⁻¹)
Danube Drava catchment (n = 54)							
Mean	0.096	0.454	0.031	1.006	1.593	0.377	0.178
Median	0.010	0.065	0.010	0.781	1.039	0.262	0.066
Min	0.003	0.020	0.001	0.037	0.130	0.054	0.005
Max	1.209	12.810	0.331	3.957	16.958	3.287	2.568
IQR	0.056	0.172	0.021	0.844	1.122	0.276	0.122
CVR, %	72	103	78	67	67	73	116
Bid Bosut catchment (n = 28)							
Mean	1.191	0.425	0.120	1.623	3.356	0.979	0.858
Median	0.370	0.071	0.030	0.793	1.725	0.887	0.763
Min	0.005	0.020	0.004	0.088	0.286	0.261	0.246
Max	10.653	3.624	0.779	9.341	20.054	3.302	2.647
IQR	1.001	0.129	0.124	1.365	3.020	0.611	0.332
CVR, %	134	107	114	88	85	53	34
River Spačva catchment (n = 24)							
Mean	0.248	0.053	0.016	0.694	1.001	0.598	0.320
Median	0.013	0.025	0.009	0.470	0.742	0.433	0.193
Min	0.005	0.020	0.002	0.126	0.134	0.091	0.005
Max	3.039	0.286	0.103	3.311	6.365	2.382	1.053
IQR	0.088	0.024	0.014	0.616	0.657	0.654	0.414
CVR, %	90	27	96	77	75	85	111

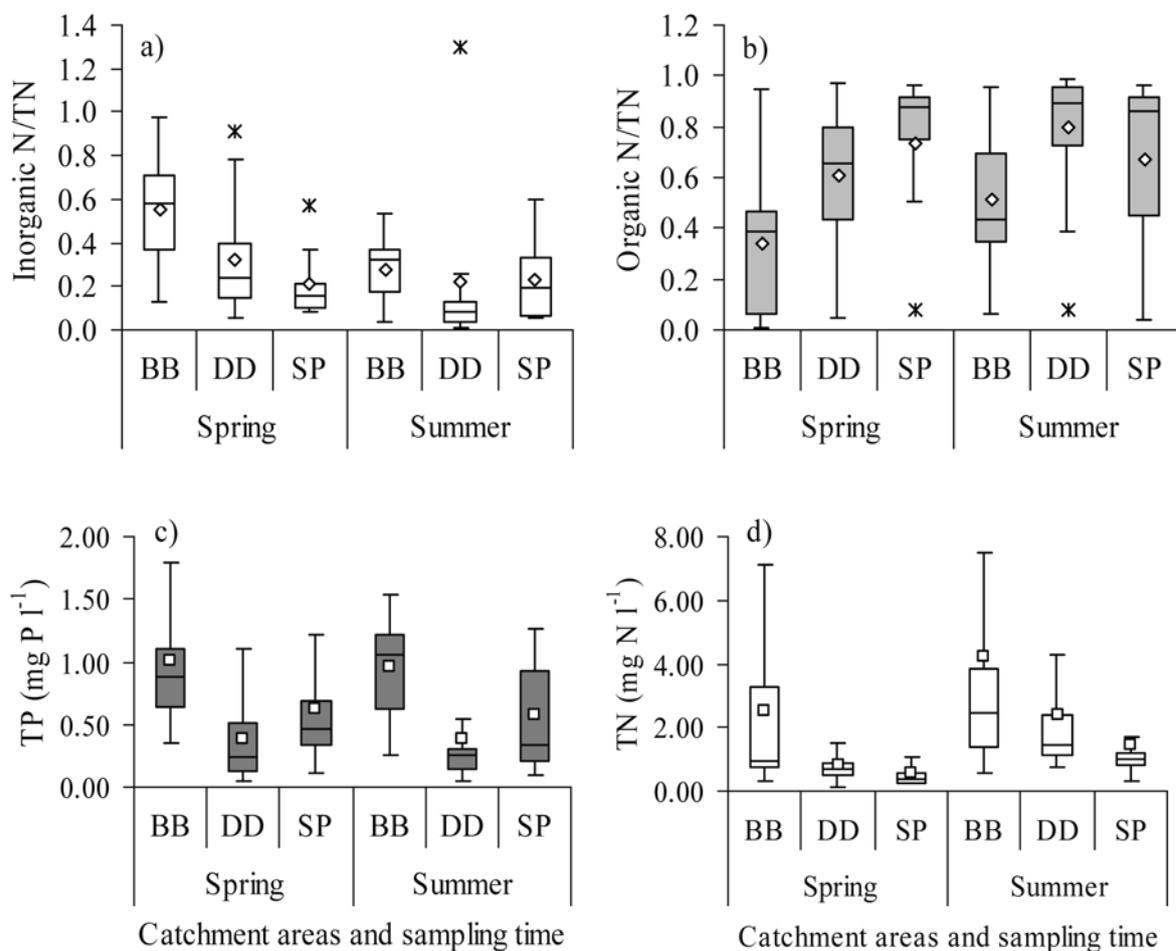


Fig. 2. Seasonal and spatial variation in the proportions of (a) TN as inorganic N ($\text{NH}_4+\text{NO}_3+\text{NO}_2$) and (b) TN as organic N (TKN- NH_4). Concentrations of TP (c) and TN (d) in spring and summer samples from the Danube-Drava (DD), the Bid-Bosut (BB) agricultural catchments and the river Spačva (SP) forested catchment. Boxplots show median (line) and mean (point), quartiles, non-outlier range and outliers for each catchment area and season.

$p < 0.0001$, $p < 0.0001$ and $p = 0.04$, respectively) while higher values of Chl-*a*, TKN, TN concentrations and TN/TP ratio were observed in the summer ($p = 0.02$, $p < 0.0001$, $p < 0.0001$ and $p < 0.0001$, respectively). Likewise, in channels within predominantly forested watershed, in the river Spačva catchment, there were significantly higher values in SD, SO_4 concentrations and COD_{Mn} in the spring ($p = 0.002$, $p = 0.02$ and $p = 0.02$, respectively) while Chl-*a*, TKN, TN concentrations and TN/TP ratio were higher in the summer ($p = 0.002$, $p = 0.01$, $p = 0.008$ and $p = 0.02$, respectively). In the Bid-Bosut catchment, $\text{NO}_3\text{-N}$, $\text{NO}_2\text{-N}$, SO_4 concentrations and pH were higher in the spring ($p = 0.002$, $p = 0.02$, $p = 0.002$ and 0.005 , respectively), whereas TKN concentrations were higher in the summer ($p = 0.002$).

The relative importance of inorganic and organic N species to total N in the investigated watercourses displayed marked regional and seasonal variation (Fig. 2a and b). Average dissolved inorganic nitrogen contribution to total nitrogen concentrations ranged from 21 and 22% in the river Spačva and the Danube-Drava catchments, up to 55% in the Bid-Bosut catchment. Organic nitrogen contributed from an average of 34% in the Bid-Bosut catchment up to 74 and 80% of TN, in the river Spačva and the Danube-Drava catchment. There was a strong correlation between TN and $\text{NH}_4\text{-N}$ ($r = 0.53$, $p < 0.001$) and TN and TKN ($r = 0.71$, $p < 0.001$) which is not surprising since ammonium and organic N are the most abundant forms of N in the investigated channels. Total $\text{NH}_4\text{-N}$ concentrations measured in surface waters were typically lower than

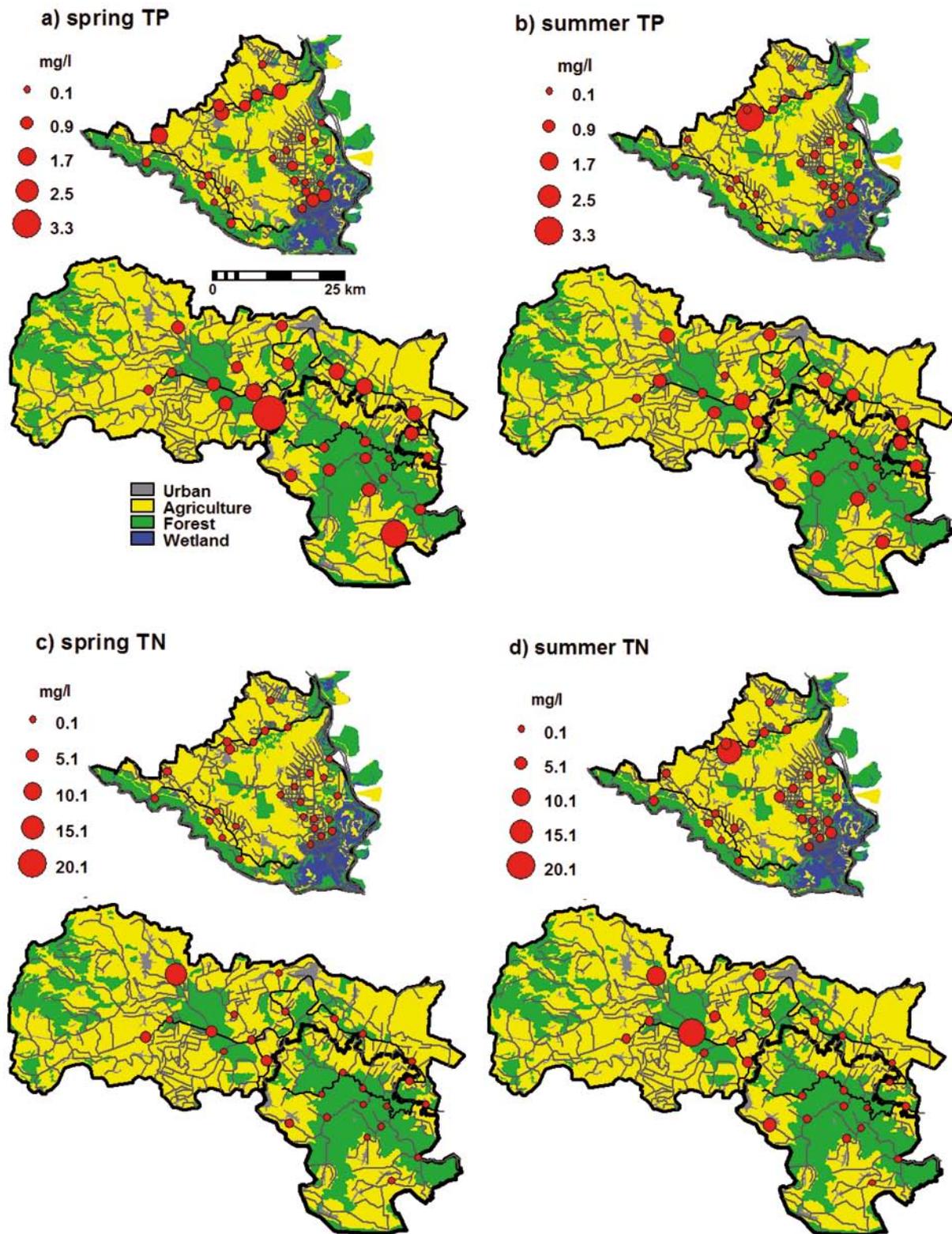


Fig. 3. The geographical distribution of spring TP (a) and TN (c) and summer TP (b) and TN (d) concentrations in watercourses of the investigated area.

0.25 mg N l⁻¹ (in 74 % of all samples) indicating good status of the investigated waters. In 15 % of the samples, NH₄-N concentrations exceeded the upper limit of 0.6 mg N l⁻¹ (Regulations on quality standards for waters 2010), indicating very bad water quality. The highest NH₄-N concentration measured was 10.7 mg N l⁻¹ (in the Biđ-Bosut catchment). As for nitrates, only 2 % of the measurements exceeded the maximum allowed concentration of 3 mg l⁻¹ NO₃-N defined by National regulations (Regulations on quality standards for waters 2010). The highest measured concentration of NO₃-N was 12.8 mg N l⁻¹ at a site in the Danube-Drava catchment, which is considerably influenced by wastewaters. In 90 % of the samples, NO₃-N concentrations were lower than 0.5 mg l⁻¹ NO₃-N indicating very good status.

TN concentrations varied among catchments and sampling periods (Figs 2c, 3c and d). Water quality based on TN concentrations worsened towards the summer (90th percentile according to WFD, EU 2000). The status changed from moderate (spring) to poor (summer) in the Biđ-Bosut catchment (with significant agricultural land use); from high (spring) to moderate (summer) in the Danube-Drava catchment area (also agricultural land use); and from very high (spring) to good (summer) in the forested catchment (the river Spačva catchment). Maximal TN concentrations (16.96 mg l⁻¹) were measured at a site significantly influenced by waste water outlet in the Danube-Drava

catchment, where water quality according to TN deteriorated from good in the spring (90th percentile of 2.54 mg l⁻¹) to bad in the summer (90th percentile of 10.27 mg l⁻¹). Seasonal changes in total phosphorus (TP) concentrations were very similar in all catchments (Figs 2b, 3a and b). TP concentrations in the investigated channels varied from 0.38 mg l⁻¹ (average for the Danube-Drava catchment) to 0.98 mg l⁻¹ (average for the Biđ-Bosut catchment), with maximal values of more than 3 mg l⁻¹ in both catchments (Table 3). Likewise, water quality according to TP concentrations was moderate in the Danube-Drava catchment and very bad in the Biđ-Bosut catchment (Regulations on quality standards for waters 2010). Also, the 90th percentile of o-PO₄ concentrations (1.19, 0.9 and 0.31 mg P l⁻¹ in the Biđ-Bosut, the river Spačva and the Danube-Drava catchment) indicated poor to very bad water quality in the investigated watercourses.

Nutrient ratios were used to evaluate the nutrient limiting primary production. The increase in summer TN concentrations resulted in an increase of summer TN/TP ratio. The most conspicuous change was observed in the Danube-Drava catchment where the TN/TP ratio indicated mainly N-limited conditions during spring (mean and median TN/TP ratios 3.0 and 2.6, respectively), and balanced supply of N and P during summer sampling (mean and median TN/TP ratios 8.6 and 6.3, respectively). In Fig. 4, an assessment can be made of which of these two major nutrients may be

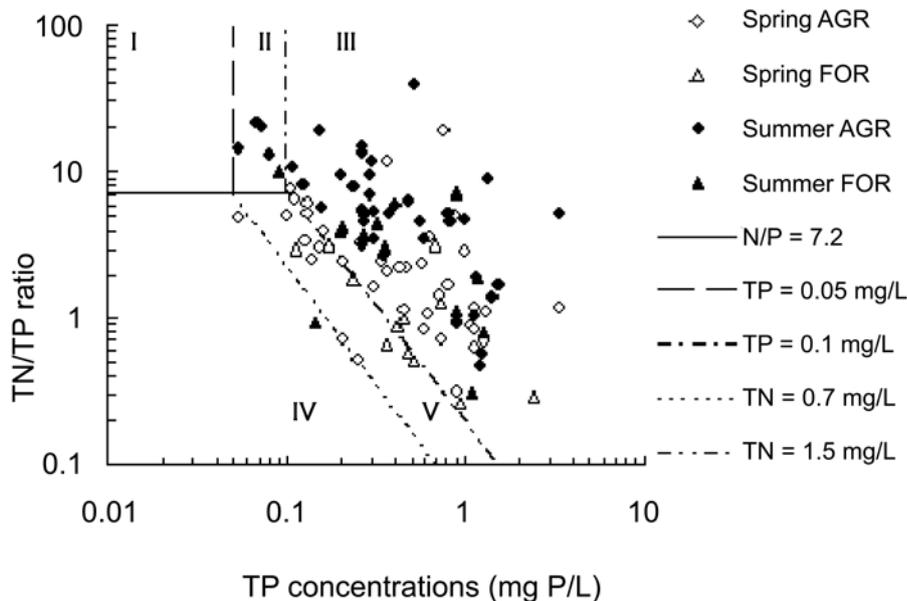


Fig. 4. Total nitrogen per total phosphorus ratio (TN/TP ratio) vs. total phosphorus concentration (TP) in forested (FOR, $n = 24$) and agricultural (AGR, $n = 82$) catchments in spring and summer samples. The lines indicate potential for N or P limitation. Zone I: P is likely to be limiting, zone II: P may be limiting for part of the growing season, zone III: neither N nor P is likely to be limiting, zone IV: N is likely to be limiting, Zone V: N may be limiting for part of the growing season (modified from Mainstone et al. 1995).

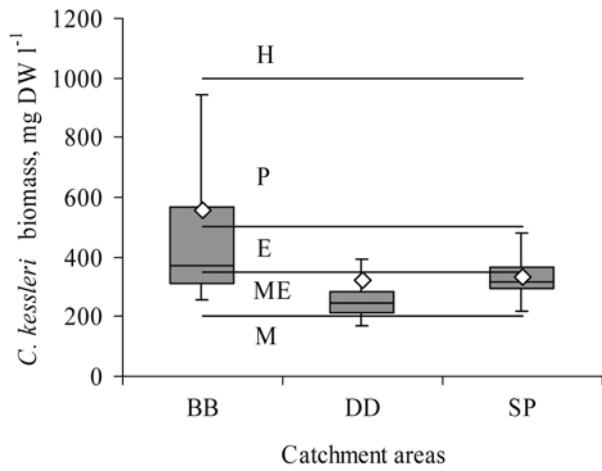


Fig. 5. Biomass integral of *C. kessleri*, mg dry weight l⁻¹ (mg DW l⁻¹), in spring and summer control water samples from the Danube-Drava (DD) and the Biđ-Bosut (BB) agricultural catchment areas, as well as from the river Spačva (SP) forested catchment area. Boxplots show median (line), mean (point), quartiles (boxes), non-outlier range (whiskers) and outliers. Borderlines of trophic conditions for mesotrophy (M), mesoeutrophy (ME), eutrophy (E), polytropy (P) and hypertrophy (H) are based on Žáková (1986).

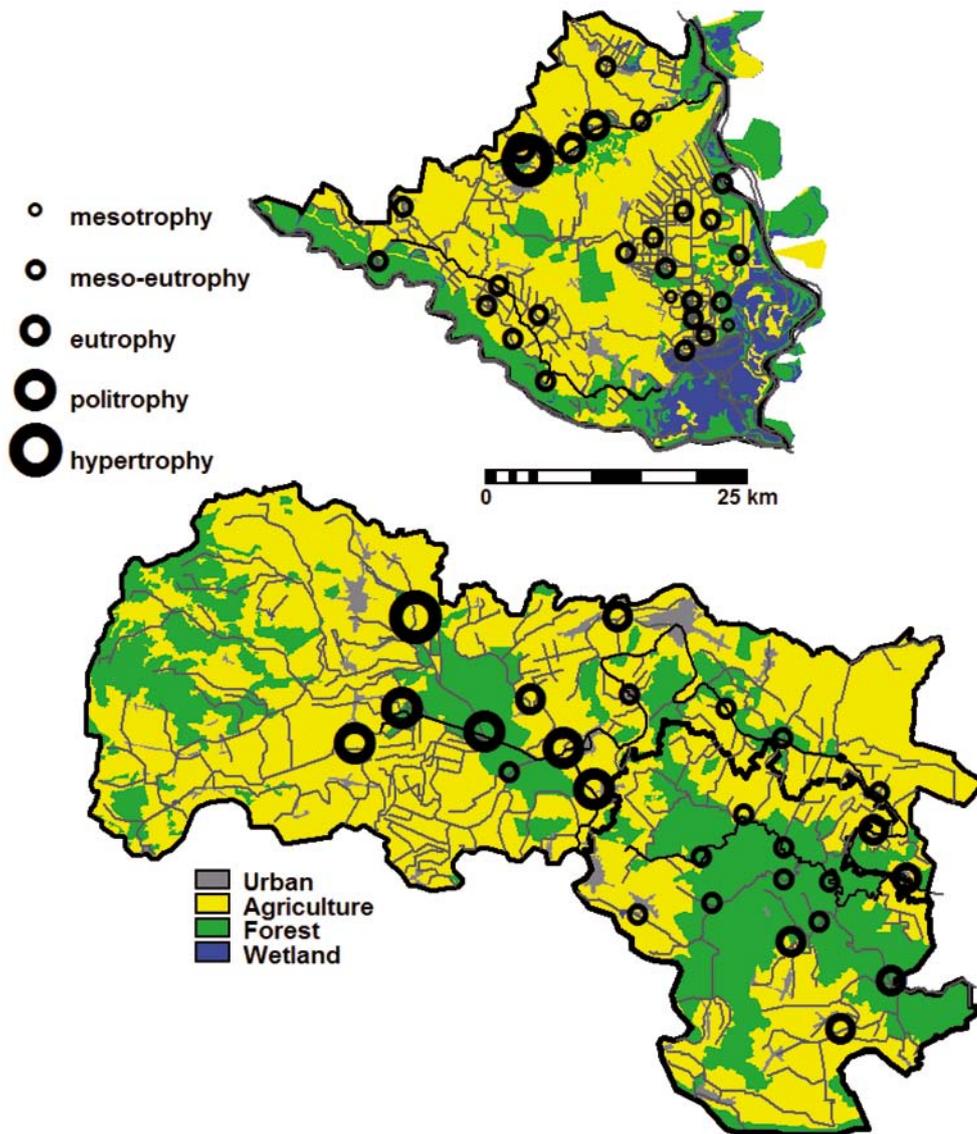


Fig. 6. The geographical distribution of the trophic conditions in the investigated area based on the results of bioassay (Integral of *C. kessleri* biomass in mg DW l⁻¹).

limiting primary production in waters of investigated forested and agricultural catchments. Fig. 4 plots N/P ratio (using total nitrogen and total phosphorus concentrations) against the total phosphorus concentrations of watercourses draining forested and agricultural catchments during spring and summer sampling. According to Mainstone et al. (1995), the graph has been divided into five zones based on a threshold N/P ratio and TP concentrations, as well as TN concentrations (plotted as a function of N/P ratio and TP thresholds). The graph indicates that neither N nor P is likely to be limiting algal growth in most of the summer samples regardless of N/P ratios. In addition, phosphorus is in surplus to algal requirements, while N may be limiting growth in part of the growing season (some spring samples).

Algal bioassay

The algal bioassay (Figs 5 and 6) showed that the growth potential differed significantly among catchments ($\chi^2 = 35.12$, $p < 0.0001$) while there was no significant difference between spring and summer samples (Wilcoxon test, $p > 0.05$). The results of multiple pair-wise analyses confirmed that trophic potential was significantly higher ($p = 0.002$ and $p = 0.02$) in the Bid-Bosut catchment than in the Danube-Drava and the river Spačva catchment.

The highest values of *C. kessleri* biomass were determined at sites with high values of nutrients (i.e. sites in the Danube-Drava catchment significantly influenced by wastewaters, Fig. 6). Mean and median values of *C. kessleri* biomass (556 and 374 mg DW l⁻¹) in water samples from the Bid-Bosut catchment (the highest percentage of arable land) pointed out to mostly eutrophic and polytrophic conditions (high eutrophication). In the surface water samples from the intensive agricultural land use in the Danube-Drava and samples from the forested land use in the river Spačva catchment, mean and median values of *C. kessleri* biomass (245 and 316 mg DW l⁻¹, 332 and 316 mg DW l⁻¹) indicated mesoeutrophic conditions (Figs 5 and 6).

In order to determine whether, and to what degree, bioassay results are related to nutrient concentrations in the original water samples, correlation coefficients were calculated. Statistically significant correlations (Kendal τ , $p < 0.05$) were found for the control growth rate of *C. kessleri* (bioassay without enrichment) with TN (0.19), NH₄-N (0.30), TP (0.33) and o-PO₄ (0.39) concentrations. Therefore, the algal growth potential in these waters appears to be determined by the amount of available forms of both nitrogen and phosphorus.

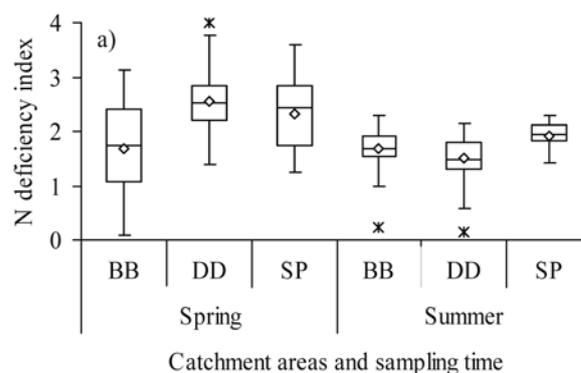


Fig. 7. Nitrogen deficiency index of *C. kessleri* in spring and summer water samples from the Danube-Drava (DD), the Bid-Bosut (BB) agricultural catchments and from the river Spačva (SP) forested catchment. Boxplots show median (line), mean (point), quartiles (boxes), non-outlier range (whiskers) and outliers.

In nutrient enrichment experiments, the N deficiency index was highest in spring samples from the Danube-Drava catchment and decreased with increasing total nitrogen concentrations in the summer (Figs 2d and 7).

N deficiency was very rare in the Spačva River catchment (statistically significant N deficiency was determined only in 4% of water samples) and occurred mainly in spring water samples. Similarly, in the Danube-Drava agricultural catchment 15% of spring water samples showed statistically significant N deficiency, as compared with no deficiency determined in summer samples. The Bid-Bosut catchment showed no signs of N deficiency. A non-parametric correlation analysis, which is not sensitive to outliers, indicated a significant ($p < 0.05$) negative correlation between N deficiency and NH₄-N (Kendall $\tau = -0.22$), KN ($\tau = -0.34$), TN ($\tau = -0.42$) concentrations, and the TN/TP ratio ($\tau = -0.27$). Phosphorus deficiency was occasionally determined (P deficiency index ranged from 0.02 to 2.3, with mean and median 0.7 and 0.6) and has not been designated as significant (results not shown).

Discussion

The observed variability in many of the physical and chemical constituents, in watercourses of eastern Croatia, can be attributed to the significant influence of both catchment land use and sampling time. The physical and chemical properties of water in lotic ecosystems are determined by climate, topography, soil

characteristics and land use of the terrestrial watershed (Turner et al. 2003, Johnson et al. 2009, Rothwell et al. 2010). Also, nutrient enrichment has been accelerated in many lotic systems because of increased nutrient loading from anthropogenic activities. According to Dodds & Oakes (2008), land use characteristics were most responsible for water quality variations among watersheds. Therefore, in a river basin various regions are expected to have different background concentrations of nutrients. On the other hand, nutrient levels are considered to be saturating in most lowland rivers for most of the time (Hilton et al. 2006). A large increase in nutrient concentrations was observed in the middle Danube River due to high loading from the Tisa, Drava, and Sava tributaries. On the other hand, a gradual increase of nutrient concentrations was observed in the upper and lower Danube due to lower loading from tributaries (Van Gils & Bendow 2000). Accordingly, in terms of potential ecological impact, a good water quality is needed in all tributaries in order to obtain a good quality in the main branch of large rivers.

Preliminary recommendations for threshold nutrient concentrations to be used as representing 'good status' in the recipients of the Danube Basin are between 1–1.5 mg N l⁻¹ for total N, and 0.02–0.08 mg P l⁻¹ for total P (Buijs 2003). Therefore, in relation to investigating ecological quality of watercourses in eastern Croatia, a section of Pannonian plain Danube River Basin, an analysis of phosphorus concentrations showed that 95 % of water samples from the investigated catchments failed to meet these environmental quality standards. Also, the mean total phosphorus concentration was found to greatly exceed the mesotrophic-eutrophic boundary of 0.075 mg l⁻¹ as proposed by Dodds et al. (1998). As for total nitrogen, one third of water samples from the investigated catchment failed to meet environmental quality standards or exceeded the mesotrophic-eutrophic boundary level of 1.5 mg l⁻¹ (Dodds et al. 1998).

In the investigated area within the triangle between Drava and Danube rivers, land use reflects underlying geology with a predominance of sustainable agriculture, whereas the catchment Biđ-Bosut with the river Spačva sub catchment has a majority of woodland areas, with traditional agriculture and associated activities also being relevant. The land use impact on water quality parameters in the investigated watercourses was most pronounced on concentrations of total and available phosphorus and ammonium. Unpolluted waters contain small amounts of ammonia and ammonia compounds, usually <0.1 mg N l⁻¹ (Regulations on

quality standards for waters 2010). Higher concentrations could be an indication of organic pollution such as from animal or human sewage and fertilizer run-off. Ammonia is, therefore, a useful indicator of organic pollution. In the investigated area, P inputs come primarily from three types of sources: sewage wastewater, septic tanks, and agriculture. Accordingly, in flowing waters of the Biđ-Bosut catchment (the highest percentage of arable land) water quality is very bad with exceedingly high phosphorus concentrations. Filipović et al. (2011) also reported stronger contamination of waters of eastern Biđ and Berava (Bosut tributaries) with nitrogen from ammonia, and a stronger pollution of groundwater with phosphorus. A possible explanation could be in the geology of the catchment, which is dominated by fertile alluvial soils that can become saturated with nutrients. N and P once moved from the soil could increase nutrient concentrations in runoff and recipient waterways to values above natural background concentrations. Therefore, as the capacity of the soil to hold P decreases, the concentrations of P in nearby watercourses increase. Besides, slow flowing watercourses accumulate phosphorus through internal sediment storage due to longer retention times.

Because both N and P have different spatial and temporal dynamics, different sources and different routes to watercourses, their relative contribution differs substantially from watershed to watershed (Smith et al. 1999). According to Mayer et al. (2002), higher percentages of rural and urban land use in the watershed, causes elevated N concentrations in rivers while predominantly forested watersheds have relatively low concentrations of riverine nitrate. Forest watercourses are thus expected to have a higher water quality. Accordingly, only moderate signs of distortion resulting from anthropogenic activity were observed in flowing waters of forested catchment, in the river Spačva basin. In most of the investigated watercourses, inorganic and organic N displayed contrasting seasonal patterns, with higher concentrations of organic forms in the summer. Organic nitrogen is naturally subjected to the seasonal fluctuations of the biological community because they are usually formed in water by phytoplankton and bacteria, and cycled within the food chain. Also, increased concentrations of organic nitrogen could indicate pollution of surface waters. Other studies have also observed higher summer concentrations of organic N in streams draining both forested and agricultural catchments (Arheimer et al. 1996, Turner et al. 2003). According to Chapman et al. (2001), higher summer concentrations of organic forms of nutrients are probably reflecting an increased

production within the channel rather than an increase in allochthonous inputs.

Based on the low N/P ratios and the overall high TP concentrations, nitrogen is expected to limit primary production in watercourses of both agricultural and forested catchment areas at the beginning of the growing season. Nitrogen limitation of freshwaters may be found in surface waters with high P concentrations due to anthropogenic activities or due to P rich soils (Flaig & Havens 1994, Havens et al. 2003). In particular, N limitation is induced by substantial amounts of non-treated domestic wastewater or animal waste, which have low N/P ratios and high concentrations of P (Welch et al. 1992). However, there is also a reason to suspect that N may not be limiting in eastern Croatia watercourses with high P concentrations since background dissolved inorganic nitrogen concentrations are also moderately high (more than 60 % of sites had dissolved inorganic nitrogen well above limiting level, $> 0.2 \text{ mg N l}^{-1}$). The results indicate that the N/P ratio is not a reliable indicator of nutrient limitation since ambient concentrations were obviously sufficient to preclude nutrient limitation. This could also be the cause of the weak relationship between nutrient concentrations and algal biomass in the investigated watercourses. In fact, phytoplankton in flowing waters receives much less attention because they are low in abundance. Also, in unusually slow flowing embayments, phytoplankton concentrations can be more than forty times that in the main stem of the river (Reynolds & Descy 1996). However, highly variable factors other than nutrients, such as shading by macrophyte vegetation, water level fluctuations or grazing intensity may provide an explanation for the lack of predictive nutrient algal relationship.

The algal bioassay showed greater N deficiency index in spring water samples of the forested catchment in the Spačva basin and of the Danube-Drava agricultural catchment. On the other hand, N deficiency was not determined in summer samples. P deficiency was rarely determined. Therefore, N deficiency is connected with the background N concentrations and not P surplus. In the Danube-Drava agricultural catchment, as expected, nitrogen deficiency of *C. kessleri* growth decreased from spring to summer following the N availability gradient. A higher degree of N limitation in spring samples can indicate that N was indeed deficient at the start of the growing season since plants may have removed available N at higher rates than they removed P (Verhoeven et al. 1990). In addition, P can be mobilized from the sediments. Thus, the nutrient enrichment experiment confirmed that nu-

trients are not limiting for algal growth in the studied watercourses.

In the algal growth potential test, watercourses of the Danube-Drava (agricultural land use) and the river Spačva (forested land use) catchments showed a lower trophic potential than watercourses of the Biđ-Bosut catchment (the highest percentage of arable land). The Biđ-Bosut watershed is rural and more populated with highly represented traditional agriculture. Therefore, septic tank inputs can quickly be transported to watercourses through hydrological events. On the other hand, because of the reduced intensity of agricultural production (during the war and post-war period from 1991 to 1998, and since land is still contaminated with mines and unexploded ordnance), a long-term water quality research in the Danube-Drava catchment area shows a positive trend in water quality improvement (Barčić & Panić 2011, Barišić-Lasović 2011). Nevertheless, the authors also state that it is certainly not a permanent condition. Also, one reason is the relative size of catchment areas, the Biđ-Bosut catchment is twice as large, and so the area under cultivation is larger. These results are in agreement with measured nutrient concentrations in the water samples and in the nutrient enrichment experiment.

The importance of measuring algal growth potential is in the difference between nutrients determined by chemical analysis and nutrients that are available for algal growth. By defrosting the water sample particulate-bound, N and P are lysed from particles. This leads to more dissolved nutrients in bioassay water than in the river water initially. Therefore, bioassays evaluate the effect of water as a complex of substances on the development of an experimental alga where increased biomass indicates increased nutrition and production potential, whereas decreased biomass indicates the opposite. Other advantages of miniaturized nutrient enrichment bioassays are in their ability to process a large number of samples faster and with greater reliability through direct measurement of algal biomass. When comparing algal growth potentials from a number of widely different water sources there are also advantages in using a single species of alga. *C. kessleri* belongs to a group of ubiquitous algal species. It is a unicellular green alga whose growth can be measured easily and accurately and which responds to growth substances uniformly (Lukavský 1992). On the other hand, using a single species bioassay may or may not be useful for determining nutrient limitation of *in situ* phytoplankton since different phytoplankton communities have different nutrient requirements. An additional limitation of the bioassay is that it is con-

ducted under laboratory conditions rather than field conditions and it is performed over a number of days under controlled conditions. Because of this, factors other than nutrient limitation may be minimised or magnified (Holland et al. 2004). Also, in the treatments with no nutrient amendment, nutrient limitation is induced over the course of time as nutrients gradually become depleted. Nevertheless, some algae are capable of concentrating certain nutrients in excess of their current need when they are grown in media with excess nutrients; therefore, they need to be starved prior to the experiment. This intracellular nutrient storage may reduce bioavailability of resources involved in algal growth limitation. Therefore, these facts must be taken into account in selecting the culture media, in determining the type and amount of algae to use, etc. A few other limitations of this simple experimental procedure emerged. The bioassay focuses on two nutrient concentrations (added and control), which may or may not be applicable to the physical environment conditions. The identification of statistical differences between treatments may not have a biologically significant effect. Quantitative estimates of the magnitude of nutrient limitation (e.g., Downing et al. 1999, and Osenberg et al. 2002) can be more productive since they can show how much the system might respond to changes in nutrients. Nevertheless, nutrient enrichment bioassays only indicate limitation under the conditions in which they are run. Other factors such as temperature, light, and grazing also need to be accounted for (Elser & Kimmel 1986, Beardall et al. 2001).

Conclusion

Nutrient loading from catchments with different land uses exhibits considerable uncertainties, making temporal and spatial monitoring of water quality essential for predicting the effects of either nutrient increases or reductions. Since temporal variations may have resulted in much of the variation of water quality parameters, seasonal influences must be considered when spatial patterns of water quality in a river basin are being examined. Overall, in the river Spačva catchment only moderate signs of distortion resulting from anthropogenic activity were observed in the flowing waters of a mainly forested catchment. On the other hand, agriculture in eastern Croatia is recognised as a potential source of nutrient pollution to watercourses and represents the most significant risk for their eutrophication. Watercourses draining the agricultural and rural watersheds of the Biđ-Bosut catchment are

severely damaged or at high risk of eutrophication, while some watercourses draining agricultural watersheds in the Danube-Drava catchment even show nutrient deficiency (primarily nitrogen). In order to protect the environment, there is a need to improve the quality of ecologically sensitive agricultural watercourses since good water quality is needed in both small and large rivers. In order to reverse the effects of eutrophication it is becoming increasingly crucial to better understand if, where and how much N and P is limiting production in freshwater ecosystems (Elser et al. 2007). Nutrient enrichment bioassay can be useful in assessing trophic loads and nutrient limitation of watercourses, by providing detailed information that is necessary to mitigate the impact of eutrophication, with the aim of sustaining a better water quality from the Danube and Sava rivers.

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GENERAL DISCUSSION

The question what limits primary production is essential to aquatic ecology. This question is interlaced with difficulties and generalization. Firstly, what is primary production? Primary production by its definition is the synthesis and storage of organic molecules during the growth and reproduction of photosynthetic organisms. It can mean gross photosynthesis, the rate of cell division or the accumulation of cell material. Secondly, are factors that limit production rate limiting (temperature, irradiance, carbon, nutrients or toxic substances) or yield limiting (nutrients: nitrogen, phosphorus, and silicate in some occasions specific for diatoms, or perhaps trace minerals). Planktonic algae, as the dominant primary producers, play an essential role in the aquatic environment. They are the basic link and a key functional group of organisms in aquatic food chains. The rate of algal biomass accumulation may be determined by the supply of nutrients while the accumulation of algal biomass may be determined by, e.g., zooplankton grazing. The importance of limiting factors also changes from season to season, as well as from one water body to another. Responses to the initial question are also clearly dependent to the species and the time scale considered. Nevertheless, algal biomass has increased in many waters, flowing, and standing, resulting in problems from decreased transparency to the proliferation of harmful algal blooms. A common strategy to reduce and control the appearance of algal blooms is to reduce nutrient

loads entering aquatic ecosystems. Therefore, it is essential to obtain reliable information, and in particular, to identify nutrients that can be control and will limit further development of phytoplankton. Algae are especially suitable for bioassays because of their sensitivity to environmental pollution and their abundance in aquatic systems. In addition, they reflect only the properties of the ambient water.

Influence of hydrologic régime

In considerations of water quality problems, riverine floodplains are part of the landscape that has been largely ignored. Floodplains are flood-dependent ecosystems that are integral parts of the river. In river-floodplain ecosystems, hydrological régime is the most influential environmental variable that determines the existence, production, and interactions of biota (Junk et al., 1989; Rennella and Quirós, 2006). It varies with the climatic zone and geomorphological features of the river and its catchment areas. The size of water bodies, their location, and hydrological connectivity with the main river affect physical and chemical conditions within the floodplain (Furch and Junk, 1993; Tockner et al., 1999; Junk and Wantzen, 2004). Studies of various river floodplains in the last two decades have shown a causal relationship between alternations of hydrological conditions and the functioning of these ecosystems.

In temperate regions, the flood pulse of large river floodplains is less predictable, and floods often occur during the growing season. Flood protection has eliminated most large river floodplains; along the Rhine River and on the upper Danube River only small remnants of the formerly extended floodplains are left and are now under the protection. Major floodplains exist on the middle and lower Danube and in the Danube Delta (Čížková et al., 2013). The characteristic ecology of floodplain channels and lakes in the investigated Danube floodplain area in eastern Croatia (Middle Danube) strongly relates to variations in frequency and duration of inundation. Based on hydrological records, the spring season and the beginning of summer is characterized by higher water level of the Danube River, while the beginning of autumn (September and October), as well as the entire winter, is characterized by lower water levels (Mihaljević et al. 1999). The high-water period can extend into the summer months when the water from melting snow combines with water from heavy summer precipitations in the Danube catchment area, especially in the Alps and Carpathians. Such heavy floods occurred, e.g., in 2002 and 2005 (Peršić et al., 2010).

According to most regional climate change models, the summer maximal temperatures are likely to increase. Annual precipitation, on the other hand, is expected to increase in northern Europe, but decrease in most of southern Europe. Consequently, the frequency and intensity of summer droughts will most likely increase (Čížková et al., 2013). Because of the extremely low water level of the Danube River, during the investigated period in 2003, there was no regular alternations of high and low water periods specific for the wetland area in Nature Park Kopački rit. During these drought conditions, sufficient nutrients were available to support algal growth and none of the analysed nutrients was limiting growth (Chapter 1). Furthermore, the results

emphasized the importance of ammonium as a primary nitrogen source for phytoplankton in shallow wetland waters. The established alkaline values of pH and low values of dissolved oxygen in shallow wetland waters resulted in high ammonium concentrations and internal loading of phosphorus (Kinnear and Garnett, 1999; Kisand and Nöges, 2003; Søndergaard et al., 2003). As dry conditions continued, nutrient enrichment bioassay indicated N and P limitation of similar magnitude suggesting possible co-limitation. According to Junk et al. (2013), summer drought is probably the most significant stressful effect of changing climate on river floodplains and wetlands in temperate zone.

The abundance of nutrients determined during flooding conditions, on the other hand, came from the inflow of flooding waters and wetland sediment re-suspension (Kisand and Nöges, 2003). At the same time, the highest values of the trophic state were determined in algal growth potential bioassay when water was sampled during flooding conditions.

Since nutrient concentrations in the Danube wetland waters, in great part, depend on flood dynamics, further investigations were aimed at the comparison of trophic conditions and nutrient deficiency during regular periodic flooding (Chapter 2) and prolonged flooding conditions (Chapter 3). In Chapter 2, it was shown that changes in N/P ratios resulted from an independent effect of inter-annual variability in the water level. In addition, fluctuations of the Danube water level differently influenced physical and chemical characteristics of the investigated floodplain lake and channel waters. Consistent actual N deficiency in lake samples determined by nutrient enrichment bioassay corresponded with DIN/TP ratios observed in lake water. In the experiment in which the channel water samples were enriched, the intensity of limitation significantly decreased during the low water period.

During high water level, N limitation was found in most of the samples. These results are in agreement with the conditions observed in the channel water. Therefore, N limitation predominated in these riparian floodplains.

As for trophic conditions according to Carlson (1977), they changed from hypertrophy (in low water period) to eutrophy (in high water period) and reflected the importance of the hydrological régime as a factor, which can affect the trophic status. On the other hand, the trophic conditions based on the values of *C. kessleri* total biomass in water samples from both sites were lower in the dry period, and increased as the water level increased.

Why did *C. kessleri* show a greater growth potential of investigated waters and N limited conditions in the same time? In this experiment, the rate of accumulated *C. kessleri* biomass was determined by the supply of available nutrients during exponential growth while the accumulation of *C. kessleri* biomass, or the final yield, was determined by the total amount of nutrients present in the investigated waters. Besides, algae can maintain growth higher than expected if they have recently had a high nutrient input. This may be the reason why observed growth sometimes exceeds predicted growth at low dissolved nitrogen concentrations.

A decrease in connectivity between the floodplain lake and its parent river (i.e., absence of flooding in 2003) caused a decrease in total nitrogen concentrations. Consequently, nitrogen fixation increased (dominance of cyanobacteria in 2003), but without a significant effect on bioavailable nitrogen concentrations (NO_3 and NH_4). On the other hand, an increase in the duration of hydrological connectivity provided a continuous input of high amount of total nitrogen. However, flooding conditions reflected in reduced ammonium-N and orthophosphate concentrations, as well as reduced phytoplankton biomass. The

inverse relationship between Danube water level and trophic status based on phytoplankton Chl-a and transparency, and positive relationship between Danube water level and the trophic status based on nutrients suggest that hydrological connectivity is a dominant factor, which influences eutrophication process in floodplain areas. Therefore, the characteristic ecology of floodplain channels and lakes strongly relates not only to variations in frequency of floods (Chapters 1 and 2) but also to variations in duration of flooding (Chapter 3).

In the Neotropical riverine floodplains (Furch and Junk, 1993; Thomaz et al., 2007), and floodplains of northern temperate zone (Tockner et al., 1999; 2000; Van der Nat et al., 2002; Langhans and Tockner, 2006; Pithart et al., 2007; Mihaljević et al., 2010) spatial heterogeneity of water chemistry and phytoplankton biomass appears with decreasing water levels. This heterogeneity of aquatic habitats is emphasized by the following surface separation of water bodies. Water entering and exiting the floodplain forms a characteristic relief. That is why in the floodplain area one can find dozens of different habitats. The most accurate classifications of aquatic habitats with respect to geomorphological, hydrological, and ecological analyses has been made for the Rhone River (Amoros et al., 1982; Castella et al., 1984; Ward and Stanford 1995), and the Danube River (Ward and Tockner, 2001; Ward et al., 2002; Schwarz, 2005). According to Tockner et al. (1998) and Ward and Tockner (2001), floodplains of the Danube River, without their natural flood disturbances, have a tendency towards geographical and temporal uniformity with a resulting reduction in biodiversity. Based on hydrological typology, the connectivity gradient in the investigated river floodplain system extended from the main river flow (Eupotamal A) to the edge of the floodplain (Paleopotamal). Therefore, spatial variability of limnological characteristics as

one of the main features of river-floodplain system is greatly influenced by fluctuations of the water level. However, we know little about the temporal and spatial distribution of productivity within floodplains, as well as what controls its distribution (Ahearn et al., 2006).

In Chapter 3, among the investigated sites, a gradient was observed in physical and chemical conditions from the main channel (Eupotamal A) towards the marginal part of the floodplain (Paleopotamal). The abiotic and biotic characteristics (e.g., N-NO₃ concentrations, lower transparency, higher Chl-a concentrations) of the Danube River and its active side-arms (Eupotamal waters) suggests that, at these sites, the light limitation, rather than nutrient limitation, is more likely the growth regulator. As reported by Hamilton and Lewis (1990) and Knowlton and Jones (1997), light limitation of algal growth prevails in floodplain waters with high connectivity. On the other hand, higher transparency, lower depth, and lower N-NO₃ concentrations in Parapotamal and Paleopotamal waters are consistent with possible N limitation of algal growth. Therefore, the long inundation periods along with the regular pulsing connectivity will control the input of nutrients and significantly affect spatial heterogeneity of river floodplain system.

According to the nutrient enrichment bioassay, inorganic nutrients in Eupotamal waters were sufficient to support the higher growth rate of *C. kessleri* while potential N limitation was determined in Parapotamal and Paleopotamal waters that had N/P ratio < 14. Furthermore, a spatial expansion of N limitation was observed as flooding continued. For example, at the beginning of the flood period N limitation was determined only in Paleopotamal waters, while, at the end of the flood period, N limitation was determined along the lateral dimension from the main stream of the Danube River (Eupotamal A) towards the edge of

floodplain (Paleopotamal). As explained in Chapter 3, the water level was high, but the hydrological action was probably low. Therefore, Danube water was not entering the floodplain. This could have enabled biota to deplete nitrate imported during the flooding making the floodplain N limited.

Distribution of nutrients in different habitat types of the Danube River floodplain, therefore, strongly depends on the retention time of floodwaters and subsequent nutrient uptake by phytoplankton, bacterioplankton, as well as macrophytes. Hence, this approach to the typology of Danube River floodplain habitat types should be further developed using biological, geomorphologic, and other hydrodynamic parameters, in addition to, hydrological connectivity.

Influence of land use

The physical and chemical properties of water in lotic ecosystems are according to numerous authors (e.g., Turner et al. 2003, Johnson et al. 2009, Rothwell et al. 2010), determined by climate, topography, soil characteristics and land use of the terrestrial watershed. According to Dodds and Oakes (2008), land use characteristics are the most responsible for water quality variations among watersheds. However, nutrient enrichment has accelerated in many lotic systems because of increased nutrient loading from anthropogenic activities. In Chapter 4, the observed variability in many of the physical and chemical constituents was attributed to the significant influence of both catchment land use and sampling time. Accordingly, water quality of waters in the catchment with the highest percentage of arable land was determined to be very bad with extremely high phosphorus concentrations. On the other hand, only moderate signs of distortion resulting from anthropogenic activity were observed in

flowing waters of forested catchments. However, watercourses draining agricultural watershed in the Danube-Drava catchment showed even nutrient deficiency (primarily nitrogen).

Based on the low N/P ratios and the overall high TP concentrations, nitrogen was expected to limit primary production in watercourses of both rural and forested catchment areas. Nevertheless, there was also a reason to suspect that N may not be limiting in eastern Croatia watercourses. Furthermore, the results indicated that N/P ratio was not a reliable indicator of nutrient deficiency since ambient concentrations were sufficient to preclude nutrient limitation.

Historically, N has been considered to be the primary growth-limiting nutrient in marine ecosystems, and phosphorus has been considered to be the principal limiting nutrient in freshwaters. However, this paradigm has recently been called into question since there is evidence of both N and P limitation in lakes and streams (Elser et al. 1990; Elser et al. 2007; Lewis and Wurtsbaugh 2008), as well as frequent phosphorus limitation in marine waters (Downing et al. 1999; Elser et al. 2007). In addition, Downing et al. (1999) observed in bioassay experiments that phosphorus limitation was strongest in unpolluted waters while N limitation was more frequent in relatively polluted waters.

Therefore, N deficiency was connected with the background N concentrations and not P surplus. In addition, the results showed significant N limitation (N deficiency) only occasionally in spring water samples and no nutrient deficiency in summer water samples of forested catchments. Similarly, in the Danube-Drava agricultural catchment 15% of spring water samples showed to be N limited, as compared with no deficiency determined in summer samples. In all samples, P deficiency was exceptionally rare. In the Danube-Drava agricultural catchment, as expected, nitrogen limitation of *C. kessleri* growth decreased

from spring to summer following the N availability gradient. Higher level of N deficiency in spring samples indicated that N was indeed deficient at the start of the growing season since plants may have removed available N at higher rates than they removed P (Verhoeven et al. 1990). In addition, P can be mobilized from the sediments. Thus, nutrient enrichment experiment confirmed that nutrients were not limiting the algal growth in studied watercourses. Furthermore, in the algal growth potential test, watercourses of the Danube-Drava (agricultural land use) and the Spačva River (forested land use) catchments showed a lower trophic potential than watercourses of the Biđ-Bosut catchment (the highest percentage of arable land).

Large rivers and their tributaries along with floodplains all over Europe tend to be affected by large-scale eutrophication occurring under the impact of agricultural and forestry management of their catchments, effluents from human settlements and industry, and atmospheric deposition. We now know that a proper understanding of nutrients, which limit phytoplankton growth, is a prerequisite for successful eutrophication control. The problem of water pollution in lowland forest and wetland ecosystems stands out in particular because these areas are usually located in the near-pristine environment. In these areas, governments should invest more in measures that prevent impact of extreme floods, droughts and pollution to successfully overcome global climate change. Assessment of resources limiting algal growth and evaluation of trophic status of rivers and wetlands, which present hydrological irregularities, must incorporate different approaches in order to provide acceptable understanding of the problem.

Advantages, limitations, and implications of algal bioassay

Finally, at the end of this discussion, the synthesis of this thesis core issue is carried out, focusing on

the advantages, limitations, and implications of algal bioassay procedure.

Some studies have shown that instantaneous measurements of nutrient concentrations are not reliable predictors of growth under nonequilibrium conditions (Sommer, 1985; Grover, 1991). An accurate determination of phytoplankton nutrient status, while possible in laboratory cultures, showed to be extremely difficult in natural populations. In the real environment conditions, the phytoplankton can be dominated by species that can be difficult to grow in laboratory cultures or poorly represented (Tilman et al., 1982). Because phytoplankton represent a community rather than a single alga, different algal species may dominate at different nutrient concentrations. For example, freshwater cyanobacteria often prevail at N/P ratios from five to ten, while a high N/P ratio (>29) stimulates growth of green algae (Schindler, 1977; Smith, 1982). Therefore, using a single species bioassay may or may not be useful for determining nutrient limitation of *in situ* phytoplankton.

In the laboratory, growth conditions are controlled by constant temperature, light, defined chemical concentrations, and known preconditioning. These types of results are fundamental measurements that can inform us about physiological and community ecology. Algal bioassays are simple and allow observing multiple generations. Only a few algal species have been found suitable for algal bioassays. A unicellular green alga *C. kessleri* that was used in these studies belongs to a group of ubiquitous algal species and is amendable to laboratory cultivation. This alga has a wide tolerance to different environmental conditions and its growth can be measured easily and accurately (Lukavský, 1992). Nevertheless, algae cultivated in laboratories are often “weed” species with high growth rates and may not be representative of populations found in investigated waters. However, when comparing algal growth

potentials from a number of widely varying water sources there are advantages in using single species bioassays.

The miniaturization of the algal growth bioassay also has several advantages, and one of the main advantages of miniaturization is the ability to process a large number of samples faster and with greater reliability through a direct measurement of algal biomass, by measuring the optical density directly in microplates using a plate reader. Therefore, microplate-based assays offer the largest advantages in terms of cost, simplicity and the large amounts of replicates. However, during the development of the experimental procedure for nutrient enrichment bioassay, a few limitations of this simple experimental procedure emerged.

Firstly, in the treatments with no nutrient amendment, nutrient deficiency is increased over the course of time as nutrients gradually become depleted. In addition, some algae are capable of concentrating certain nutrients in excess of their current demand (when they are in media with excess nutrients). This intracellular nutrient storage may reduce bioavailability of resources involved in algal growth limitation. Therefore, they need to be starved prior to the experiment. These facts must be taken into account in selecting the culture media, in determining the type and amount of algae to use, etc.

Secondly, bioassay focuses on control sample and added nutrient concentrations, which may or may not be applicable to the physical environment conditions. The detection of statistical differences between treatments may not have biologically significant effect. However, quantitative estimates of the nutrient deficiency indicator (e.g., Downing et al., 1999; Osenberg et al., 2002) may be more beneficial since these estimates show how much the system might respond to changes in nutrients. To control or manage eutrophication, we must be able to quantify nutrient loading and to predict the

response of individual water bodies to changes in nutrient concentrations.

Thirdly, laboratory bioassays are performed over a number of days under controlled laboratory conditions rather than field conditions, that is, they lack nutrient resupply from the water column, suspended particles or sediment. Because of this, factors other than nutrient limitation may be minimised or magnified (Holland et al. 2004). Therefore, extrapolating the results obtained by laboratory bioassay to time or parts of water bodies, other than the time and place of sampling, should be considered with caution.

Nevertheless, the importance of measuring algal growth potential in bioassay remains in the difference that is determined between nutrients measured by chemical analysis and nutrients

actually available for algal growth. For example, by defrosting the water sample, particulate-bound N and P are lysed from particles. This leads to more dissolved nutrients in bioassay water than in the lake or river water initially. Therefore, bioassay evaluates the impact water, as a mixture of substances, has on the development of an experimental alga. As a result, increased biomass indicates increased nutritional conditions and yield capacity, whereas decreased biomass indicates the opposite. At best, bioassay probably only provides us with the knowledge of potentially limiting nutrients if growth is to continue in the absence of limitation by other factors. Still, factors that need to be accounted for are temperature, light, and zooplankton grazing (Elser and Kimmel, 1986; Beardall et al., 2001).

CONCLUSIONS

The main aim of this thesis was to provide information on the temporal and spatial variation of resource limitation on algal growth rates, in order to create a scientific basis for evaluating regional strategies of nutrient management in the investigated catchments.

Trophic conditions based on algal growth potential were inversely related to trophic conditions based on phytoplankton chlorophyll-*a* concentrations (**Chapters 1-3**). Therefore, in defining the trophic state both parameters should be considered as complementary. Additionally, algal biomass and the overall ecosystem productivity were controlled not only by the type, but also by the intensity of nutrient limitation. The basis of eutrophication management is often the limiting nutrient concept. Thus, the degree of algal growth response to nutrient enrichment led to a sharper definition of the concept of nutrient limitation by providing a biologically meaningful measure that is comparable across studies.

The location of floodplain water bodies regarding the river (spatial dimension) and hydrological

connectivity between the river and its floodplain (temporal dimension) along with the retention time of floodwaters and nutrient uptake by biota were the most significant factors that influenced nutrient dynamics in the Danube river floodplain system (**Chapter 2 and 3**). A spatial gradient of phytoplankton chlorophyll-*a* concentration was observed during the stagnating water flow while uniformity of chlorophyll-*a* concentrations among habitat types followed the increase of water level. In addition, a gradual decrease in algal growth potential was determined from the main river channel towards the edge of floodplain with the subsequent increase in the degree of nitrogen limitation. Additionally, nitrogen limitation expanded spatially as inundation continued (**Chapter 3**). However, detected nitrogen limitation throughout the floodplain more likely indicated that eutrophic water bodies have been over-fertilized with phosphorus.

In order to provide appropriate identification of the problem when assessing water quality and trophic status of rivers and wetlands, both characterised by

In addition to contributing to our basic knowledge of the structure and functioning of aquatic ecosystems, studies on nutrient limitation have a direct application to water quality management. Moreover, systematic studies involving water quality assessment at catchment scale are necessary for the development of integrated water quality management. Because financial cost and the type of nutrient reduction strategies vary considerably with the targeted nutrient, scientific foundations are needed for management decisions concerning eutrophication control.

hydrological irregularities, different approaches must be combined. Nutrient enrichment bioassays constitute a useful tool to provide complementary and necessary information to draw the strategies for water quality management.

In **Chapter 4**, temporal variations resulted in many of the variations in water quality parameters. Nutrient loading from catchments with different land uses exhibited considerable uncertainties, making temporal and spatial monitoring of water quality essential for predicting the effects of either nutrient increases or reductions. Therefore, seasonal influences must be considered when spatial patterns of water quality in a river basin are being examined.

To conclude, an understanding of the variability of factors that control algal growth is globally important. In addition to contributing to the basic knowledge of the structure and functioning of aquatic ecosystems, studies of nutrient limitation have a direct application to water quality management. Furthermore, systematic studies involving water quality assessment at catchment scale are necessary for the development of integrated water quality management. Because financial cost of nutrient reduction strategies varies considerably with the targeted nutrient, good scientific foundations are needed for management decisions concerning eutrophication control.

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SAŽETAK

Iako se većina istraživanja eutrofikacije bazira na lentičkim sustavima, sve veći problem postaje obogaćivanje hranjivim tvarima lotičkih sustava. Unos hranjivih tvari odraz je načina korištenja zemljišta u slivu (ratarstvo, stočarstvo, šumarstvo itd.), a glavni problem eutrofikacije rijeka predstavlja unos fosfora i dušika. Zbog smanjenog potencijala razrjeđenja (uslijed klimatskih promjena, regulacije vodotoka i sl.), unos fosfora kao i dušika u mnogim rijekama i njihovim pritocima Dunavskog sliva na kritičnoj je razini. Poimanje ograničavanja primarne produkcije dušikom i fosforom smatra se osnovom za istraživanje eutrofikacije, a podrazumijeva da je jedan ključni element ograničavajući čimbenik (limitirajući nutrijent) rasta algi u ekosustavu, da je rast algi proporcionalan dostupnosti tog elementa i da se kontroliranje eutrofikacije može postići ograničavanjem unosa tog ključnog elementa. Jačina limitacije nutrijentima u vodama Istočne Slavonije i Baranje određivana je u laboratorijskim uvjetima usporedbom prirasta laboratorijski uzgajane kulture algi bez i s dodanim hranjivim solima. Dodatak hranjive soli, koja je ujedno i ograničavajući čimbenik rasta testiranih algi, uvjetovala je značajno povećanje prirasta njihove biomase. Kao rezultat dobivena je mjerljiva vrijednost ograničavajućeg djelovanja određene hranjive tvari, koju je moguće uspoređivati s rezultatima drugih istraživanja.

Veći dio istraživanja ove doktorske disertacije proveden je u poplavnom području rijeke Dunav u sjeveroistočnoj Hrvatskoj. U **prvom i trećem poglavlju** istraživanja poplavnog područja obuhvatila su postaje raspoređene poprečno od glavnog toka rijeke Dunav prema rubnom dijelu njenog poplavnog područja u Parku prirode Kopački rit. U **drugom poglavlju** istraživanja su usmjerena na rubni dio Parka prirode Kopački rit uz nasip Dunav-Drava, a obuhvatila su postaje koje predstavljaju različita vodena staništa. U **četvrtom poglavlju** ove doktorske disertacije istraživanja kvalitete voda i procjene eutrofikacije obuhvatila su područje slivova baranjskih vodotoka u kojem dominiraju obradive površine sa intenzivnom poljoprivrednom proizvodnjom te vodotoke nizinskih poplavnih šuma slivnog područja Biđ-Bosut.

U **prvom poglavlju**, na temelju potencijala rasta alga (AGP) u vodama poplavnog područja Parka prirode Kopački rit, utvrđeni su mezotrofni do eutrofni uvjeti. Statistički značajna korelacija utvrđena je između AGP-a *C. kessleri* i koncentracije nitrata u istraživanim uzorcima vode. Omjer dušika i fosfora pokazao je da je dušik potencijalni ograničavajući čimbenik rasta algi. Značajno niže utvrđene vrijednosti TSI_{TN} od TSI_{SD} , TSI_{Chla} i TSI_{TP} također su ukazale na značaj dušika kao ograničavajućeg čimbenika primarne produkcije. Da bi utvrdili jačinu limi-

tacije nutrijentima, uspoređivan je prirast laboratorijski uzgajane kulture algi sa i bez dodanih hranjivih tvari tijekom 7 dana inkubacije. Dodatak hranjive tvari, koja je ujedno i ograničavajući čimbenik rasta testiranih algi, uvjetovao je značajno povećanje prirasta njihove biomase. Kao rezultat dobivena je mjerljiva vrijednost ograničavajućeg djelovanja određene hranjive tvari ($\Delta\mu$, d^{-1}).

U **drugom** su **poglavlju** istraživane varijacije u N i P limitaciji tijekom sušnog (2003.) i poplavnog razdoblja (2004.) u poplavnom području Parka prirode Kopački rit. Laboratorijskim biotestom utvrđena je promjena od nelimitirajućih uvjeta (tijekom sušnog razdoblja) ka N limitaciji (tijekom razdoblja plavljenja) u Kopačevskom kanalu, kao i stalna N limitacija na postajama Sakadaškog jezera. Omjer otopljenog anorganskog dušika i ukupnog fosfora bio je u korelaciji s rezultatima biotesta. Samo je ponekad utvrđena i limitacija fosforom. Hipertrofni (tijekom sušnog razdoblja) i eutrofni uvjeti (tijekom razdoblja plavljenja) ukazali su na važnost fluktuacija vodostaja kao čimbenika koji utječe na eutrofikaciju istraživanih lokaliteta u poplavnom području.

U **trećem** je **poglavlju** utvrđeno da dugotrajna plavljenja utječu na koncentracije hranjivih soli, osobito nitrata te značajno određuju prostornu heterogenost područja. Prema hidrološkom režimu i smanjenju povezanosti između uzorkovanih postaja u poplavnom području i rijeke Dunav, razlikovali su se različiti tipovi vodenih staništa (eupotamal, parapotamal i paleopotamal). Heterogenost vodenih staništa u poplavnom području prema koncentracijama Chl-a utvrđena je tijekom stagnirajućeg vodostaja, dok

je do efekta homogenizacije došlo porastom vodostaja tijekom plavljenja. Biotest je pokazao da nutrijenti u vodama Eupotamala (N/P omjer 16-22) podržavaju optimalan prirast *C. kessleri*, dok je potencijalna N limitacija utvrđena u vodama Parapotamala i Paleopotamala (N/P <14). Duljim zadržavanjem poplavnih voda utvrđeno je prostorno širenje N limitacije prema glavnom toku rijeke. Položaj vodnih tijela u poplavnom području u odnosu na glavni tok rijeke (prostorna dimenzija) i hidrološka povezanost između rijeke i njenog poplavnog područja (vremenska dimenzija), zajedno s trajanjem plavljenja i biološkim unosom, najznačajniji su čimbenici koji utječu na dinamiku nutrijenata u poplavnom području Dunava.

U **četvrtom** je **poglavlju** istraživan utjecaj korištenja zemljišta u porječju na kvalitetu voda u poljoprivrednom i šumskom području Istočne Hrvatske. S obzirom na nizak omjer koncentracija dušika i fosfora kao i visoke koncentracije fosfora, dušik je bio očekivani ograničavajući čimbenik rasta algi. Međutim, rezultati su pokazali da N/P omjer nije vjerodostojan pokazatelj limitacije nutrijenata. Metodom biotesta utvrđeno je da su vodotoci ruralnog i poljoprivrednog područja (porječje Biđ-Bosut) opterećeni nutrijentima. Umjereno opterećenje nutrijentima utvrđeno je u vodotocima s pretežno šumskim porječjem (Spačvanski bazen), dok je u vodotocima Baranjskih slivova povremeno utvrđena i limitacija dušikom.

Dobiveni rezultati ekoloških i fizioloških istraživanja omogućit će implementaciju metode biotesta za procjenu stanja trofije kao nužnog preduvjeta definiranja mjera za smanjenje opterećenja voda hranjivim tvarima.



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