Science of the Total Environment 573 (2016) 1460–1471

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Determination of the sources of nitrate and the microbiological sources of pollution in the Sava River Basin

Janja Vrzel [a,b], Branka Vuković-Gačić [c], Stoimir Kolarević [c], Zoran Gačić [d], Margareta Kračun-Kolarević [e], Jovana Kostić [d], Mustafa Aborgiba [c], Andreas Farnleitner [f], Georg Reischer [f], Rita Linke [f], Momir Paunović [e], Nives Ogrinc [b,g,*]

[a] Department of Geography, University of Munich, Luisenstraße 37, 80333 Munich, Germany
[b] Jožef Stefan International Postgraduate School, Jamova ulica 39, 1000 Ljubljana, Slovenia
[c] University of Belgrade, Faculty of Biology, Chair of Microbiology, Center for Genotoxicology and Ecogenotoxicology, Studentski trg 16, 11000 Belgrade, Serbia
[d] University of Belgrade, Institute for Multidisciplinary Research, Kneza Višeslava 1, 11030 Belgrade, Serbia
[e] University of Belgrade, Institute for Biological Research "Siniša Stanković", Bulevar despota Stefana 142, 11060 Belgrade, Serbia
[f] Vienna University of Technology, Institute for Chemical Engineering, Research Group Environmental Microbiology and Molecular Ecology, The Interuniversity Cooperation Centre Water & Health, Gumpendorfer Straße 1a, 1060 Vienna, Austria
[g] Department of Environmental Sciences, Jožef Stefan Institute, Jamova ulica 39, 1000 Ljubljana, Slovenia

HIGHLIGHTS

- There are spatial and temporal differences in nutrient concentrations in the Sava River Basin.
- The combination of soil nitrification and admixture of nitrate from wastewater and manure are the main sources of nitrate in autumn.
- Microbiological analyses showed the existence of hot spots of fecal pollution.
- Microbial source tracking analyses confirmed that the pollution is human associated.

GRAPHICAL ABSTRACT

ARTICLE INFO

Article history:
Received 14 June 2016
Received in revised form 29 July 2016
Accepted 29 July 2016
Available online 10 August 2016

Keywords:
Nutrients

ABSTRACT

Coupled measurements of nitrate (NO_3^-), nitrogen (N), and oxygen (O) isotopic composition ($\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$) were used to investigate the sources and processes of N cycling, while the microbial source tracking (MST) method was used to identify microbiological pollution in the surface water of the Sava River Basin (SRB) in autumn in 2014 and 2015 during high and low water discharge. Atmospheric nitrate deposition or nitrate-containing fertilizers were found not to be significant sources of riverine nitrate in the SRB. The ranges of isotope values suggest that NO_3^- in the SRB derives from soil nitrification, sewage, and/or manure, which were further supported by MST analysis. Microbiological indicators show the existence of hotspots of fecal pollution

* Corresponding author at: Jožef Stefan International Postgraduate School, Jamova ulica 39, 1000 Ljubljana, Slovenia.
E-mail addresses: j.vrzel@iggf.geo.uni-muenchen.de (J. Vrzel), brankavg@bio.bg.ac.rs (B. Vuković-Gačić), stoimirk@bio.bg.ac.rs (S. Kolarević), zorga@imsi.rs (Z. Gačić), margareta.kracun@ibiss.bg.ac.rs (M. Kračun-Kolarević), kosticjovana@hotmail.com (J. Kostić), mustafab@bio.bg.ac.rs (M. Aborgiba), andreas.farnleitner@wavenet.at (A. Farnleitner), georg.reischer@tuwien.ac.at (G. Reischer), rita.linke@tuwien.ac.at (R. Linke), mpaunovi@ibiss.bg.ac.rs (M. Paunović), nives.ogrinc@ijs.si (N. Ogrinc).

http://dx.doi.org/10.1016/j.scitotenv.2016.07.213

Stable isotopes
Microbial source tracking
Fecal indicators
Sava River Basin

in the SRB, which are human associated. Long-term observations indicate persistent fecal contamination at selected locations caused by continuous discharge of untreated wastewaters into the SRB.

1. Introduction

During the last two decades, much effort has been invested into understanding the nutrient cycles, heightened by problems associated with global, regional and local environmental problems (Heatwaite, 2010). These problems result in part from increased fluxes of nutrients such as nitrogen (N) and phosphorous (P) compounds into water bodies that can lead to eutrophication, causing ecological changes including loss of plant and animal species. In addition, these fluxes can influence the quality of water for human consumption and other purposes. In many river catchments the main source of N pollution is run-off from agricultural land, although discharges from wastewater treatment works can also be significant. According to the European Environmental Agency current concentrations of nitrate are still above what might be considered to be 'background' or natural levels, except in the northern European countries (EEA, 2015). Thus control of these nutrient discharges is needed to reduce pollution levels in water bodies.

For solving problems related to loading of nutrients such as N, tools are needed for identifying sources of N, often a challenging task. Isotope techniques constitute a promising tool for determining sources of nitrate (NO_3^-) in surface water and could help to identify the processes that NO_3^- has undergone in the aquatic system. This is possible because the isotopic signature in NO_3^- from fertilizer, atmospheric, soil and manure-derived sources differs sufficiently to enable an unambiguous distinction to be made between them (Hebert and Wassenaar, 2001; Chang et al., 2002; Mayer et al., 2002; Voss et al., 2006). However, the origin of NO_3^- must be linked to the entire N cycle, since values of $\delta^{15}N$ of NO_3^- can be biased due to the mixing of different NO_3^- sources or to isotope fractionation occurring during various processes (nitrification, denitrification, assimilation, remineralization) (Kendall et al., 2008). The dual isotope approach, based on the determination of $\delta^{15}N$ and $\delta^{18}O$ values of NO_3^-, provides more conclusive information for tracing sources of NO_3^- in water (Widory et al., 2004; Seiler, 2005; Xue et al., 2009). However, in the case of discriminating between sewage and manure the $\delta^{15}N$ and $\delta^{18}O$ values of NO_3^- overlap and this approach could not be used to differentiate between these two sources. The contamination of water bodies by sewage or manure is generally determined using the detection of Fecal Indicator Bacteria (Kirschner et al., 2014). Specific differentiation between sources of fecal contamination is of particular importance, because the risk to humans is usually considered higher from human fecal contamination (sewage) than from animal fecal contamination. New approaches based on PCR quantification and biomarker analysis could be used to track microbiological sources of contamination. The human-associated fecal marker BacHum (Kildare et al., 2007) and a recently modified version of the HF183II (Green et al., 2014) could be used to track human associated fecal pollution, while the ruminant-associated BacR qPCR assay (Reischer et al., 2006) and the pig-associated Pig2Bac qPCR assay (Mieszkin et al., 2009) are the methods for detecting animal fecal pollution sources. The contamination of water by fecal pollution leads to exposure to pathogens via drinking water production, recreation or irrigation. However, monitoring the microbiological quality of surface waters is quite neglected despite its importance for human health (Kittinger et al., 2013).

In the case of the Sava River Basin (SRB), many of the settlements situated on the river banks discharge large quantities of untreated or improperly treated wastewaters directly into surface waters (Kapetanović et al., 2015; Aborgiba et al., 2016). On the other hand, in the middle and lower course, the Sava River flows through regions of intense agriculture: the Slavonija in Croatia, the Bosanska Posavina and the Semberija in Bosnia and Herzegovina and the Srem in Serbia, together cover an area >100,000 km^2 (Paunović et al., 2012). The results of long-term trend analysis show a reduction of NO_3^- at the majority of locations in the SRB, ranging from 8 to 58%, the highest being observed at Litija in Slovenia from 2006 to 2011 and the lowest at Županja in Croatia from 2003 to 2011 (Vrzel and Ogrinc, 2015). In addition, a switch from a negative to a positive trend in phosphate (PO_4^{3-}) concentration was observed at Sremska Mitrovica indicating that this location has been more susceptible to nutrient inputs, mainly from wastewater effluent discharges. This assumption was further supported by the investigation performed in autumn 2006. At this location and in the Sava River near Belgrade, elevated $\delta^{15}N_{NO3}$ values of up to +25.5‰ were determined (Ogrinc et al., 2008). The high $\delta^{15}N_{NO3}$ values obtained fall within the range of animal waste and sewage (Aravena et al., 1993; Fogg et al., 1998; McClelland and Valiela, 1998; Wankel et al., 2006), and were only found in autumn 2006 during extremely low water discharge. Due to the use of water for irrigation, evaluation of the microbiological quality of the Sava River becomes essential for further river management. One of the pathways of exposure to contaminated water that must not be neglected is flooding. The study of Casteel et al. (2006) indicated that flooding can result in extensive fecal contamination of nearby agricultural soil with pathogens.

The aim of the present study was to assess the sources of NO_3^- using a combination of the N and O stable isotope approach and the level of microbial pollution in the SRB under different hydrological conditions – in 2014 during high water levels and in 2015 during low water levels. This study was performed to fill the gaps in the information related to riverine nutrient cycling and to evaluate whether the isotopic composition of riverine nitrate provides source or process information, or a combination of both. Related work performed in 2005 and 2006 published by Ogrinc et al. (2008) quantifies the sources of riverine N throughout the SRB using only stable isotope composition of N in NO_3^-, while to our knowledge no research including biomarker analysis to track microbiological sources of contamination has been performed in the whole SRB. Thus, this interdisciplinary study taking into account biogeochemical and molecular approaches has been applied for the first time in the SRB and was used to identify the hotspots of pollution, while microbial source tracking was used to identify the major sources of pollution.

2. Materials and methods

2.1. Study site

The main characteristics of the Sava watershed are summarized according to the report of the International Sava River Basin Commission (ISRBC, 2011). The River Sava is 990 km long, including the 45 km Sava Dolinka headwater rising at Zelenci, Slovenia. It is the greatest tributary of the Danube in terms of volume of water, and the second-largest after Tisza in terms of its catchment area of 97,713 km^2. The Sava flows through four countries in the Balkan Peninsula: Slovenia, Croatia, along the northern border of Bosnia and Hercegovina, and through Serbia. Climatic conditions and physical features, such as morphology, geology, pedology and vegetation, of the SRB are highly heterogeneous. The Sava flows through a variety of landscape types, including alpine, karstic, deep river valleys and shallow Pannonian flats. The average bottom slope of 15.8% qualifies the Sava as a typical lowland or middle course river starting in the central flow in Slovenia.

The SRB is divided into three climatic areas: Alpine, Pannonian and Continental. In the Alpine areas, in Slovenia, the mean annual precipitation is the highest (2000–3000 mm/y) and the mean annual temperature the lowest (6 °C). On the other hand, in the Continental area, in

Serbia, annual precipitation decreases to around 660 mm/y and the mean annual temperature increases to about 13 °C. The average discharge of the Sava increases downstream from 84 m^3/s at Ljubljana to 255 m^3/s at Zagreb, to 1722 m^3/s at Belgrade. Further, the increase gradient of solar radiation was observed in the SRB from 3713 MJ m^{-2} determined in the Alpine region, through 4013 MJ m^{-2} in Continental region to 4135 MJ m^{-2} in Pannonian region (http://solarelectricityhandbook.com/solar-irradiance.html).

The large retention areas of the Sava are one of the most effective flood control systems in Europe. Its management is seen as an international model for sustainable flood management. The backwater effect of the Iron Gate Dam (Djerdap I) during low flow of Sava is observed almost up to the town of Šabac at 105 km from Belgrade.

The geology of the River Sava basin is diverse. The most important geological characteristic influencing the regime of water and sediment of the SRB is the presence of the karst regions in the southern part of the river basin. The rest of the river basin, between the External Dinarides and the border of the Sava catchment belongs to the Inner Dinarides zone and Pannonian basin. In this zone the following lithological units are present: sandstone, marls, claystones, intrusive and extrusive igneous rocks (diabase, spilite, dacite, andesite etc.), metamorphic rocks (serpentinite, phylite, argiloshist etc.). The main aquifers are formed in alluvial deposits representing the large reserves of groundwater (Ogrinc et al., 2010). According to Harmonized World Soil database, the soils with the largest extent are the Cambisols (weakly to moderately developed soils) that cover 46.4% of the basin. Other important soil groups are the Luvisols, Leptosols, Podzoluvisols (leached soils) and Fluvisols. Geology and soils play an important role in sediment formation in the SRB. Previous research indicates that soil was the dominant source of particulate organic matter (Ogrinc et al., 2008). A rather sharp transition from a gravel-bed river (at the Upper Sava) to a sand-bed river (at the Middle Sava) is present in the SRB. Grain size distribution shows the presence of silt or clay and except at the Šabac (location 11) a uniform composition of sand particles was observed. However no clear correlation between the part of fine fraction and location was obtained, although in the lower, flat part of the river the percentage of fine particles in sediments can reach up to 90% of the total sediment content (Globevnik et al., 2010).

Land use of the SRB reflects the differences in relief, climate and stream flow. The greatest population density is located near large cities. Three capitals are located near the Sava: Ljubljana, Zagreb and Belgrade. Agriculture is the dominant activity in the Croatian (40%) and Serbian (~100%) parts of the watershed, while the upper part, in Slovenia, is mainly covered by forest (>50%). The Bosnian part of the watershed is dominated by valleys and hills, with about 30% agricultural area and 30% forest. For these four countries, the basin catchment area comprises 60–70% of their land and is the source of >80% of total available water. Due to important hydropower generation, industrial and agricultural activities, and a high population density, especially near large cities (Ljubljana, Zagreb, Belgrade), strong anthropogenic influences on water quality are expected.

2.2. Sampling

2.2.1. Sample collection

Two sampling campaign at the SRB, performed within the scope of the GLOBAQUA project (Navarro-Ortega et al., 2015), are presented in this paper and cover the period of the high and the low water discharge in September 2014 and 2015, respectively, at 12 locations from its source in Slovenia to the confluence with the Danube in Serbia (Fig. 1). At some sampling stretches two sampling locations were selected (marked as 'a') in order to obtain a representative water body for further investigation. Thus, altogether 17 locations were selected in 2014, while samples from 15 locations were obtained in 2015. For microbiological characterization additional samples were collected from 4 wastewater outlets detected in 2015 at the following locations: Radovljica – location 2 (RWW), Zagreb – location 6 (ZWW), Sremska Mitrovica - location 10a (SMWW) and Šabac – location 11 (ŠWW).

Discharge and water quality data for long-term periods 2006–2012, 2003–2012 and 1989–2012 were provided by the Environmental Agency of the Republic Slovenia (ARSO; URL: http://www.arso.gov.si/), Hrvatske vode (URL: http://www.voda.hr) and the Republic Hydrometeorological Services of Serbia (http://www.hidmet.gov.rs).

Hydrologic balance depends primarily on climatic conditions and physical features of the catchments and its spatial distribution is heterogeneous. In general, Sava has the largest discharge in spring and the lowest in late summer. However, seasonal and annual maxima and minima of discharge vary according to the locations (ISRBC, 2011).

2.2.2. Field measurements

Temperature (T), pH, electrical conductivity (ElCo) and dissolved oxygen (DO) were measured in the field. T and DO were measured directly in the stream, using a VTV OXI model 91 m. ElCo was measured using a Corning 316 m with a two point calibration at 0 μS and 1413 μS. The precisions of DO saturation and ElCo measurements were within ±5%. Because pH is sensitive to degassing and warming, water samples were collected in a large volume, air-tight container and pH was measured at least twice to verify electrode stability, using a Corning 315 high sensitivity pH meter coupled with an Orion Ross electrode. The pH meter was calibrated on the NBS scale using two buffers (pH 4.10 and 6.97) with a reproducibility of ±0.02 pH units.

Sample aliquots collected for chemical analysis were passed through 0.45 μm membrane filters into bottles and kept refrigerated until analysed. Samples for anions, total alkalinity and stable isotope analysis of nitrate ($\delta^{15}N_{NO3}$, $\delta^{18}O_{NO3}$) were collected in HDPE bottles. Samples for determining the stable isotope composition of oxygen ($\delta^{18}O$) were stored in HDPE bottles without filtering. Samples for carbon isotope analyses of dissolved inorganic carbon ($\delta^{13}C_{DIC}$) were capped in glass serum vials filled with no headspace. Particulate material for stable C and N isotopic analyses was collected on precombusted Whatman glass fiber filters (GF/F) by filtering 1–2.5 L of water using a pump connected to a Teflon filter holder. Filters were wrapped in aluminum foil.

The samples for microbiological analysis were collected within 10 days of survey at approximate the same time of the day. In all cases, water samples were collected in sterile, 1 L glass bottles from about 30 cm below the water surface. Samples were kept in dark, cool boxes prior to analysis and analysed within 3 h of arrival in the laboratory.

2.3. Chemical and stable isotope analysis

2.3.1. Chemical analysis

Total alkalinity (TA) was determined within 24 h of sample collection by the Gran titration method, with a precision of ±1%. Concentrations of nitrate (NO_3^-), nitrite (NO_2^-), ammonium (NH_4^+) and phosphate (PO_4^{3-}) were analysed using standard colorimetric methods (Grasshoff et al., 1983) with a precision of measurement ±3%. Concentrations of dissolved organic carbon (DOC) were measured with a Shimadzu TOC-5000 instrument with an analytical precision of ±2%.

2.3.2. Stable isotope analysis

The isotopic composition of oxygen ($\delta^{18}O$) was determined by standard water–CO_2 equilibration technique (Epstein and Mayeda, 1953) on a Multiflow continuous flow isotope ratio mass spectrometer (Isoprime GV Instruments).

Isotope analyses of nitrogen and oxygen of NO_3^- were carried out by Hydroisotop GmbH, Schweitenkirchen, Germany using the denitrifier method (Sigman et al., 2001; Casciotti et al., 2002), which is based on the isotopic analysis of nitrous oxide (N_2O) produced by denitrifying *Pseudomonas* strains. The measurements were performed on a Gas

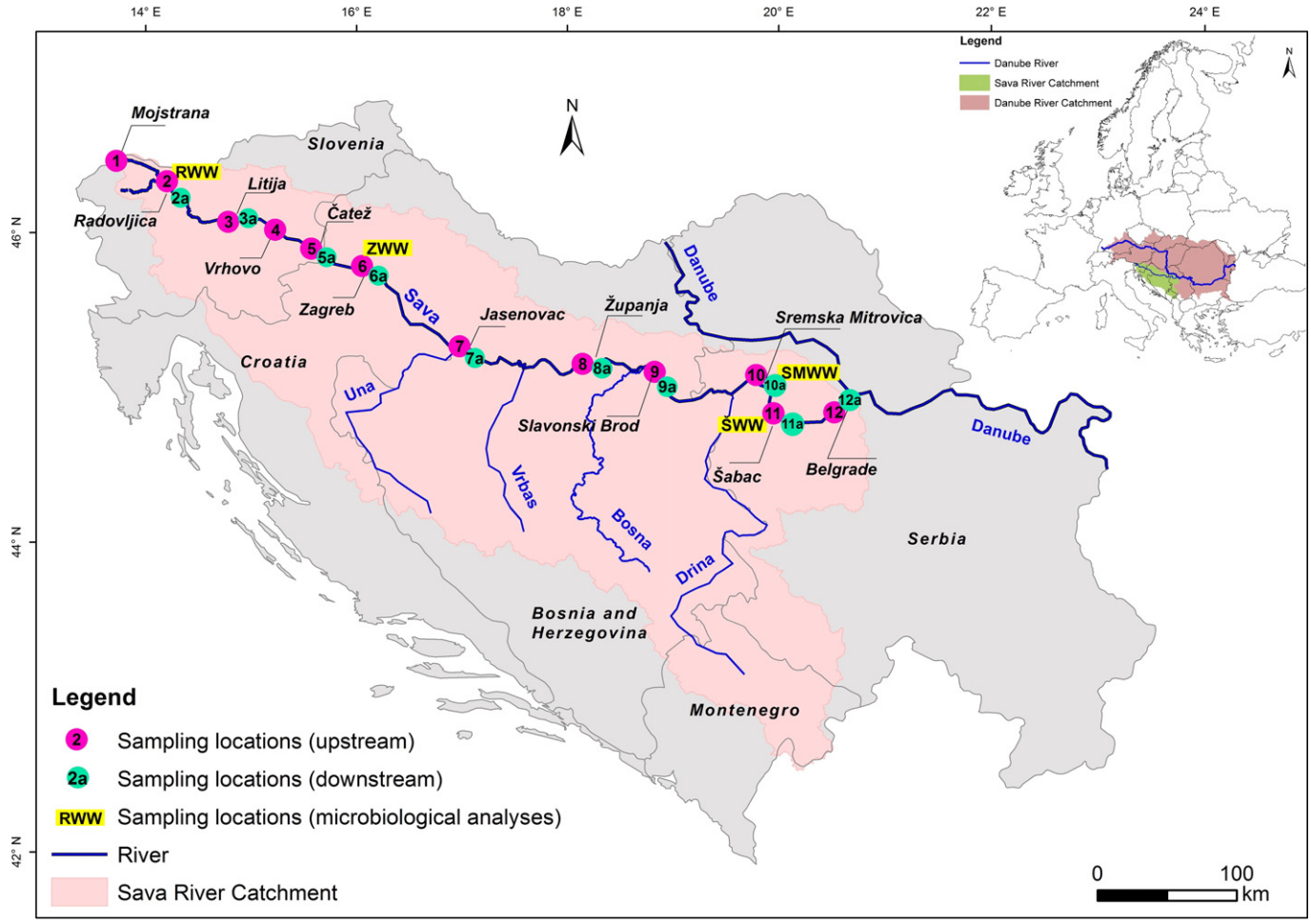

Fig. 1. Map of selected monitoring locations in the Sava River Basin in September 2014 and 2015.

Bench (Thermo Finnigan Gas Bench II) coupled to an isotope ratio mass spectrometer Delta Plus XP.

$\delta^{13}C_{DIC}$ values were determined after extraction as CO_2 in glass septum tubes (VACUTAINER Septum Tubes, Labco Limited, UK) by Europa Scientific 20–20 mass spectrometer with an ANCA-TG preparation module for trace gas samples using the procedure described by Ogrinc et al. (2008). The samples collected on Whatman GF/F filters for carbon and nitrogen isotopic analysis ($\delta^{15}N_{TPN}$ and $\delta^{13}C_{POC}$) were first dried at 40 °C. $\delta^{13}C_{POC}$ values were determined after treatment with 1 M HCl to remove carbonate minerals, while $\delta^{15}N_{TPN}$ values were determined on the bulk samples without acidification. $\delta^{13}C_{POC}$ and $\delta^{15}N_{TPN}$ values were measured with a Europa Scientific 20–20 mass spectrometer with an ANCA-SL preparation module.

All stable isotope results are reported using conventional delta (δ)-notation in per mil (‰) relative to a suitable standard: for oxygen VSMOW standard was used, while for carbon and nitrogen VPDB and AIR standards were used, respectively. The results were calibrated against the following international reference materials: IAEA-CH-7 (polyethylene; $\delta^{13}C = -32.15 \pm 0.05$‰) and IAEA-CH-6 (sucrose; $\delta^{13}C = -10.45 \pm 0.03$‰) for carbon; IAEA-N-1 (ammonium sulfate; $\delta^{15}N = 0.4 \pm 0.2$‰) and IAEA-N-2 (ammonium sulfate; $\delta^{15}N = 20.3 \pm 0.2$‰) for nitrogen; IAEA-NO-3 (potasium nitrate; $\delta^{15}N = 4.7 \pm 0.2$‰ and $\delta^{18}O = 25.6 \pm 0.4$‰) for nitrogen and oxygen; SLAP2 (Standard Light Antarctic Precipitation; $\delta^{18}O = -55.50 \pm 0.02$‰) and GISP (Greenland Ice Sheet Precipitation; $\delta^{18}O = -24.76 \pm 0.09$‰) for oxygen. Reproducibility of the measurements was ±0.1‰ for $\delta^{18}O$, ±0.2‰ for $\delta^{13}C_{DIC}$ and ±0.3‰ for $\delta^{15}N$ and $\delta^{18}O$ in NO_3^-. The mean standard deviations for replicate POM samples were higher: 0.4‰ and 0.5‰ for $\delta^{13}C_{POC}$ and $\delta^{15}N_{TPN}$, respectively, perhaps reflecting more subsampling error in these particulate samples.

2.4. Microbiological analysis

2.4.1. Detection of total coliforms, Escherichia coli and enterococci

Defined Substrate Technology (DST) was used to detect total coliforms (TC), *E. coli* (EC) and enterococci (FE). This enables isolation and identification of certain groups of bacteria by enzymatic hydrolysis of specific substrates (Buckalew et al., 2006). Quantification was performed with Colilert Quanti-Tray 2000 system, which provides a Most Probable Number (MPN) result, based on color/fluorescence change in 97 wells. For coliform bacteria two dilutions were analysed (1:10 and 1:1000) while for enterococci only one dilution was analysed (1:10). Powdered reagents Colilert-18 and *E*-enterolert were used for cultivation of coliforms and enterococci respectively. A diluted water sample was mixed with powdered reagents and poured into Quanty-Tray, a sterile plastic disposable 97-well tray. For coliforms, trays were incubated at 37 °C for at least 18 h. Enterococci were incubated for at least 24 h at 44 °C.

After incubation, an appearance of yellow color in wells was indicative of the presence of total coliforms. Fluorescence in wells, under UV illumination (365 nm) was used as an indication of the presence of *E. coli*, in Colilert, and enterococci in Enterolert-E. The number of positive wells was scored and converted to MPN using tables provided by the manufacturer. Assessment of the water quality was based on the total

coliforms, *E. coli* and enterococci classification scheme suggested by Kirschner et al. (2009) and was expressed as MPN/100 mL.

2.4.2. Detection of presumptive Clostridium perfringens by the membrane filtration method

Before the filtration, water samples were pasteurized for 15 min at 60 °C. *C. perfringens* (CP) were enumerated by membrane filtration and incubation on Tryptose Sulphite Cycloserine (TSC) media according to ISO 14189:2013 and the number of cells expressed as CFU/100 mL of sample.

2.4.3. Microbial fecal source tracking

2.4.3.1. Sample preparation and DNA extraction. Water samples for MST were collected, only during the 2015 survey from all 15 studied locations. Triplicate subsamples, between 150 and 200 mL, were filtered through 0.2 μm polycarbonate filters. Filtration volume was. Filters were immediately frozen at −20 °C. Clean filters were frozen and stored alongside the sample filters as filtration controls. DNA was extracted from two series of filters by a phenol-chloroform extraction combined with bead-beating (Reischer et al., 2008). DNA was dissolved in 100 μL Tris buffer. Extraction controls were routinely run alongside each extraction batch. Prior to the qPCR assays, DNA concentration in the extracts was measured by the PicoGreen assay according to the manufacturer's instructions (QuantiFluor® dsDNA Sytem, Promega, Madison, WI, USA) on a Anthos Multimode Fluorometer Zenyth 3100 (UK-Biochrom Ltd.).

2.4.3.2. qPCR quality assurance and inhibition control. Sample DNA was diluted 1:4 and 1:16 and the AllBac assay (Layton et al., 2006) applied to ensure the presence of amplifiable bacterial DNA and the absence of inhibition. An internal amplification control was also included in the AllBac assay.

2.4.3.3. Microbial fecal source tracking assays. The human-associated fecal marker BacHum (Kildare et al., 2007) and a recently modified version of the HF183II (Green et al., 2014) were enumerated by quantitative PCR (qPCR), to indicate human associated fecal pollution. The ruminant-associated BacR qPCR assay (Reischer et al., 2006) and the pig-associated Pig2Bac qPCR assay (Mieszkin et al., 2009) were included as methods for detecting animal fecal pollution sources. All these qPCR assays were adapted to run on the Rotor-Gene Q thermocycler with the Rotor-Gene Multiplex PCR mastermix (Qiagen Inc.). Quantification was achieved by running plasmid standard dilution series of known concentration. No template controls (containing no DNA) were applied in all instrument runs.

For the AllBac qPCR reaction the mixture was composed of 2.5 μL of the 1:4 or 1:16 sample DNA dilution, 600 nmol/L primer AllBac296f, 600 nmol/L primer AllBac412r, 25 nmol/L TaqMan MGB AllBac375Bhqr, 0.4 μg/μL of bovine serum albumin (Roche, Vienna, Austria), 7.5 μL of Rotor-Gene Multiplex PCR Kit (total volume of 15 μL). An internal amplification control was included in the AllBac qPCR reaction mixture comprised 500 nmol/L primer IPC-ntb2-F, 500 nmol/L primer IPC-ntb2-R, 200 nmol/L probe IPC-ntb2-P and IAC template (1000 copies/2.5 μL).

For the BacHum qPCR reaction, the mixture was composed of the following: 2.5 μL of the 1:4 sample DNA dilution, 400 nmol/L primer BacHum-160f, 400 nmol/L primer BacHum-241r, 80 nmol/L TaqMan probe BacHum-193p, 0.4 μg/μL of bovine serum albumin (Roche, Vienna, Austria), 7.5 μL of Rotor-Gene Multiplex PCR Kit (total volume of 15 μL).

For the HF183II qPCR reaction, the mixture was composed of the following: 2.5 μL of the 1:4 DNA dilution, 1 μmol/L primer HF183, 1 μmol/L primer BacR287, 80 nmol/L TaqMan probe BacP234MGB, 0.4 μg/μL of bovine serum albumin (Roche, Vienna, Austria) and 7.5 μL of Rotor-Gene Multiplex PCR Kit (total volume of 15 μL).

For the BacR qPCR reaction, the mixture was composed of the following: 2.5 μL of 1:4 sample DNA dilution, 100 nmol/L primer BacR_f, 500 nmol/L BacR_r, 100 nmol/L TaqMan MGB probe BacR_p, 0.4 μg/μL of bovine serum albumin (Roche, Vienna, Austria) and 7.5 μL of Rotor-Gene Multiplex PCR Kit (total volume of 15 μL).

For the Pig-2-Bac qPCR reaction, the mixture was composed of the following: 2.5 μL of 1:4 sample DNA dilution, 300 nmol/L primer Pig-2-Bac41F, 300 nmol/L Pig-2-Bac163R, 200 nmol/L TaqMan MGB probe Pig-2-Bac113MGB, 0.4 μg/μL of bovine serum albumin (Roche, Vienna, Austria) and 7.5 μL of Rotor-Gene Multiplex PCR Kit (total volume of 15 μL).

The PCR program was as follows: 95 °C for 5 min, 45 cycles of 95 °C for 30 s (AllBac) and for 15 s (all other assays), 60 °C for 45 s (AllBac) and 60 s (all other assays).

All reactions were performed in duplicate. A total of seven 10-fold serial dilutions of plasmid standard (1–10^6 gene copies) were run in duplicate as well as a no-template control. The results of quantitative

Table 1
Physico-chemical, nutrient and stable isotope parameters in late summer 2014.

Sampling site				Physico-chemical parameters					Nutrients					Isotopes					
Code	Date	Latitude (N)	Longitude (E)	T °C	pH	O_2 %	ElCo μS/cm	TA meq/kg	NO_3^- mg L^{-1}	NO_2^-	NH_4^+	PO_4^{3-}	DOC mg L^{-1} C	$\delta^{18}O$ ‰	$\delta^{15}N_{NO3}$	$\delta^{18}O_{NO3}$	$\delta^{15}N_{TPN}$	$\delta^{13}C_{DIC}$	$\delta^{13}C_{POC}$
2	01/09/2014	46.34	14.17	10.84	7.70	93.3	277	1.747	2.11	0.07	0.09	3.2	3.2	−10.0	0.1	11.1	1.0	−7.9	−26.7
2a	01/09/2014	46.29	14.26	11.52	8.44	91.2	286	1.900	2.33	0.05	0.15	0.10	2.5	−10.2	1.1	10.6	2.9	−9.1	−26.5
3	02/09/2014	46.06	14.81	12.24	7.55	96.8	279	n.a.	3.73	n.a.	n.a.	n.a.	1.6	−9.6	3.7	9.4	1.1	−8.4	−26.5
3a	02/09/2014	46.07	14.85	13.53	7.81	82.6	290	2.177	3.27	0.06	0.1	1.6	2.4	−9.3	4.7	9.8	1.1	−11.1	−26.1
5	03/09/2014	45.90	15.63	14.07	7.85	130	340	1.825	3.77	0.17	0.1	10.6	2.9	−9.3	3.4	9.0	2.2	−11.6	−27.3
5a	03/09/2014	45.87	15.65	13.33	7.73	117	381	2.194	1.69	0.04	0.35	0.48	2.7	−9.1	3.7	8.9	2.9	−12.3	−26.6
6	04/09/2014	45.78	16.00	13.98	7.52	96.8	361	2.150	1.77	0.04	0.76	<0.06	3.6	−9.1	4.5	9.4	4.0	−12.4	−26.8
6a	04/09/2014	45.76	16.05	14.12	7.96	97.4	363	2.358	2.53	0.06	0.09	1.6	2.5	−9.1	4.9	11.9	3.5	−12.0	−27.2
7	05/09/2014	45.26	16.89	15.06	8.14	68.3	283	2.415	3.58	0.04	0.05	6.8	4.8	−8.8	5.0	10.8	6.4	−13.1	−26.9
7a	05/09/2014	45.25	16.95	14.32	7.99	78.2	246	2.274	3.22	0.05	<0.02	0.16	2.5	−9.0	4.3	11.1	0.3	−12.9	−27.2
8	06/09/2014	45.15	18.01	15.25	7.66	81.6	320	2.038	1.82	0.07	<0.02	0.16	3.6	−9.0	3.1	8.1	3.5	−11.8	−26.1
8a	06/09/2014	45.13	18.09	15.84	7.65	76.2	352	2.338	0.73	0.24	0.19	<0.06	2.9	−8.8	4.2	8.6	3.6	−12.9	−26.4
9	07/09/2014	45.08	18.69	15.73	7.55	74.0	350	1.982	3.22	0.04	0.2	1.4	3.0	−8.8	3.2	8.5	4.5	−12.3	−25.7
9a	07/09/2014	45.02	18.74	16.12	7.95	75.2	367	1.880	2.55	0.05	0.2	2.3	3.9	−8.9	4.2	10.0	4.1	−11.7	−25.8
10	08/09/2014	45.97	19.60	16.49	7.61	79.8	327	1.912	3.52	0.05	0.07	1.3	3.0	−8.9	4.7	9.9	3.8	−12.6	−26.2
10a	08/09/2014	44.91	19.75	16.66	8.71	80.0	320	2.501	3.60	0.05	0.06	1.5	4.8	−8.9	4.5	10.1	4.8	−12.6	−25.7
12	09/09/2014	44.77	20.81	17.26	7.57	77.5	340	2.343	2.26	0.06	0.13	0.8	3.0	−8.9	4.4	8.8	5.6	−11.3	−26.8
12a	09/09/2014	44.81	20.44	17.16	8.03	76.2	334	2.762	3.86	0.05	0.06	0.9	2.6	−8.9	4.6	9.9	3.8	−12.2	−26.4

n.a. – not analysed.

Table 2
Physico-chemical, nutrient and stable isotope parameters in late summer 2015.

Sampling site				Physico-chemical parameters					Nutrients					Isotopes					
Code	Date	Latitude (N)	Longitude (E)	T °C	pH	O_2 %	ElCo µS/cm	TA meq/kg	NO_3^-	NO_2^-	NH_4^+	PO_4^{3-}	DOC	$\delta^{18}O$	$\delta^{15}N_{NO3}$	$\delta^{18}O_{NO3}$	$\delta^{15}N_{TPN}$	$\delta^{13}C_{DIC}$	$\delta^{13}C_{POC}$
									mg L^{-1}				mg L^{-1} C	‰					
1	01/09/2015	46.49	13.74	9.9	7.10	80.7	210	2.921	1.44	<0.02	<0.05	n.a.	1.41	−10.82	−2.7	8.9	4.1	−8.2	−26.9
2	01/09/2015	46.34	14.17	14.5	8.20	103	230	2.908	2.92	0.034	0.158	n.a.	1.66	−10.02	1.6	8.9	0.0	−7.8	−27.2
3	02/09/2015	46.06	14.81	17.6	8.80	101	260	3.790	6.48	n.a.	n.a.	0.108	1.93	−9.29	3.4	9.6	9.3	−10.2	−28.7
3a	02/09/2015	46.07	14.85	17.7	7.62	94.6	100	3.882	6.69	0.128	<0.05	0.154	1.93	−9.30	4.0	8.8	4.3	−10.2	−29.2
4	02/09/2015	46.04	15.24	20.2	7.82	96.6	280	3.843	6.53	0.034	0.086	0.024	2.12	−9.21	4.5	10.1	n.a.	−10.2	−28.3
5	03/09/2015	45.90	15.63	23.1	7.75	106	320	3.924	4.57	0.032	<0.05	n.a.	2.14	−9.35	5.0	7.1	4.0	−10.0	−29.4
6	04/09/2015	45.78	16.00	22.6	7.72	107	285	4.077	4.23	0.03	<0.05	n.a.	2.32	−9.22	6.1	7.5	5.5	−10.2	−29.4
7	05/09/2015	45.26	16.89	23.8	7.41	69.0	270	4.080	5.39	0.106	<0.05	0.151	2.84	−9.25	4.2	10.9	5.9	−10.8	−28.6
8	05/09/2015	45.15	18.01	24.8	7.83	100	336	3.969	4.32	0.032	<0.05	0.094	2.78	−9.31	6.6	10.1	6.8	−10.3	−27.3
9	06/09/2015	45.08	18.69	24.1	8.32	82.2	427	3.716	4.56	0.034	0.053	0.127	3.08	−9.26	6.7	9.5	6.0	−10.5	−25.6
10	07/09/2015	44.97	19.60	23.1	8.22	84.9	325	3.405	2.52	0.036	<0.05	n.a.	2.53	−9.53	5.7	11.0	5.7	−10.1	−28.1
10a	07/09/2015	44.91	19.75	22.6	8.48	89.2	293	3.229	2.11	0.022	<0.05	n.a.	2.29	−9.69	5.6	8.0	7.5	−10.4	−29.5
11	08/09/2015	44.75	19.72	22.4	8.08	94.2	336	3.389	2.21	0.02	<0.05	0.372	2.58	−9.59	6.1	8.1	7.7	−10.5	−29.2
11a	08/09/2015	44.74	19.82	22.5	8.94	115	313	3.525	2.05	0.022	<0.05	0.088	2.43	−9.65	4.8	9.7	1.0	−10.5	−29.1
12	09/09/2015	44.77	20.81	23.7	8.44	85.9	324	3.635	2.42	<0.02	0.088	0.087	2.40	−9.65	4.7	7.4	4.5	−10.0	−27.5

n.a. – not analysed.

microbial source tracking were expressed as marker equivalents (ME), taking account of the filtration volume and the DNA sample dilution to yield the result (Reischer et al., 2006, 2008).

2.5. Computational methods/statistics

Considering that the data were in line for usage of parametric tests, differences of the variables between the sampling sites were investigated by one-way ANOVA followed by Duncan's multiple range test. A 95% confidence interval was used for the confidence level. Based on the physical and chemical parameters studied, Euclidian distances were calculated and used to construct an Unweighted Pair-Group Method with Arithmetic mean (UPGMA) phenogram to reveal overall similarity between the sites studied in 2014 and 2015. Logarithmic transformations (log + 1) of microbiological data were performed by calculating log10 after addition of 1 to a given value. All statistical analyses were performed using Statistica 6.0 Software (StatSoft, Inc.).

3. Results and discussion

3.1. Distribution of nutrients in the Sava River Basin

3.1.1. Long-term nutrient trends

Long-term nutrient trends are presented in Vrzel and Ogrinc (2015). Here only a brief summary is reported for the same locations, but using the new location numbers from Fig. 1. Concentrations of both nutrients (NO_3^- and PO_4^{3-}) were correlated significantly with flow at all locations, except at location 10. A dilution effect was detected at locations 2, 3, 4, and 8 while, at location, 9, a continuous input of NO_3^- into the riverine system was indicated. Variations in rainfall and temperature were found to contribute greatly to the monthly variation in NO_3^- concentration.

Temporal variability in nutrient concentrations was controlled by riverine flow and indicated that the highest NO_3^- concentrations were usually observed during winter/autumn when leaching of soil from slopes and banks was more intensive. The lowest NO_3^- concentrations observed during the summer were related to the limiting leaching

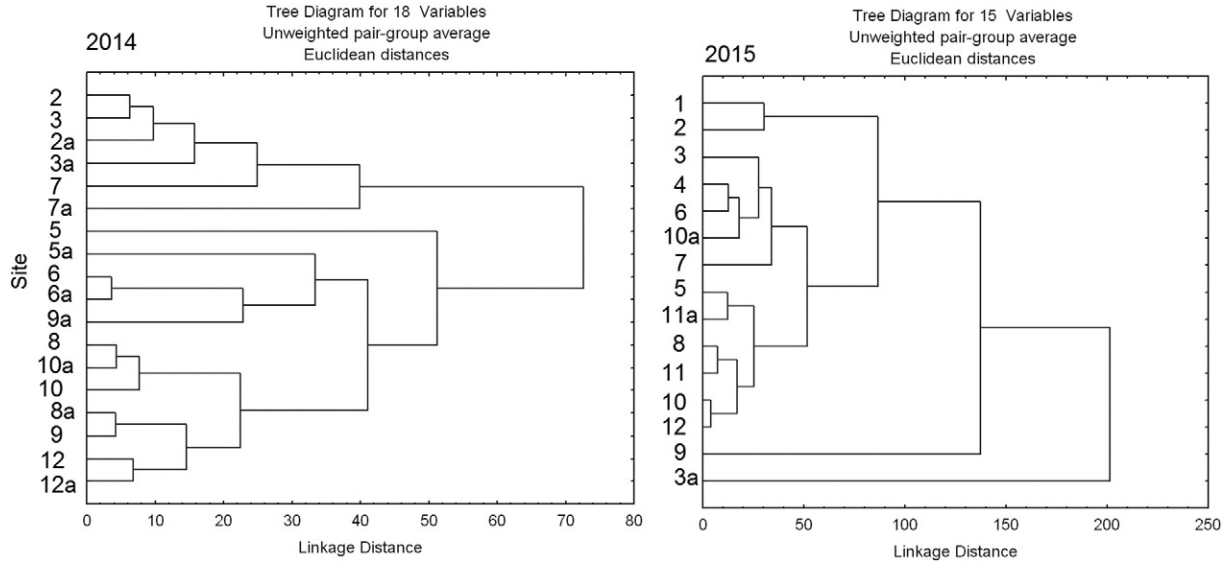

Fig. 2. UPGMA (Unweighted Pair-Group Method with Arithmetic mean) tree derived from Euclidian distances for the sites studied in 2014 and 2015 and based on physical and chemical parameters.

from the catchment and reduction of NO_3^- caused by phytoplankton uptake. Occurrence of denitrification was unlikely, since hypoxic/anoxic conditions are usually not present in the river. On the other hand, during the summer the highest PO_4^{3-} concentrations were related to low flow conditions and to the presence of reductive conditions releasing inorganic P from sediments into the water column.

The results of trend analysis show a reduction of nutrients at the majority of selected locations, ranging from 6 to 58% (Vrzel and Ogrinc, 2015). The trend in PO_4^{3-} concentrations exhibits different behavior. No significant trend was observed at locations 3 and 10 while, at locations 4 to 9, trends show significant decline in PO_4^{3-} concentrations with reductions in PO_4^{3-} concentrations of 18–34%. Although there was no significant trend in PO_4^{3-} concentration at location 10, the switch from a negative slope in the regression line for location 9 to a positive slope for location 10 suggests that inputs of PO_4^{3-} had been increasing downstream. This is possible, given that location 10 has been more susceptible to nutrient inputs, mainly from wastewater effluent discharges (Ogrinc et al., 2008; Ogrinc et al., 2015).

3.1.2. Nutrients in late summer in 2014 and 2015

The data regarding environmental parameters in late summer 2014 and 2015 are presented in Tables 1 and 2. The water temperature (T) usually increased with distance from the source (location 1) (Ogrinc et al., 2008). Statistically significant differences in the temperature of water, T, between locations was observed ($p < 0.0001$) with the lowest value of T always observed at location 1 and the highest usually found in the lower reaches of the Sava (18.4 °C at location 12 in 2014 and 24.8 °C at location 8 in 2015). A statistically significant difference in water temperature was also observed between 2014 and 2015 ($p < 0.0001$) with the highest value observed in 2015.

Specific conductivity (ElCo) ranged from 210 to 427 μS cm^{-1}, with the lowest and highest values observed in 2015 at locations 1 and 9, respectively. Significant differences in ElCo were observed between sampling locations ($p = 0.003$) in 2015. No statistically significant temporal differences in ElCo were observed in 2015 compared to 2014 ($p = 0.066$).

pH ranged from 7.10 to 8.94, with the lowest and highest values obtained in 2015 at locations 1 and 11, respectively. No significant spatial ($p = 0.652$ in 2014 and $p = 0.507$ in 2015) and yearly ($p = 0.1627$) differences were observed in pH values.

Concentrations of NO_3^- ranged from the limits of detection (LOD) determined at location 1 to 6.69 mg L^{-1} at location 3 (Tables 1 and 2). Significant differences between NO_3^- concentrations were observed between 2014 and 2015 ($p = 0.0213$), with higher values observed in 2015, but no significant differences between sampling locations were observed in 2014 and 2015.

The measured NH_4^+ concentrations were between <0.05 and 0.76 mg L^{-1}, with the highest values observed in 2015. Concentrations of NO_2^- were very low, ranging from 0.02 to 0.24 mg L^{-1}. NO_2^- is the intermediate inorganic species of the interconversion of NO_3^- and NH_4^+. In an aquatic ecosystem, NO_2^- is rapidly converted to NO_3^- under oxidizing conditions. NO_3^- is, therefore, usually far more abundant in the aquatic environment. This explains the relatively high concentrations of NO_3^- compared to those of NO_2^- during the whole study period (Tables 1 and 2). The concentrations of PO_4^{3-} ranged from 0.02 to 10.6 mg L^{-1} and were, on average, higher in 2014. Spatial and year differences were not significant in the cases of NH_4^+, NO_2^-, and PO_4^{3-}.

DOC concentrations ranged from 1.41 to 4.83 mg C L^{-1} and were statistically higher in 2014 than in 2015. Spatial distribution indicated that observed values were statistically higher in the lower part of the Sava River in 2014 and 2015.

Values of $\delta^{15}N_{NO3}$ for riverine samples ranged from −2.7 to 6.7‰ (Tables 1 and 2) and displayed significant differences between those in 2014 and 2015 (higher values in 2015; $p = 0.0492$) and also between headwater/mouth locations (higher values at the mouth; $p = 0.054$). $\delta^{18}O_{NO3}$ values were quite uniform, with an average value of 9.4 ± 1.2‰. No significant differences were observed in relation to year of sampling or sample location.

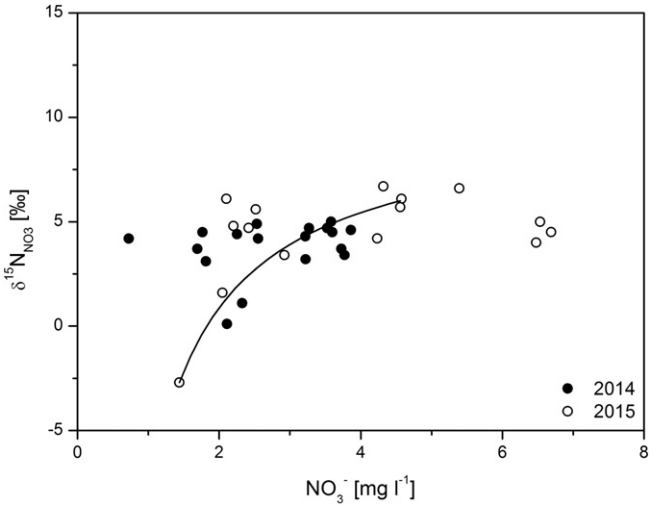

Fig. 3. The relation between $\delta^{15}N_{NO3}$ values and NO_3^- concentrations in the Sava River Basin. The following isotope-mixing curve was used to describe the observed data: $\delta^{15}N = \frac{\delta^{15}N_1 \times f \times N_1 + \delta^{15}N_2 \times (1-f) \times N_2}{N}$ where N, N_1 and N_2 are concentrations of NO_3^- in the sample, two end-members and $\delta^{15}N$, $\delta^{15}N_1$ and $\delta^{15}N_2$ are corresponding $\delta^{15}N$ values. The first end-member, with an NO_3^- concentration of 1.44 mg L^{-1} and $\delta^{15}N_{NO3}$ of −2.7‰, was chosen for the sample observed in a pristine area, while the second end-member was selected from location 9 where the highest $\delta^{15}N_{NO3}$ value of 6.7‰ was observed with NO_3^- concentration of 4.56 mg L^{-1}.

Values of $\delta^{13}C_{DIC}$ ranged from −13.1 to −7.8‰ and of TA from 1.747 to 4.080 meq/kg. Statistically significant spatial differences were observed in both parameters in 2014 ($p < 0.001$) and 2015 ($p < 0.001$). Locations 1 and 2, with higher values of $\delta^{13}C_{DIC}$ and lower TA, differed significantly from other locations. The higher values of $\delta^{13}C_{DIC}$ indicate that dissolution of carbonates prevails while, at the downstream locations, degradation of organic matter appeared to be more important. The difference from measurements at other locations in the SRB was not significant in 2015 and relatively similar $\delta^{13}C_{DIC}$ values were observed in the whole lower part of the River Sava (average $\delta^{13}C_{DIC}$ −10.3 ± 0.7‰). A similar finding was observed in the study performed in 2005 and 2006 (Ogrinc et al., 2008).

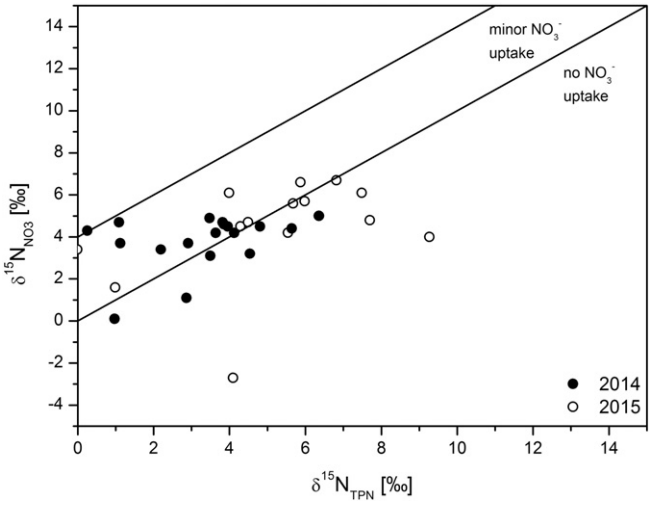

Fig. 4. Values of $\delta^{15}N_{NO3}$ versus $\delta^{15}N_{PN}$ for the Sava River Basin. The diagonal lines indicate the expected $\delta^{15}N$ values if there was no isotopic fractionation during uptake (lower line), and the expected $\delta^{15}N$ values if the fractionation was 4‰ (upper line), a typical fraction for uptake of NO_3^- (Finlay and Kendall, 2007). The further the data plot below the lower line, the less likelihood of NO_3^- uptake occurring.

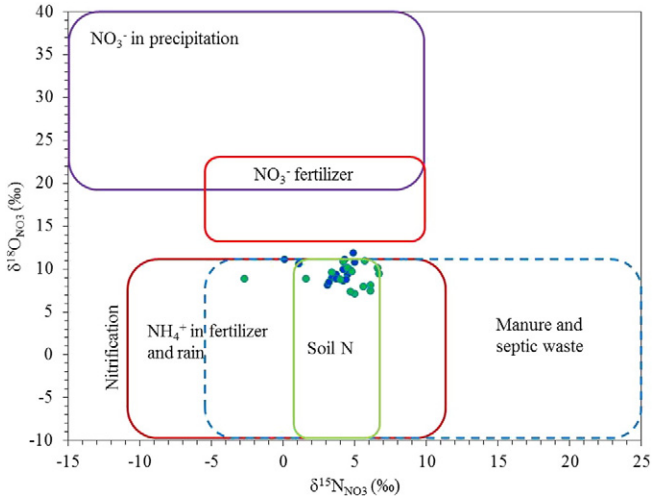

Fig. 5. $\delta^{15}N$ and $\delta^{18}O$ values of riverine nitrate in the Sava River Basin (adopted by Kendall et al., 2008). Ranges of isotope compositions for four major nitrate sources are indicated by boxes: (a) atmospheric nitrate deposition, (b) nitrate-containing fertilizers, (c) nitrate derived from nitrification e.g. in soils, and (d) nitrate in manure and/or sewage.

Values of $\delta^{13}C_{POC}$ ranged from −29.5 to −25.6‰ and of $\delta^{15}N_{PN}$ from 0.0 to 9.3‰. No significant differences were observed in $\delta^{15}N_{PN}$ values in relation to sampling location, while significant differences in $\delta^{13}C_{POC}$ values were observed in 2015, where location 9 differed significantly from locations 5, 6, and 7.

The differences between 2014 and 2015 sampling periods in TA, $\delta^{13}C_{DIC}$, $\delta^{13}C_{POC}$ and $\delta^{15}N_{TPN}$ values ($p = 0.038$, $p < 0.001$, $p < 0.001$ and $p = 0.012$, respectively) were statistically significant, with lower $\delta^{13}C_{DIC}$ and higher TA, $\delta^{13}C_{POC}$ and $\delta^{15}N_{TPN}$ values obtained in 2014.

The UPGMA phenogram derived from the matrix of Euclidian distances for the sites studied in 2014 and 2015 is shown in Fig. 2. With some exceptions, in both years the grouping of the sites belonging to upper, middle and lower stretches of the river is observed, supporting our previous observation in 2005 and 2006 (Markovics et al., 2010) where only chemical composition of the Sava River and its tributaries were investigated. In 2014 the major driving forces for distances among the sites were water temperature, concentrations of NO_3^- and DOC, $\delta^{13}C_{DIC}$ and $\delta^{15}N_{NO3}$ values, while in 2015 the concentrations of NO_3^- and $\delta^{15}N_{NO3}$ had the highest impact. In 2015 the locations 3a and 9 differed most distinctly from the other sites.

3.1.2.1. Determination of nitrate sources. A clear trend of increasing $\delta^{15}N_{NO3}$ values with increasing NO_3^- concentrations is evident from Fig. 3, suggesting that riverine nitrate in River Sava contained contributions from at least two different sources: one source generating low concentrations and $\delta^{15}N_{NO3}$ values below 2‰, and another with higher concentrations and a $\delta^{15}N_{NO3}$ value above 6‰. An isotope mixing line with two different riverine end-members was therefore constructed. The first end-member, with an NO_3^- concentration of 1.44 mg L^{-1} and $\delta^{15}N_{NO3}$ of −2.7‰, was chosen from the sample observed in a pristine area at the source of the River Sava (location 1; Fig. 1), while an NO_3^- concentration of 4.56 mg L^{-1} and a $\delta^{15}N_{NO3}$ of 6.7‰ were selected as the second source, with the highest $\delta^{15}N_{NO3}$ value observed at location 9 (Table 2). The results indicate that most of the data fall on the mixing line in 2014, while higher deviations from the mixing line were observed in 2015.

The low NO_3^- concentrations and low $\delta^{15}N_{NO3}$ values observed at locations 1 and 2 in 2014 and 2015 were indicators of NO_3^- leaching from pristine soils, the river in the upper watershed draining mainly a forested catchment (Harrington et al., 1998; Voss et al., 2006). $\delta^{15}N_{NO3}$ values range from −3 to 5‰ for NO_3^- leaching from non-fertilized soils (Fogg et al., 1998; Mayer et al., 2002) while, on the other hand, NO_3^- originating from animal manure and sewage generally shows a $\delta^{15}N_{NO3}$ value >7‰ (Aravena et al., 1993; Fogg et al., 1998; McClelland and Valiela, 1998; Wankel et al., 2006). The highest $\delta^{15}N_{NO3}$ value of 6.7‰ was observed at location 9 in 2015, but higher values could not be detected, as was the case in autumn 2006, when the river discharge was even lower than in 2015 (Ogrinc et al., 2008).

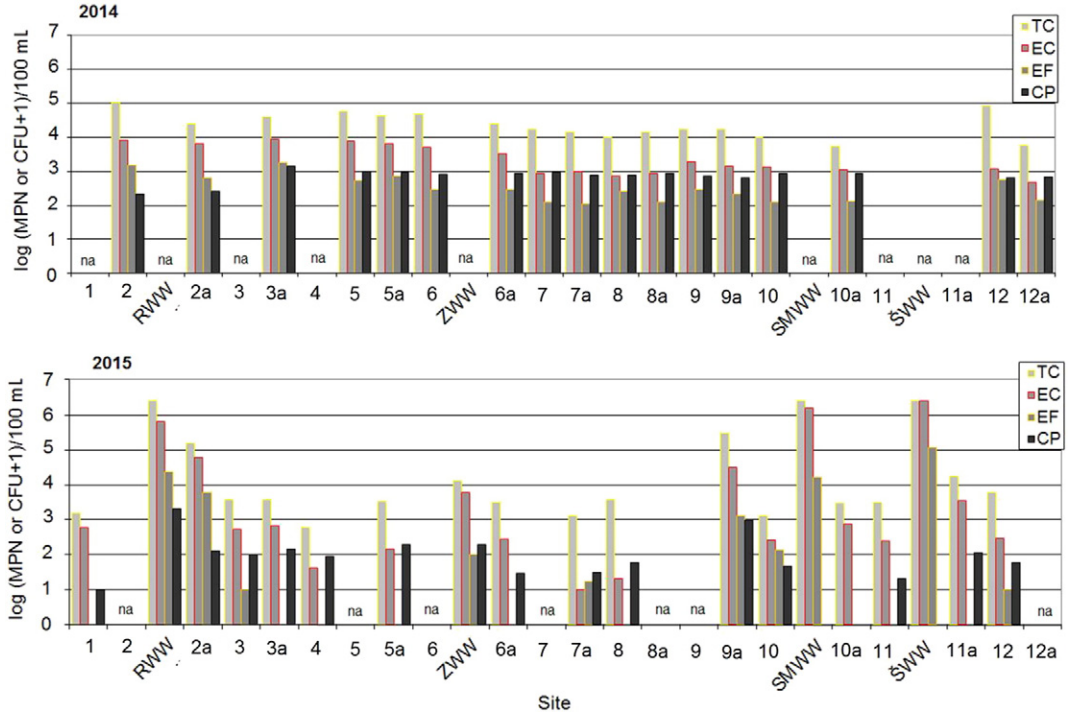

Fig. 6. Numbers of indicator bacteria (log (MPN or CFU + 1) / 100 mL) measured in water samples of the Sava River and in wastewater samples (WW) collected during the survey; TC - total coliforms, EC – *E. coli*, EF – enterococci, CP – *C. perfringens*; n.a. – not assessed.

The increase in $\delta^{15}N_{NO3}$ above the values expected from simple mixing indicates the consumption of NO_3^- by photosynthetic uptake or denitrification processes. Denitrification was not an important process, since neither hypoxic nor anoxic conditions were present in the River Sava. Thus, the stable isotope data and the decrease in NO_3^- concentrations observed at these locations (Tables 1 and 2) indicate higher assimilation rates of NO_3^-. Plotting values of $\delta^{15}N_{NO3}$ against those of $\delta^{15}N_{TPN}$ provides a useful means for determining whether NO_3^- uptake could be significant for various sites, since the $\delta^{15}N$ of algae should always be equal or lower than that of the N source (Finlay and Kendall, 2007). In Fig. 4 are two diagonal lines that indicate the $\delta^{15}N$ values expected if there was no isotopic fractionation during uptake (lower line) and, likewise, the expected values of $\delta^{15}N$ if the fractionation was 4‰ (upper line), a typical fraction for uptake of NO_3^- (Finlay and Kendall, 2007). As shown in Fig. 4, no locations show differences in $\Delta\delta^{15}N$ as high as 4‰. Most of the data plot near the lower diagonal line defined by $\delta^{15}N_{TPN} = \delta^{15}N_{NO3}$, consistent with minor uptake of local nitrate, while the data below the lower diagonal line indicate the insignificant uptake of NO_3^-.

In addition, and based on values of $\delta^{13}C_{POC}$ (Tables 1 and 2), the presence of phytoplankton was detected. The $\delta^{13}C$ value of phytoplankton was estimated from the $\delta^{13}C_{DIC}$ and a C isotope discrimination of 21‰ on photosynthetic C assimilation (Mook and Tan, 1991; Hellings et al., 1999; Hoffman and Bronk, 2006). The mean values of $\delta^{13}C$ for phytoplankton in the lower part of River Sava from location 3 could be estimated to be -33.6 ± 0.6‰ in 2014 and -31.3 ± 0.2‰ in 2015. Our previous investigation indicated that, at 59% of the locations, the main source of POC is derived from soil OC (Ogrinc et al., 2008). Thus, it was assumed that two main sources dominated the POC in River Sava in the autumns of 2014 and 2015: soil and phytoplankton. The proportion of these two sources was estimated using isotope mass balance, taking into account two end-members: the average $\delta^{13}C$ value of −26‰ for soil OC and the calculated mean $\delta^{13}C$ values for phytoplankton. The calculations indicate that >90% of POC is of soil origin at all locations in 2014, while in 2015 phytoplankton was found to be the main source of POC, accounting for >60% at locations 3, 5, 6, 10 and 11.

The data plotted below the mixing line indicate the additional source of NO_3^- in the River Sava, which was more pronounced in 2015 at locations 3, 5, 7 and could be related to nitrification. Since nitrification leaves an imprint on $\delta^{18}O_{NO3}$ values, these values were used to identify the origin of NO_3^- derived from nitrification processes in manure, sewage, or soils (Fig. 5). Based on the assumption that $\delta^{18}O_{NO3}$ of microbially-produced NO_3^- is determined by the two oxygens derived from H_2O and one from atmospheric O_2 (Hollocher, 1984; Anderson and Levine, 1986; Böhlke et al., 1997; Durka et al., 1994; Kendall, 1998; Wassenaar, 1995; Mayer et al., 2001), the expected range of $\delta^{18}O$ values of NO_3^- produced by nitrification can be calculated from the known $\delta^{18}O$ values for atmospheric oxygen and ambient water by the equation (Mayer et al., 2001): $\delta^{18}O_{NO3} = 2/3\ \delta^{18}O$ (in water) $+ 1/3\ \delta^{18}O\text{-}O_2$. The atmospheric oxygen is known to have a $\delta^{18}O$ value of 23.5‰ (Kroopnick and Craig, 1972), while the ambient water is expected to display an isotope value similar to that in the river water, as shown in Tables 1 and 2. The calculated $\delta^{18}O_{NO3}$ values for nitrate from nitrification were around 2‰ and differed significantly from those of our measured data with an average value of 9.4 ± 1.2‰ (Tables 1 and 2). However, $\delta^{18}O$ values of microbially-produced NO_3^- have been observed to be up to 5‰ higher than the calculated values (Aravena et al., 1993; Kendall, 1998; Mayer et al., 2001) and thus more closely related to our data. Further, it is possible to conclude that atmospheric deposition, with high $\delta^{18}O_{NO3}$ values between 25 and 70‰ (e.g. Durka et al., 1994; Kendall, 1998), does not contribute significantly to river NO_3^-, nor do the nitrates originating from fertilizers, with $\delta^{18}O_{NO3}$ values of ~+22‰ (Fig. 5). This is in line with studies carried out on USA and German rivers (Mayer et al., 2002; Johannsen et al., 2008). The absence of a trend of increasing $\delta^{18}O_{NO3}$ values with increasing agricultural land-use could be due to two reasons, the uptake of fertilizer-nitrate by crops and microbes and the subsequent re-mineralization and nitrification, producing nitrate with $\delta^{18}O_{NO3}$ values below +15‰ (Mayer et al., 2001) and thus changing the original oxygen isotope ratio of the fertilizer-nitrate. NO_3^- derived from nitrification of ammonium or urea-based fertilizers is also characterized by $\delta^{18}O_{NO3}$ values of less than +15‰, since nitrogen from ammonium or urea-containing fertilizers must be nitrified before it can contribute to riverine nitrate.

3.2. Microbiology

3.2.1. Comparative data for the whole Sava River

To compile long-term data for the whole Sava River, the same strategy proposed by Kirschner et al. (2009) for the Danube River was used. The data from two whole river surveys (2014 and 2015) and long-term data from the TransNational Monitoring Network (TNMN) of the International Commission for the Protection of the Danube River (ICPDR) were integrated. The data from TNMN are available at the http://www.icpdr.org/main/activities-projects/tnmn-transnational-

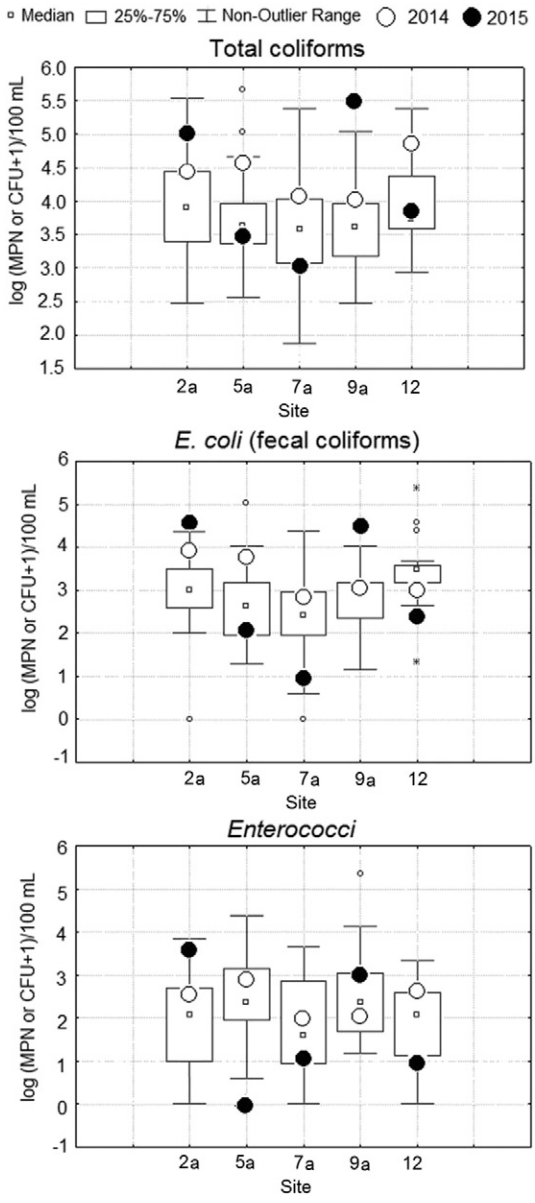

Fig. 7. Long term monitoring data on the numbers of the indicator bacteria (log (MPN-CFU + 1) / 100 mL) at 5 sites used in routine monitoring (Box-Whiskers); white circles – samples collected in 2014, black circles – samples collected in 2015.

monitoring-network. The dataset comprised five years of monthly monitoring (the only period available in the database was 2000–2004) from four TNMN locations: Jesenice (SI), Jasenice (HR), Jasenovac (HR) and Županja (HR). For Belgrade, pooled data obtained during five years of monthly monitoring (2010–2014) and provided by the Belgrade City Institute of Public Health were used. Selected locations overlap or are within 5 km of the locations studied in the whole river sampling campaigns: Jesenice (SI) - Litija, Jasenice (HR) - Čatež, Jasenovac - Jasenovac, Županja – Županja and Belgrade – Belgrade.

The long-term monitoring data are summarized in Fig. 6. For all three groups of bacteria, the majority of values showed grouping within the class II (moderate pollution) and class III (critical pollution). It is alarming that, at the locations 3a, 9a and 12, over 50% of the samples were critically polluted, indicating continuous presence of fecal pollution at these locations and is consistent with our previous results (Kapetanović et al., 2015). The lowest medians were observed at location 7a.

3.2.2. Sampling survey in 2014 and 2015

Primarily, the focus was on microbiological indicators of fecal pollution (*E. coli*, fecal enterococci), which are used as surrogates for the possible presence of intestinal pathogens (Kirschner et al., 2014). The numbers of total coliforms were assessed, as this group is still included in the national regulations of Serbia in assessing the ecological status of surface waters. We have also quantified *C. perfringens* as a potential indicator for point source emissions from wastewater (Sorensen et al., 1989). The results of the two whole river surveys are summarized in Fig. 6.

During the survey 2014, the numbers of total coliforms ranged from 3.73 (location 10a) to 5.03 (location 2) log (MPN + 1) / 100 mL. As indicated in Fig. 7, samples were scattered within the classes II and III (moderate to critical pollution). The numbers of *E. coli* ranged from 2.84 (location 8a) to 3.94 log (MPN + 1) / 100 mL (location 3a). The numbers of enterococci ranged from 2.03 (location 8a) to 3.26 (location 3a) log (MPN + 1) / 100 mL. The numbers of *C. perfringens* ranged from 2.03 (location 5a) to 3.13 (location 3a) log (CFU + 1) / 100 mL.

In 2015, the river samples were scattered within the classes I and II (slight to moderate pollution). The numbers of total coliforms ranged from 2.79 (location 4) to 5.49 (location 9a) log (MPN + 1) / 100 mL. The numbers of *E. coli* ranged from 1.0 (location 7a) to 4.51 log (MPN + 1) / 100 mL (location 9a). At 9 out of 15 locations the numbers of enterococci were below the limit of detection (<1 log (MPN + 1) / 100 mL), the highest numbers being recorded at location 9a (3.12 log (MPN + 1) / 100 mL). The numbers of CP ranged from 0 (location 10a) to 3.98 (location 9a) times log (CFU + 1) / 100 mL. By all monitored indicators, samples collected from the WW discharge points at locations 2, 10 and 11 were excessively contaminated. The effects were evident downstream at the location 11a, but especially at the location 2a.

For comparison, data obtained within the surveys were included into Fig. 8. At locations 5a and 7a a shift towards the upper quartile of the range was observed for samples collected in 2014 while a shift towards the lower quartile of the range was observed for samples collected in 2015. On the other hand, at locations 2a and 9a, the ones heavily impacted by wastewaters, we have noted the opposite situation. High water velocity, in combination with high water level, in 2014 resulted in homogeneity of water quality along the river. This is also valid for other parameters, since no statistically significant differences between sampling locations were found in pH, concentration of O_2, ElCo, concentrations of NO_2^-, NH_4^+, PO_4^{3-} or DOC and $\delta^{18}O_{NO3}$ values. Therefore, in 2014, the hotspots of pollution were hard to identify. In contrast, in 2015 we found WW outlets at 4 locations, but the effect was only evident at location 2a. It was interesting that a critical level of pollution was found at location 9a, although the direct point of discharge was not defined. This observation could be further supported by the high NO_3^- and DOC concentrations and the highest $\delta^{15}N_{NO3}$ value of 6.7‰ and the $\delta^{13}C_{POC}$ value of −25.6‰. According to the statistical evaluation this location differed most from the other sites in 2015 (Fig. 2). Besides the point discharges of WW, diffuse pollution, originating from households of the nearby located villages, was considered as a possible pathway of contamination. Also in the middle and lower sections of the river, the use of animal manure as a fertilizer in agriculture is commonly employed without strict legislative regulations, which should not be neglected.

Since the standard indicators, *E. coli* and enterococci, cannot provide any reliable information regarding the origin of fecal pollution (Kirschner et al., 2014), we have employed the MST technique to identify the major source of pollution. Quantitative PCR (qPCR)-based assays for the analysis of general-, human-, wastewater-, or animal-associated genetic *Bacteroidetes* fecal markers have gained increasing popularity in the field of fecal pollution analysis and microbial source tracking (MST) during recent years (Mayer et al., 2016). The human-associated fecal markers BacHum and HF183II were detected at 12 and 13 locations, respectively. The ruminant-associated BacR and the pig-associated Pig-2-Bac markers were not detected in any of the samples. As indicated in Table 3, high correlation was observed between the standard indicators (coliforms and enterococci) and human associated fecal markers. This is consistent with previous studies that support the fact that fecal pollution based on qPCR quantification can be performed with at least equal precision compared with traditional ISO-based cultivation techniques (Stapleton et al., 2009; Mayer et al., 2016). The lowest correlation was observed with numbers of *C. perfringens*. Among the indicators studied, *C. perfringens* numbers show the lowest degree of variation along the river, as reported earlier (Byamukama et al., 2005).

4. Conclusions

The combined use of the isotope compositions of N and O in NO_3^- provides evidence that NO_3^- is affected primarily by two different

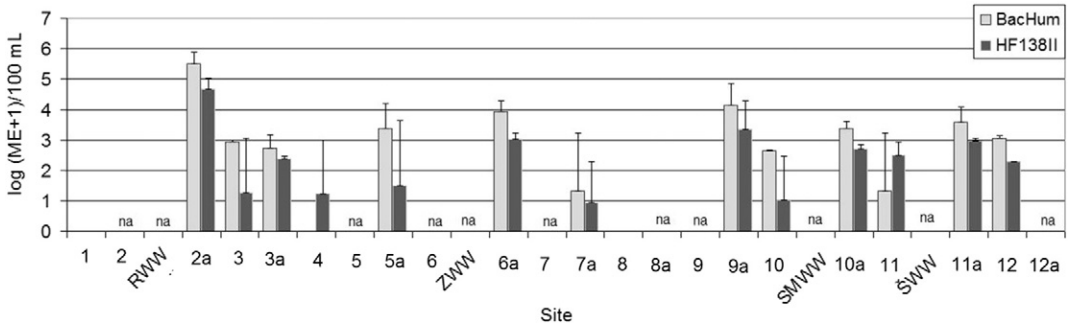

Fig. 8. Concentration of the human-associated fecal markers BacHum and HF183II expressed as log (ME + 1) / 100 mL, values are means ± SD obtained from two filter series; n.a. – not assessed.

Table 3
Correlation between the standard indicators (TC, EC, EF and CP) and the human-associated fecal markers BacHum and HF183II monitored. Marked correlations are significant for $p < 0.003$ (p value adjusted for multiple comparisons).

Spearman	EC		EF		CP		BacHum		HF138II	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*
TC	0.63	0.012	0.28	0.318	0.64	0.010	0.65	0.009	0.60	0.018
EC			0.26	0.349	0.29	0.301	0.70	0.004	0.70	0.004
EF					0.30	0.278	0.36	0.187	0.19	0.508
CP							0.40	0.135	0.32	0.252
BacHum									0.89	**0.000**

sources: one source originating from pristine soils, and another originating from wastewater and manure, although processes such as phytoplankton assimilation and nitrification have also been observed, especially in autumn 2015. According to the analyses of the $\delta^{15}N_{NO3}$ and $\delta^{15}N_{TPN}$ values the minor and/or zero uptake of local nitrate was detected in most locations. Particulate organic carbon (POC) is mainly of soil origin in 2014 while, in 2015, phytoplankton was found to be the main source of POC at locations 3, 5, 6, 10 and 11. According to the $\delta^{18}O_{NO3}$ values it is possible to conclude that atmospheric deposition does not contribute significantly to river NO_3^- nor do the nitrates originating from commercial fertilizers. We therefore suggest that the comparatively low isotopic enrichment factors for nitrogen and oxygen arise from a combination of soil nitrification and an admixture of NO_3^- from wastewater and manure. This assumption could be further confirmed by microbiological analyses. These analyses comprised analysis of the standard indicators of fecal pollution and microbial source tracking (MST) analyses based on the human-associated BacHum and HF183II, the ruminant-associated BacR and the pig-associated Pig2Bac genetic Bacteroidetes fecal markers. The results reveal the existence of hotspots of fecal pollution of human associated origin in the SRB. Long-term data at selected locations indicated persistent fecal contamination, showing that the locations are impacted by continuous discharge of untreated wastewaters. This study describes the present nutrient and microbiological pollution status in the SRB, and indicates the urgent need for effective wastewater treatment plants in water management.

Acknowledgements

This study was conducted within the EU Seventh Framework Programme – GLOBAQUA (managing the effects of multiple stressors on aquatic ecosystem under water scarcity) under Grant agreement no. 603629-ENV-2013-6.2.1. It also represents a part of the activities within the projects funded by the Ministry of Education, Science and Technological Development of the Republic of Serbia projects no. 173045 and 173025 and the bilateral project Serbia and Austria: Implementation of Microbial Source Tracking (MST) method for assessment of faecal pollution in the Sava River and its relation (potential relationship) to the presence of genotoxic agents. The research was conducted partially within the PhD study of Janja Vrzel financed by the European Social Fund no. C2130-12-000070. We acknowledge the staff of Hrvatske vode and the Republic Hydrometeorological Service of Serbia, who helped us to obtain the discharge and water quality data, and the International Commission for the Protection of the Danube River (ICPDR) for data obtained from the Transnational Monitoring Network. The authors thank Prof. R. Pain for linguistic corrections.

References

Aborgiba, M., Kostić, J., Kolarević, S., Kračun-Kolarević, M., Elbahi, S., Knežević-Vukčević, J., Lenhardt, M., Paunovć, M., Gačić, Z., Vuković-Gačić, B., 2016. Flooding modifies the genotoxic effects of pollution on a worm, a mussel and two fish species from the Sava River. Sci. Total Environ. 540, 358–367.

Anderson, I.C., Levine, J.S., 1986. Relative rates of nitric oxide and nitrous oxide production by nitrifiers, denitrifiers, and nitrate respirers. Appl. Environ. Microbiol. 51, 938–945.

Aravena, R., Evans, M.L., Cherry, J.A., 1993. Stable isotopes of oxygen and nitrogen in source identification of nitrate from septic systems. Ground Water 31, 180–186.

Böhlke, J.K., Ericksen, G.E., Revesz, K., 1997. Stable isotope evidence for an atmospheric origin of desert nitrate deposits in northern Chile and southern California, USA. Chem. Geol. 136, 135–152.

Buckalew, D.W., Hartman, L.J., Grimsley, G.A., Martin, A.E., Register, K.M., 2006. A long-term study comparing membrane filtration with Colilert defined substrates in detecting fecal coliforms and *Escherichia coli* in natural waters. J. Environ. Manag. 80, 191–197.

Byamukama, D., Mach, R.L., Kansiime, F., Manafi, M., Farnleitner, A.H., 2005. Discrimination efficacy of fecal pollution detection in different aquatic habitats of a high-altitude tropical country, using presumptive coliforms, *Escherichia coli*, and *Clostridium perfringens* spores. Appl. Environ. Microbiol. 71, 65–71.

Casciotti, K.L., Sigman, D.M., Hastings, M., Böhlke, J.K., Hilkert, A., 2002. Measurement of the oxygen isotopic composition of nitrate in seawater and freshwater using the denitrifier method. Anal. Chem. 74, 4905–4912.

Casteel, M.J., Sobsey, M.D., Mueller, J.P., 2006. Fecal contamination of agricultural soils before and after hurricane-associated flooding in North Carolina. J. Environ. Sci. Health A 41, 173–184.

Chang, C.C.Y., Kendall, C., Silva, S.R., Battaglin, W.A., Campbell, D.H., 2002. Nitrate stable isotopes: tools for determining nitrate sources among different land use in the Mississippi River Basin. Can. J. Fish. Aquat. Sci. 59, 1874–1885.

Durka, W., Schulze, E.D., Gebauer, G., Voerkeliust, S., 1994. Effects of forest decline on uptake and leaching of deposited nitrate determination from ^{15}N and ^{18}O measurements. Nature 372, 765–767.

Epstein, S., Mayeda, T., 1953. Variations of ^{18}O content of waters from natural sources. Geochim. Cosmochim. Acta 4, 213–224.

European Environment Agency (EEA), 2015. Indicator Assessment, Prod-ID: IND-8-en, CSI 020, WAT 003. Nutrients in Freshwater.

Finlay, J.C., Kendall, C., 2007. Stable isotope tracing of temporal and spatial variability in organic matter sources to freshwater ecosystems, chapter 10. In: Michener, R.H., Lajtha, K. (Eds.), Stable Isotopes in Ecology and Environmental Science, second ed. Blackwell Publishing, Oxford, pp. 283–333.

Fogg, G.E., Rolston, D.E., Decker, D.L., Louie, D.T., Grismer, M.E., 1998. Spatial variation in nitrogen isotope values beneath nitrate contamination sources. Ground Water 36, 418–426.

Globevnik, L., Mikoš, M., Padežnik, M., Petan, S., Petkovšek, A., Vidmar, A., Milačič, R., Ščančar, J., Heath, E., Ogrinc, N., Brilly, M., 2010. SARIB: chapter 13. In: Brilly, M. (Ed.), Hydrological Processes of the Danube River Basin: Perspectives From the Danubian Countries. Springer, Dordrecht, Heidelberg, pp. 389–428 http://dx.doi.org/10.1007/978-90-481-3423-6.

Grasshoff, K., Ehrhardt, K., Ehrhardt, M., 1983. Methods of Seawater Analysis. Verlag Chemie, New York.

Green, H.C., Haugland, R.A., Varma, M., Millen, H.T., Borchardt, M.A., Field, K.G., Walters, W.A., Knight, R., Kelty, C.A., Shanks, O.C., 2014. Improved HF183 quantitative real-time PCR assay for characterization of human fecal pollution in ambient surface water samples. Appl. Environ. Microbiol. 80, 3086–3094.

Harrington, R.R., Kennedy, B.P., Chamberlain, C.P., Blum, J.D., Folt, C.L., 1998. ^{15}N enrichment in agricultural catchments: field patterns and applications to tracking Atlantic salmon (*Salmo salar*). Chem. Geol. 147, 281–294.

Heatwaite, A.L., 2010. Multiple stressors on water availability at global to catchment scales: understanding human impact on nutrient cycles to protect water quality and water availability in the long term. Freshw. Biol. 55, 241–257.

Hebert, C.G., Wassenaar, L.I., 2001. Stable nitrogen isotopes in waterfowl feathers reflect agricultural land use in western Canada. Environ. Sci. Technol. 35, 3482–3487.

Hellings, L., Dehairs, F., Tacks, M., Keppens, E., Baeyens, W., 1999. Origin and fate of organic carbon in the freshwater part of the Scheldt estuary as traced by stable carbon isotope composition. Biogeochemisty 47, 167–186.

Hoffman, J.C., Bronk, D.A., 2006. Interannual variation in stable carbon and nitrogen isotope biogeochemistry of the Mattaponi River, Virginia. Limnol. Oceanogr. 51, 2319–2332.

Hollocher, T.C., 1984. Source of the oxygen atoms of nitrate in the oxidation of nitrite by *Nitrobacter agilis* and evidence against a P–O–N anhydride mechanism in oxidative phosphorylation. Arch. Biochem. Biophys. 233, 721–727.

ISRBC, 2011. International Sava River Basin Commission. Sava River Basin Management Plan. Draft Version 6.2. 241 pages http://www.savacommission.org/srbmp/.

Johannsen, A., Dähnke, K., Emeis, K., 2008. Isotopic composition of nitrate in five German rivers discharging into the North Sea. Org. Geochem. 39, 1678–1689.

Kapetanović, D., Smrzlić, I.V., Valić, D., Teskeredžić, E., Kolarević, S., Sunjog, K., Tomović, J., Kračun-Kolarević, M., Knežević-Vukčević, J., Paunović, M., Gačić, Z., Vuković-Gačić, B., 2015. Microbial characterisation of the Sava River. In: Milačič, R., Ščančar, J., Paunović, M. (Eds.), The Sava River. Springer Berlin Heidelberg, Berlin, pp. 201–228.

Kendall, C., 1998. Tracing sources and cycling of nitrate in catchments. In: Kendall, C., McDonnell, J.J. (Eds.), Isotope Tracers in Catchment Hydrology. Elsevier, Amsterdam, pp. 519–576.

Kendall, C., Elliott, E.M., Wankel, S.D., 2008. Tracing anthropogenic inputs of nitrogen to ecosystems. In: Michener, R., Lajtha, K. (Eds.), Stable Isotopes in Ecology and Environmental Science, second ed. Blackwell Publishing Ltd, Oxford, pp. 375–449.

Kildare, B.J., Leutenegger, C.M., McSwain, B.S., Bambic, D.G., Rajal, V.B., Wuertz, S., 2007. 16S rRNA-based assays for quantitative detection of universal, human-, cow-, and dog-specific fecal *Bacteroidales*: a Bayesian approach. Water Res. 41, 3701–3715.

Kirschner, A.K., Kavka, G., Reischer, G.H., Sommer, R., Blaschke, A.P., Stevenson, M., Vierheilig, J., Mach, R.L., Farnleitner, A.H., 2014. Microbiological water quality of the Danube River: status quo and future perspectives. In: Liska, I. (Ed.), The Danube River Basin. Springer Berlin Heidelberg, Berlin, pp. 439–468.

Kirschner, A.K.T., Kavka, G., Velimirov, B., Mach, R.L., Sommer, R., Farnleitner, A.H., 2009. Microbiological water quality along the Danube River: integrating data from two

whole-river surveys and a transnational monitoring network. Water Res. 43, 3673–3684.
Kittinger, C., Marth, E., Reinthaler, F.F., Zarfel, G., Pichler-Semmelrock, F., Mascher, W., Mascher, F., 2013. Water quality assessment of a central European river—does the directive 2000/60/EC cover all the needs for a comprehensive classification? Sci. Total Environ. 447, 424–429.
Kroopnick, P., Craig, H., 1972. Atmospheric oxygen: isotopic composition and solubility fractionation. Science 175, 54–55.
Layton, A., McKay, L., Williams, D., Garrett, V., Gentry, R., Sayler, G., 2006. Development of bacteroides 16S rRNA gene TaqMan-based real-time PCR assays for estimation of total, human, and bovine fecal pollution in water. Appl. Environ. Microbiol. 72, 4214–4224.
Markovics, R., Kanduč, T., Szramek, K., Globočanin, D., Milačič, R., Ogrinc, N., 2010. Chemical dynamics of the Sava riverine system. J. Environ. Monit. 12, 2165–2176.
Mayer, R.E., Bofill-Mas, S., Egle, L., Reischer, G.H., Schade, M., Fernandez-Cassi, X., 2016. Occurrence of human-associated bacteroidetes genetic source tracking markers in raw and treated wastewater of municipal and domestic origin and comparison to standard and alternative indicators of faecal pollution. Water Res. 90, 265–276.
Mayer, B., Bollwerk, S.M., Mansfeldt, T., Hütter, B., Veizer, J., 2001. The oxygen isotope composition of nitrate generated by nitrification in acid forest floors. Geochim. Cosmochim. Acta 65, 2743–2756.
Mayer, B., Boyer, E.W., Goodale, C., Jaworski, N.A., Breemen, N.V., Howarth, R.W., Seitzinger, S., Billen, G., Lajtha, K., Nadelhoffer, K., Van Dam, D., Hetling, L.J., Nosal, M., Paustian, K., 2002. Sources of nitrate in rivers draining sixteen watersheds in the northeastern U.S.: isotopic constraints. Biogeochemisty 57, 171–197.
McClelland, J.W., Valiela, I., 1998. Linking nitrogen in estuarine producers to land derived sources. Limnol. Oceanogr. 43, 577–585.
Mieszkin, S., Furet, J.P., Corthier, G., Gourmelon, M., 2009. Estimation of pig fecal contamination in a river catchment by real-time PCR using two pig-specific *Bacteroidales* 16S rRNA genetic markers. Appl. Environ. Microbiol. 75, 3045–3054.
Mook, W.G., Tan, F.C., 1991. Stable carbon isotopes in rivers and estuaries. In: Degens, E.T., Kampe, S., Richey, J.E. (Eds.), Biochemistry of Major World Rivers. J. Wiley and Son, Ltd, Chicester, pp. 245–264.
Navarro-Ortega, A., Acuña, V., Bellin, A., Burek, P., Cassiani, G., Choukr-Allah, R., Dolédec, S., Elosegi, A., Ferrari, F., Ginebreda, A., Grathwohl, P., Jones, C., Rault, K., Kasper Kok, P., Koundouri, P., Ludwig, R.P., Merz, R., Milacic, R., Muñoz, I., Nikulin, G., Paniconi, C., Paunović, M., Petrovic, M., Sabater, L., Sabater, S., Skoulikidis, N.T., Slob, A., Teutsch, G., Voulvoulis, N., Barceló, D., 2015. Managing the effects of multiple stressors on aquatic ecosystems under water scarcity. The GLOBAQUA project. Sci. Total Environ. 503–504, 3–9.
Ogrinc, N., Kanduč, T., Golobočanin, D., Kocman, D., Miljević, N.R., 2010. A Hydrogeochemical and Isotope Investigation of the River Sava Watershed. Nova Science Publishers Inc., New York.
Ogrinc, N., Kanduč, T., Kocman, D., 2015. Integrated approach to the evaluation of chemical dynamics and anthropogenic pollution sources in the Sava River Basin. In: Milačič, R., Ščančar, J., Paunović, M. (Eds.), The Sava River Vol. 31. Springer Berlin Heidelberg, pp. 75–94.
Ogrinc, N., Markovics, R., Kanduč, T., Walter, L.M., Hamilton, S.K., 2008. Sources and transport of carbon and nitrogen in the river Sava watershed, a major tributary of the river Danube. Appl. Geochem. 23, 3685–3698.
Paunović, M., Tomović, J., Kovačević, S., Zorić, K., Žganec, K., Simić, V., Atanacković, A., Marković, V., Kračun, M., Hudina, S., Lajtner, J., Gottstein, S., Lucić, A., 2012. Macroinvertebrates of the natural substrate of the Sava River – preliminary results. Water Resour. Manag. 2, 33–39.
Reischer, G.H., Haider, J.M., Sommer, R., Stadler, H., Keiblinger, K.M., Hornek, R., Zerobin, W., Mach, R.L., Farnleitner, A.H., 2008. Quantitative microbial faecal source tracking with sampling guided by hydrological catchment dynamics. Environ. Microbiol. 10, 2598–2608.
Reischer, G.H., Kasper, D.C., Steinborn, R., Mach, R.L., Farnleitner, A.H., 2006. Quantitative PCR method for sensitive detection of ruminant fecal pollution in freshwater and evaluation of this method in alpine karstic regions. Appl. Environ. Microbiol. 72, 5610–5614.
Seiler, R.L., 2005. Combined use of ^{15}N and ^{18}O of nitrate and ^{11}B to evaluate nitrate contamination in groundwater. Appl. Geochem. 20, 1626–1636.
Sigman, D.M., Casciotti, K.L., Andreani, M., Barford, C., Galanter, M., Böhlke, J.K., 2001. A bacterial method for the nitrogen isotopic analysis of nitrate in seawater and freshwater. Anal. Chem. 73, 4145–4153.
Sorensen, D.L., Eberl, S.G., Dicksa, R.A., 1989. *Clostridium perfringens* as a point source indicator in non-point polluted streams. Water Res. 23, 191–197.
Stapleton, C.M., Kay, D., Wyer, M.D., Davies, C., Watkins, J., Kay, C., McDonald, A.T., Porter, J., Gawler, A., 2009. Evaluating the operational utility of a bacteroidales quantitative PCR-based MST approach in determining the source of faecal indicator organisms at a UK bathing water. Water Res. 43 (19), 4888–4899.
Voss, M., Deutsch, B., Elmgren, R., Humborg, C., Kuuppo, P., Pastuszak, M., Rolff, C., Schulte, U., 2006. Source identification of nitrate by means of isotopic tracers in the Baltic Sea catchments. Biogeosciences 3, 663–676.
Vrzel, J., Ogrinc, N., 2015. Nutrient variations in the Sava River Basin. J. Soils Sediments 15, 2380–2386.
Wankel, S.D., Kendall, C., Francis, C.A., Paytan, A., 2006. Nitrogen sources and cycling in the San Francisco Bay Estuary: a nitrate dual isotope approach. Limnol. Oceanogr. 51, 1654–1664.
Wassenaar, L.I., 1995. Evaluation of the origin and fate of nitrate in the Abbotsford aquifer using the isotopes of ^{15}N and ^{18}O in NO_3^-. Appl. Geochem. 10, 391–405.
Widory, D., Kloppmann, W., Chery, L., Bonnin, J., Rochdi, H., Guinamant, J.L., 2004. Nitrate in groundwater, an isotope multi-tracer approach. J. Contam. Hydrol. 72, 165–188.
Xue, D., Botte, J., De Baets, B., Accoe, F., Nestler, A., Taylor, P., Van Cleemput, O., Berglund, M., Boeckx, P., 2009. Present limitations and future prospects of stable isotope methods for nitrate source identification in surface- and groundwater. Water Res. 43, 1159–1170.