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DOCTORAL DISSERTATION

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Supervisor:
Prof. Jelena Parlov, PhD

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Rudarsko-geološko-naftni fakultet

Patricia Buškulić

DINAMIKA NITRATA U NESATURIRANOJ I SATURIRANOJ ZONI U PRILJEVNOM PODRUČJU CRPILIŠTA VELIKA GORICA

DOKTORSKI RAD

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This dissertation contains:
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an abstract in English and an extended abstract in Croatian;
keywords in English and Croatian;
and biography of the author with a list of published papers.

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ABSTRACT

The Zagreb aquifer system hosts strategic groundwater reserves of the Republic of Croatia and supplies drinking water to one million inhabitants of the capital city and Zagreb County. Nitrate ions (NO_3^-) are one of the most common contaminants in the groundwater of the Zagreb aquifer system and elevated nitrate concentrations have been confirmed through previous research. Previous research conducted in the Zagreb area did not investigate the nitrogen (N) dynamics in the soil and unsaturated zone, the processes affecting NO_3^- infiltration into the aquifer, nor did it quantify the proportional contributions from different sources of N. Therefore, field and laboratory research, monthly sampling and analyses of groundwater and surface water, precipitation and soil water, as well as analyses of soil and various local N sources were conducted in the catchment area of the Velika Gorica well field, located on the right bank of the Sava River, in the southern part of the Zagreb aquifer system. The main goal was to define the sources and dynamics of NO_3^- in the unsaturated and saturated zone within the area of the Eutric Cambisols on the Holocene deposits, one of the most common soils developed in the area of the Zagreb aquifer system. A multi-method approach was used, integrating physico-chemical, hydrogeochemical and isotopic data (water and nitrate stable isotopes) together with analyses of molar ratios, statistical techniques and mixing models. As a starting point, research demonstrates the approach to estimate the ambient background value (ABV) of nitrate in order to assess the presence of groundwater contamination. The ABV of nitrate determined for the catchment area of the Velika Gorica well field ranged from 7.1 to 7.5 mg/L. Furthermore, a new water sample preparation method for stable isotopic analysis of nitrogen and oxygen in dissolved NO_3^- was implemented. The combination of a nitrous oxide isotopic analyzer and the new sample preparation method provides reliable measurements of $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$, with optimal stability achieved under specific conditions. Soil moisture content, pH, organic carbon availability and soil texture are important controlling factors influencing the dynamics of N species within the soil. In the deepest soil horizon within the coarse-grained Eutric Cambisols, N is present mostly as nitrites (NO_2^-) and nitrates (NO_3^-) due to soil ammonium (NH_4^+) nitrification and leaching from the upper soil horizons during intense rainfall. Under such aerobic conditions, which promote stability and the potential for transport, N migrates to deeper parts of unsaturated zone in the form of NO_2^- and NO_3^-. The isotopic composition of water and two-component mixing model showed that groundwater is predominantly recharged by the Sava River but its contribution varied spatially. The results also show the existence of a different source of recharge in the southern part of the aquifer system. Nitrate in the groundwater predominantly originates from organic sources, with nitrification as the main biogeochemical process, while denitrification was identified at sampling sites with specific anaerobic conditions. However, the Bayesian mixing model showed that contribution from mineral fertilizer exists and confirmed its significance as the source of NO_3^- and possible cause of elevated NO_3^- concentrations. Hydrogeochemical data, molar ratios and correlation analyses suggested that most part of nitrate concentrations were associated with the multiple contamination inputs, but at the same time did not clearly recognize the influence of mineral fertilizer. Therefore, the findings of this research highlighted the importance of employing a multi-method approach if NO_3^- originates from multiple sources in order to obtain reliable and comprehensive understanding of NO_3^- dynamics.

Keywords: origin and dynamics of nitrate, ambient background value, water stable isotopes, groundwater recharge, hydrogeochemistry, molar ratios, statistical analyses, nitrate stable isotopes, Bayesian mixing model, Zagreb aquifer system, soil zone, Eutric Cambisols

PROŠIRENI SAŽETAK

Zagrebački vodonosni sustav sadrži strateške zalihe podzemne vode Republike Hrvatske, te je iznimno važan izvor pitke vode za oko milijun stanovnika. Ima visoku do vrlo visoku prirodnu ranjivost, što u urbanim područjima i područjima s intenzivnijim industrijskim i poljoprivrednim aktivnostima dovodi do povišenih koncentracija onečišćivala u podzemnoj vodi. Nitrati (NO_3^-) su prepoznati kao jedni od najčešćih skupina onečišćivala podzemne vode zagrebačkog vodonosnog sustava, a povišene koncentracije nitrata u podzemnoj vodi potvrđene su kroz ranija istraživanja. Glavni antropogeni izvori nitrata povezani su s procjeđivanjem iz propusnih septičkih jama i kanalizacijske mreže, organskim stajskim gnojivom i intenzivnom uporabom anorganskih mineralnih gnojiva, dok značajni prirodni izvori uključuju atmosfersko taloženje dušika (N) i razgradnju organskog dušika iz tla. Za utvrđivanje postojanja onečišćenja podzemne vode, važno je razlikovati prirodne i antropogene utjecaje na kakvoću podzemne vode. Kako bi razlikovali prirodne i antropogeno uvjetovane koncentracije neke tvari u vodi, osmišljen je koncept ambijentalne pozadinske vrijednosti (engl. ambient background value, ABV), koji opisuje nemjerljivo poremećenu i ne više netaknutu pozadinsku koncentraciju tvari u podzemnoj vodi. Pozadinske koncentracije tvari, određene na razini regionalnih vodonosnih sustava, mogu se značajno razlikovati od pozadinskih koncentracija istih tvari određenih u homogenim područjima unutar vodonosnih sustava. Za identifikaciju izvora, te procesa koji utječu na transformaciju i dinamiku NO_3^- nužno je koristiti stabilne izotope dušika i kisika iz otopljenog nitrata ($\delta^{15}N_{NO3}$ i $\delta^{18}O_{NO3}$). Analiza $\delta^{15}N_{NO3}$ i $\delta^{18}O_{NO3}$ omogućena je razvojem različitih laboratorijskih metoda za pripremu uzoraka. Postojeće metode koje se trenutno provode u većini svjetskih laboratorija, poput bakterijske denitrifikacijske metode ili metode redukcije kadmija (azid metoda), uključuju više koraka pretvorbe ili upotrebu toksičnih kemikalija. Godine 2019., razvijena je titanij(III) redukcijska metoda za određivanje izotopnog sastava nitrata koja predstavlja metodu kemijske pretvorbe u jednom koraku primjenom reagensa Ti(III) klorid otopine koji reducira NO_3^- u N_2O plin. Metoda je jednostavnija, brža i isplativija u odnosu na ostale laboratorijske metode. Iako je izotopna analiza vrijedan alat za praćenje dinamike nitrata, procesi nitrifikacije i denitrifikacije te prisutnost više izvora nitrata kompliciraju jednoznačno određivanje podrijetla i procesa transformacije nitrata. Zbog navedenoga, kombinacijom hidrogeokemijskih analiza, analiza izotopnog sastava stabilnih izotopa vode i stabilnih izotopa nitrata, analiza molarnih omjera, statističkih metoda i modela miješanja, moguće je pouzdanije odrediti podrijetlo i dinamiku nitrata.

Ranija istraživanja bila su usmjerena na utvrđivanje podrijetla nitrata u zagrebačkom vodonosnom sustavu, procjenu trendova nitrata u podzemnoj vodi i određivanje područja s najvećim rizikom od onečišćenja nitratima. Izotopni sastav $\delta^{15}N_{NO3}$ i $\delta^{18}O_{NO3}$ bio je izmjeren dominantno na uzorcima podzemne vode te su rezultati pokazali da su nitrati u podzemnoj vodi zagrebačkog vodonosnog sustava pretežito organskog podrijetla, a većina analiza pokazala je izotopni sastav karakterističan za nitrate iz otpadnih voda. Iako su provedena istraživanja pojave dušika u podzemnoj vodi, nije istražena dinamika dušika u zoni tla i nesaturiranoj zoni, geokemijski i hidrodinamički procesi koji utječu na procjeđivanje nitrata u vodonosnik i na dinamiku nitrata u saturiranoj sredini, kao ni kvantifikacija udjela različitih izvora onečišćenja nitratima u podzemnoj vodi. Praćenje izvora i procesa koji utječu na stvaranje i transformaciju NO_3^- u podzemnoj vodi, kao i u zoni tla, važno je za bolje razumijevanje ciklusa dušika, zaštitu kakvoće vode i održivo upravljanje vodonosnim sustavom. Kako bi se, osim podrijetla, odredila i dinamika NO_3^-, potrebno je mjeriti izotopni sastav $\delta^{15}N_{NO3}$ i $\delta^{18}O_{NO3}$ i u površinskoj vodi, oborini i procjednoj vodi iz tla. Također, potrebno je provesti i analizu izotopnoga sastava ukupnog dušika u čvrstim uzorcima različitih izvora dušika, što dosad još nije napravljeno na području istraživanja.

Crpilište Velika Gorica jedno je od najvažnijih crpilišta Zagrebačke županije, koje opskrbljuje pitkom vodom Grad Zagreb i grad Veliku Goricu i nalazi se na desnom zaobalju rijeke Save u južnom dijelu zagrebačkog vodonosnog sustava. Zagrebački vodonosni sustav izgrađen je od nekonsolidiranih kvartarnih sedimenata, koji se sastoje uglavnom od pijeska, šljunka i praha ili prahovitih glina, i sadrži dva hidraulički povezana sloja, plitki i duboki. Plitki, holocenski vodonosnik izgrađen je od aluvijalnih naslaga, uglavnom šljunka i pijeska. U izravnoj je hidrauličkoj vezi s rijekom Savom, koja predstavlja glavni izvor prihranjivanja podzemne vode. Dublji, pleistocenski vodonosnik izgrađen je od jezersko-barskih naslaga s raznim udjelima šljunka, pijeska, praha i gline. Crpilište se sastoji od pet zdenaca dok se u priljevnom području crpilišta nalazi 13 piezometara koji se uglavnom koriste za praćenje kakvoće podzemne vode u plitkom vodonosniku. Unutar prve zone sanitarne zaštitne crpilišta Velika Gorica izgrađen je znanstveno istraživački poligon Rudarsko-geološko-naftnog fakulteta Sveučilišta u Zagrebu, u kojem su se provodila mjerenja za potrebe ovog istraživanja. Istraživački poligon se nalazi u eutričnom smeđem tlu na holocenskim nanosima. Debljina nesaturirane zone na istraživanom području obično varira od 5 do 8 metara i ovisi o razini podzemne vode. U gornjem dijelu nesaturirane zone određeni su sljedeći pedološki horizonti tla: A (0–0,15 m), 2B (0,15–0,55 m), 3BC (0,55–0,9 m) i C (0,9–1,17 m). Gornjih 90 centimetara profila tla uglavnom se sastoji od prahovitih i pjeskovitih materijala, s proslojcima gline, dok šljunak dominira u donjem dijelu profila, sve do razine podzemne vode. Unutar istraživačkog poligona nalazi se meteorološka postaja i pedološka jama opremljena instrumentima za promatranje i mjerenje parametara u nesaturiranoj zoni. Dominantan način korištenja zemljišta, u priljevnom području crpilišta Velika Gorica, je poljoprivredno korištenje u izmjeni s izgrađenim (urbanim) zemljištem.

Ciljevi ovog istraživanja bili su: (1) odrediti ambijentalnu pozadinsku koncentraciju nitrata u hidrogeološki homogenom području heterogenog vodonosnog sustava pod dugotrajnim utjecajem ljudske aktivnosti, (2) implementirati titanij(III) redukcijsku metodu za analizu stabilnih izotopa dušika i kisika iz otopljenog nitrata, (3) poboljšati sveobuhvatno razumijevanje dinamike nitrata u nesaturiranoj i saturiranoj zoni na području eutričnog smeđeg tla na holocenskim nanosima zagrebačkoga vodonosnika i (4) definirati dominantni prirodni oblik dušika koji se iz zone tla infiltrira u vodonosnik priljevnoga područja crpilišta Velika Gorica. U skladu s navedenim ciljevima, postavljene su sljedeće hipoteze: (1) ambijentalna pozadinska koncentracija nitrata u podzemnoj vodi priljevnoga područja crpilišta Velika Gorica je niža od 10 mg/L NO_3^-, (2) u području bez antropogenih aktivnosti, dušik se dominantno unosi iz zone tla (eutrično smeđe tlo na holocenskim nanosima) u nesaturiranu zonu u obliku nitrita i nitrata i (3) izvor nitrata u podzemnoj vodi priljevnoga područja crpilišta Velika Gorica nisu mineralna gnojiva. Kao najvažniji znanstveni doprinos treba istaknuti: unaprjeđenje poznavanja procesa transformacije dušikovih spojeva u tlu, nesaturiranoj zoni i vodonosniku; određivanje dinamike i podrijetla nitrata, kao i kvantifikaciju udjela različitih izvora dušika, primjenom stabilnih izotopa ^{15}N i ^{18}O u otopljenom nitratu i izotopa ^{15}N u potencijalnim izvorima onečišćenja nitratima; i izradu konceptualnog modela dinamike dušikovih spojeva u nesaturiranoj i saturiranoj zoni zagrebačkog vodonosnog sustava.

Zbog navedenoga, u priljevnom području crpilišta Velika Gorica, hidrogeološki homogenom području unutar zagrebačkog vodonosnog sustava, provedena su terenska i laboratorijska istraživanja, mjesečna uzorkovanja u trajanju od jedne godine, analize kakvoće podzemne i površinske vode, oborine i procjedne vode iz tla, kao i analize tla i različitih izvora dušika. Uzorci podzemne vode prikupljeni su iz 10 piezometara, a izmjerene su i razine podzemne vode u svrhu izrade karata ekvipotencijala za vrijeme visokih i niskih podzemnih voda. Uzorci površinske vode uzorkovani su iz rijeke Save na lokaciji Podsusedski most. In-situ mjerenja uključivala su: pH, električnu vodljivost, sadržaj otopljenog kisika i temperaturu. Uzorci procjedne vode iz tla prikupljeni su iz pedološke jame iz četiri horizonta tla, primjenom usisnih čašica. Satna mjerenja vlage u tlu i temperature tla provedena su pomoću TDR (engl. time domain reflectrometry) sonda, koje su instalirane u četiri horizonta tla. Meteorološka stanica, smještena blizu pedološke jame, kontinuirano je mjerila oborinu i temperaturu zraka, dok je oborina uzorkovana pomoću uzorkivača kiše. Svi tekući uzorci filtrirani su na terenu i pohranjeni u plastične HDPE (engl. high-density polyethylene) boce kako bi se obavile kemijske i izotopne analize. Čvrsti uzorci različitih izvora dušika prikupljeni su unutar područja istraživanja i uključivali su šest uzoraka organskog podrijetla i četiri uzorka mineralnog gnojiva. Organski uzorci uključivali su dva uzorka stajskog gnojiva, dva uzorka iz septičkih jama i dva uzorka otpadnih voda, dok su uzorkovana mineralna gnojiva uglavnom bila amonijska. Uzorci tla prikupljeni su do dubine od 1,2 metra po intervalima od 10 cm, blizu pedološke jame. U laboratorijima Rudarsko-geološko-naftnog fakulteta Sveučilišta u Zagrebu provedena su sljedeća mjerenja: analize glavnih aniona i kationa, analize izotopnog sastava stabilnih izotopa vode (δ^2H_{H2O} and $\delta^{18}O_{H2O}$) i stabilnih izotopa nitrata ($\delta^{15}N_{NO3}$ i $\delta^{18}O_{NO3}$) u tekućim uzorcima, kao i analize odabranih fizikalno-kemijskih parametara u uzorcima tla. Uzorci za određivanje izotopnog sastava dušika i kisika iz nitrata pripremljeni su pomoću Ti(III) redukcijske metode. Analize izotopnog sastava ukupnog dušika ($\delta^{15}N_{bulk}$) i postotka dušika (%N) u čvrstim uzorcima različitih izvora dušika provedena su u laboratoriju Fakulteta geoznanosti Sveučilišta u Barceloni.

U početnom koraku istraživanja, proveden je postupak procjene ambijentalne pozadinske koncentracije nitrata u svrhu definiranja prisutnosti onečišćenja podzemne vode. Ambijentalna pozadinska koncentracija nitrata procijenjena je korištenjem dostupnih povijesnih podataka o koncentraciji nitrata iz 12 piezometara smještenih u priljevnom području crpilišta Velika Gorica, korištenjem statističkih metoda temeljenih na modelima (iterativna 2σ tehnika, izračunata funkcija raspodjele, kumulativna frekvencijska krivulja i metoda temeljena na vjerojatnosnom grafu). Istodobno je testirana pouzdanost rezultata svake metode. Interpretacijom rezultata utvrđeno je da pozadinska koncentracija nitrata u priljevnom području crpilišta Velika Gorica iznosi od 7,1 do 7,5 mg/L, te se razlikuje od pozadinske koncentracije određene na razini zagrebačkog vodonosnog sustava. Istraživanje je pokazalo da N_2O izotopni laserski uređaj GLA451-N2OI3, u kombinaciji s novijom metodom pripreme uzoraka, omogućava pouzdana mjerenja $\delta^{15}N_{NO3}$ i $\delta^{18}O_{NO3}$. Dinamika NO_3^- unutar eutričnog smeđeg tla i dominantni prirodni oblik N koji se infiltrira u vodonosnik definirani su na osnovi karakteristika tla i procjedne vode iz tla, primjenom parametarskih i neparametarskih statističkih testova, analizom i karakterizacijom fizikalnih i fizikalno-kemijskih parametara koji utječu na dinamiku dušikovih spojeva unutar zone tla te analizom izotopnog sastava stabilnih izotopa vode i nitrata u tlu. Rezultati su pokazali da su sadržaj vlage u tlu, pH, sadržaj organskog ugljika i tekstura tla važni faktori koji kontroliraju dinamiku dušikovih spojeva u zoni tla. Unutar najdubljeg horizonta tla (C horizont), koji je krupnozrnat, dušik je prisutan najvećim dijelom u obliku nitrita (NO_2^-) i nitrata (NO_3^-) kao rezultat nitrifikacije amonij iona (NH_4^+) iz

tla i procjeđivanja iz plićih horizonata uslijed oborina dužeg trajanja i jakog intenziteta. Uslijed takvih aerobnih uvjeta, koji omogućavaju stabilnost oksidiranih oblika dušika, omogućen je transport NO_2^- i NO_3^- u dublje dijelove nesaturirane zone. Izotopni sastav vode i model miješanja pokazao je da se podzemna voda na istraživanom području pretežno prihranjuje rijekom Savom, ali to prihranjivanje prostorno varira. Također, rezultati upućuju na postojanje različitog izvora prihranjivanja u južnom dijelu vodonosnika. Dinamika nitrata, podrijetlo i procesi koji utječu na transformaciju NO_3^- u podzemnoj vodi definirani su provedbom sveobuhvatnih analiza, koje su uključivale: analize fizikalno-kemijskih svojstava, hidrogeokemijske analize, analize izotopnog sastava stabilnih izotopa nitrata, analize molarnih omjera, statističke metode i model miješanja temeljen na Bayesovoj statistici. Interpretacijom rezultata utvrđeno je da su nitrati u istraživanom području najvećim dijelom organskog podrijetla, a nitrifikacija predstavlja glavni biogeokemijski proces koji se odvija u podzemnoj vodi, dok je proces denitrifikacije utvrđen na specifičnim lokacijama uzorkovanja kao rezultat anaerobnih uvjeta. Rezultati analize izotopnog sastava dušika u čvrstim uzorcima različitih izvora onečišćenja nitratima i modela miješanja temeljenog na Bayesovoj statistici su pokazali da su mineralna gnojiva značajan izvor onečišćenja nitratima na području zagrebačkog vodonosnog sustava i mogući uzrok povišenih koncentracija NO_3^- u podzemnoj vodi. Hidrogeokemijskim analizama, molarnim omjerima i korelacijskim analizama je utvrđeno postojanje različitih izvora onečišćenja nitratima u razdoblju uzorkovanja. Međutim, tim analizama nije jasno prepoznato onečišćenje koje dolazi iz mineralnih gnojiva. Iako takve metode predstavljaju vrijedan alat, trebaju se koristiti s dozom opreza kada nitrati potječu iz više izvora onečišćenja. Kroz istraživanje je istaknuta važnost primjene većeg broja metoda u svrhu pouzdanijeg i sveobuhvatnijeg razumijevanja onečišćenja nitratima.

Ključne riječi: podrijetlo i dinamika nitrata, ambijentalna pozadinska koncentracija, stabilni izotopi vode, napajanje podzemne vode, hidrogeokemija, molarni omjeri, statističke analize, stabilni izotopi nitrata, model miješanja temeljen na Bayesovoj statistici, zagrebački vodonosni sustav, zona tla, eutrično smeđe tlo

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1. INTRODUCTION

Groundwater is the most common and valuable source of water for drinking and irrigation worldwide (Sunitha et al., 2022). Nevertheless, groundwater nitrate contamination has become a worldwide environmental problem (Rockström et al., 2009; Erisman et al., 2013; Xia et al., 2018; Romanelli et al., 2020; Matiatos et al., 2021; Balacco et al., 2023; Tabi et al., 2024) due to increasing trends of nitrates (Fenech et al., 2012; Abascal et al., 2022; Ascott et al., 2024) to levels that are potentially concerning for ecological and human health. Human impacts on environmental compartments are growing, causing a deterioration of groundwater quality and a significant threat for groundwater use in the future. Long-term ingestion of elevated nitrate concentrations in drinking water presents a significant risk on human health and cause adverse effects such as methemoglobinemia in infants (Fraser and Chilvers, 1981; Knobeloch et al., 2000; Greer and Shannon, 2005), preterm births (Sherris et al., 2021), low birth weights (Coffman et al., 2021) and colorectal cancer (Schullehner et al., 2018; Temkin et al., 2019).

To address the issue of nitrate contamination, both the European Union (98/83/EC) and the World Health Organization (WHO, 2004) have established a maximum contaminant level (MCL) of 50 mg/L as NO_3^- in drinking water. This same limit has also been established in Croatia (OG 125/2017, 39/2020, 64/2023). Furthermore, the European Union has adopted the Nitrate Directive (91/676/EEC) aimed at reducing nitrate contamination from agricultural sources and preventing further contamination, which Croatia is required to adhere to.

Dissolved nitrate ($NO_3^–$) is identified as one of the most common forms of reactive nitrogen (N) and originates from natural and/or anthropogenic sources (Xue et al., 2009; Fenech et al., 2012; Nikolenko et al., 2018; Linhoff, 2022; Mayer and Matiatos, 2024). It occurs naturally as a result of the decomposition of soil organic nitrogen and atmospheric deposition of N (Mayer et al., 2002; Torres-Martínez et al., 2020), while the main anthropogenic sources are sewage or industrial wastewaters, discharge from septic tanks, livestock manure and the intensive use of inorganic mineral fertilizers (Balderacchi et al., 2013; Tokazhanov et al., 2020; Hussien et al., 2023). In the soil, nitrate and ammonium (NH_4^+) are the main forms of inorganic N, both of which are essential for plant development and growth. In addition, $NO_3^–$ is considered as the most stable and mobile form of N species in solution (Spalding and Exner, 1993), which do not tend to adsorb to the soil particles by adsorption due to its negative charge. Ammonium is a form of N with a positive charge which tends to adsorb to the soil particles. As a result, most of the N that moves from the soil zone into the aquifer is in the form of $NO_3^–$ (Arauzo et al., 2022). In general, due to over saturation in the soil, N species are lost to groundwater through leaching and contributes to groundwater contamination (Ayiti and Babalola, 2022). Once groundwater has been contaminated with nitrates, remediation is usually difficult and lengthy (Pisciotta et al., 2015; Li and Tabassum, 2021). Nitrate removal from groundwater can be achieved through process of denitrification or dilution (Pauwels et al., 2000). Investigating nitrate is challenging because N transformation processes take place in two zones: the unsaturated zone (which comprises the soil zone) and the saturated zone.

To determine the presence of groundwater contamination, it is important to distinguish between natural and anthropogenic influences on groundwater quality (Nakić et al., 2013; de Caro et al., 2017). However, distinguishing between concentrations related to the natural processes from those indicating anthropogenic influences can often be challenging (Sellerino et al., 2019). Due to the long-term human influence on the environment, the natural composition of groundwater almost does not exist today. Therefore, since it is pointless to try to determine natural background concentrations of substances in groundwater, Reiman and Garrett (2005) introduced the concept of the ambient background value (ABV), which describes an immeasurably disturbed and no longer intact background concentration of substances in groundwater. Moreover, tracing the biogeochemical processes and sources of NO_3^- in the soil and groundwater is essential for improved understanding of N cycling, better protection of water quality and sustainable management of the aquifer (Zhao et al., 2020; Matiatos et al., 2024).

To identify the source(s) of NO_3^- contamination, as well as the processes that influence the transformations and dynamics of N, researchers commonly use nitrogen and oxygen stable isotopes of NO_3^-, namely $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ (Aravena et al., 1993; Wassenaar, 1995; Zhang et al., 2018; Esquivel-Hernández et al., 2023; Hussien et al., 2023; Matiatos et al., 2023; Rotiroti et al., 2023). Although nitrate stable isotope analyses enables the identification and tracking of NO_3^- sources, the isotopic ranges of NO_3^- sources may overlap and biogeochemical processes like nitrification and denitrification may complicate the determination of NO_3^- origin and transformations (Jung et al., 2020). Biogeochemical processes predominantly occur in the unsaturated zone and shallow aquifers, influencing NO_3^- and $\delta^{15}N_{NO3}$ values. Nitrification process involves the oxidation of NH_4^+ to NO_3^- (Kendall, 1998) and occurs under aerobic conditions with the presence of nitrifying bacteria, while denitrification generally involves the reduction of NO_3^- to N_2, N_2O or NO and occurs under anaerobic conditions in presence of the denitrifying bacteria (Brandes and Devol, 1997). Denitrification is a natural attenuation process that reduces NO_3^-concentrations and produces a linear relationship in the residual NO_3^- with a typical $\delta^{18}O_{NO3}$/ $\delta^{15}N_{NO3}$ ratios range from 1:1.3 to 1:2.1 (Böttcher et al., 1990; Fukada et al., 2003; Liu et al., 2006).

To improve the application of nitrate isotope techniques, the use of additional parameters and methods is considered important. The molar ratios analyses are recognized as a valuable tool for exploring N dynamics and sources (Zeng and Wu, 2015). The elevated Cl^- concentrations and low NO_3^-/Cl^- ratios in the groundwater samples indicate that NO_3^- was derived from manure and/or sewage (Widory et al., 2005). Samples with a low Cl^- content and high NO_3^-/Cl^- ratio (ratio equal to 1) could be associated with a mixture of agricultural inputs (Torres-Martínez et al., 2021). In addition, according to Zhao et al. (2020), higher NO_3^-/Cl^- molar ratios often suggest potential NO_3^- inputs from sources such as precipitation, fertilizer application and nitrification of soil N. According to Li et al. (2010) and Kovač et al. (2018a), a low NO_3^-/K^+ molar ratios indicate contamination by sewage effluent from the urban area. Mineral fertilizers and manure often have higher NO_3^-/K^+ molar ratios, ranging from 3 to 4 and from 10 to 12, respectively. Moreover, different statistical methods are used to estimate the NO_3^- origin. A strong positive statistical correlation between NO_3^- and Cl^- indicates an origin of nitrates from organic wastes (manure and/or sewage) (Rodvang et al., 2004; Moratalla et al., 2009; Kohn et al., 2015). However, Xue et al. (2009) argue that correlation analyses are only useful in cases where there is a single source of contamination and no fractionation. Apart from using nitrate stable isotope analyses, hydrogeochemical data, molar ratios and correlation statistical analyses, Bayesian isotope mixing models are also employed to identify and quantify nitrogen sources (Kim et al., 2014; Matiatos et al., 2023). Furthermore, the nitrification process in the soil is influenced by many factors, such as soil moisture (Ayiti and Babalola, 2022), temperature (Pastén-Zapata et al., 2014), soil pH (Cui et al., 2022), organic carbon content (Xiao et al., 2021), the presence of major oxides (Huang et al., 2016; Simonin et al., 2017; Xin et al., 2017) and heavy metals (Hamsa et al., 2017) as well as soil texture (Zhou et al., 2019). By examining these factors, the biogeochemical processes within the soil zone can be more effectively analysed and traced. Therefore, the use of various physico-chemical properties, hydrogeochemical data, molar ratios, water stable isotopes, statistical tools and mixing models, in combination with nitrate stable isotopes, can enhance the application of nitrate isotope techniques and improve the reliability of research.

Groundwater presents the primary and valuable source of potable water for water supply systems in the Republic of Croatia. The Zagreb aquifer system is very important source of drinking water for the inhabitants of the City of Zagreb and Zagreb County and contains strategic groundwater reserves of the Republic of Croatia. However, fast growth of the City of

Zagreb and development of industry have significantly affected the groundwater quality, with observed groundwater contamination over the last decades (Nakić et al., 2013). The Zagreb aquifer system shows high to very high vulnerability to contamination as evidenced by the presence of five main groups of contaminants (toxic metals, nitrates, pesticides, pharmaceuticals and chlorinated aliphatics) (Nakić et al., 2013) and elevated groundwater concentrations of NO_3^- in dominantly urban and agricultural areas (Kovač et al., 2017a). Continuous soil water, surface water and groundwater monitoring is crucial to evaluate the temporal variability in NO_3^- content, whereas the identification of NO_3^- sources and processes affecting NO_3^- dynamics will enable a better understanding of the N cycle and facilitate sustainable management and protection of the Zagreb aquifer system.

1.1 Study area

The Zagreb aquifer system is situated in the north-western part of the Republic of Croatia within the Sava River catchment and covering an area of about 350 km^2 (Figure 1A). It is an unconfined alluvial aquifer system composed of unconsolidated Quaternary sediments consisting mainly of sand, gravel and silt or silty clays. It consists of two hydraulically connected aquifers: a shallow aquifer of Holocene alluvial deposits, and a deeper aquifer of Pleistocene lacustrine-marsh deposits (Velić and Durn, 1993). The thickness of the aquifer system ranges from less than 10 meters in the western part to approximatelly 100 meters in the eastern part. In general, groundwater flows from west/northwest to east/southeast and coincides with the Sava River flow direction, which is the main source of groundwater recharge (Posavec et al., 2017; Parlov et al., 2019). The Zagreb aquifer system area is defined by three dominant pedological units: Fluvisols, Stagnosols, and Eutric Cambisols on Holocene deposits (Bogunović et al., 1998; Ružičić, 2013) (Figure 1B). Based on data from the Croatian Meteorological and Hydrological Service, the climate is classified as a humid continental climate, with an annual average precipitation of 967 mm for the period from 2001 to 2020. The monthly average precipitation is around 80 mm and is evenly distributed throughout the year. The annual average air temperature is 11.9 °C, with a minimum in January and a maximum in July (Buškulić et al., 2023b).

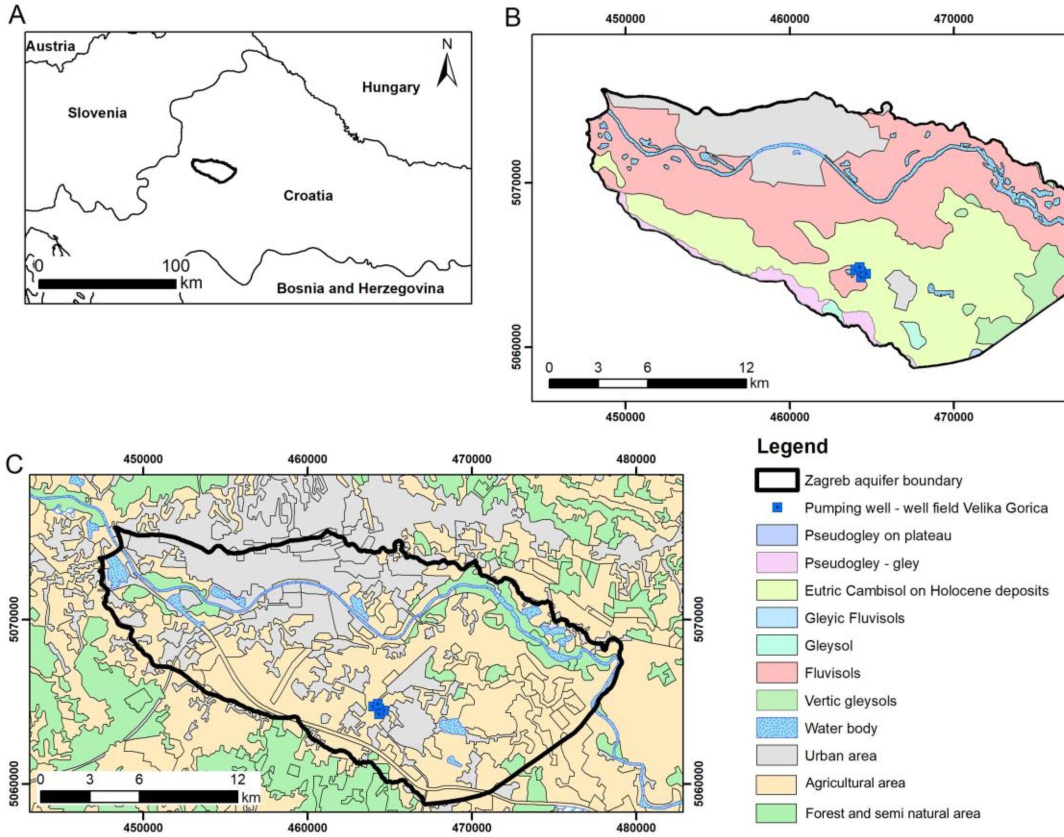

Figure 1. (A) The position of the Zagreb aquifer system in Croatia; (B) the location of the Velika Gorica well field with the soil type pattern; and (C) the location of the Velika Gorica well field with the land use pattern.

The study area represents the Velika Gorica well field, located on the right bank of the Sava River, in the southern part of the Zagreb aquifer system (Figure 1B). This well field is one of the most important well fields in the Zagreb County, consisting of five pumping wells and supplying drinking water to the capital city and the town of Velika Gorica. The monitoring wells are located in the catchment area of the Velika Gorica well field over an area of about 9 km^2 (Figure 2A) and generally used to monitor groundwater quality. Within the first sanitary protection zone of the Velika Gorica well field, there is a scientific research polygon of the Faculty of Mining, Geology, and Petroleum Engineering, University of Zagreb (Figure 2B). The research polygon consists of the meteorological station, the rain sampler and the pedological pit (Figure 2C) equipped with instruments to monitor parameters in the unsaturated zone and collect soil water samples. The unsaturated zone thickness at this site usually ranges from 5 to 8 meters and depends on the groundwater levels. According to the World Reference Base classification (FAO, 2015), the following soil horizons are identified at the top of the unsaturated zone: A (0–0.15 m), 2B (0.15–0.55 m), 3BC (0.55–0.9 m), and C (0.9–1.17 m). The upper 90 centimetres of the soil profile are predominantly composed of silty and sandy materials, with intermittent clay layers, while gravels dominate in the lower part of the unsaturated zone (Kukolja, 2018; Ružičić et al. 2019b) (see Table 1 in Buškulić et al., 2023a). The pedological pit is located within Eutric Cambisols on Holocene deposits, one of the most common soils developed in the area of the Zagreb aquifer system (Bogunović et al., 1998; Ružičić et al., 2013). The monitoring wells Lg-1, Vg-11, Vg-6 and Vg-5/2 are located in the area of Fluvisols, while all other monitoring wells are located on Eutric Cambisols on the Holocene deposits. The study area mainly consists of urban and agricultural areas (Figure 1C).

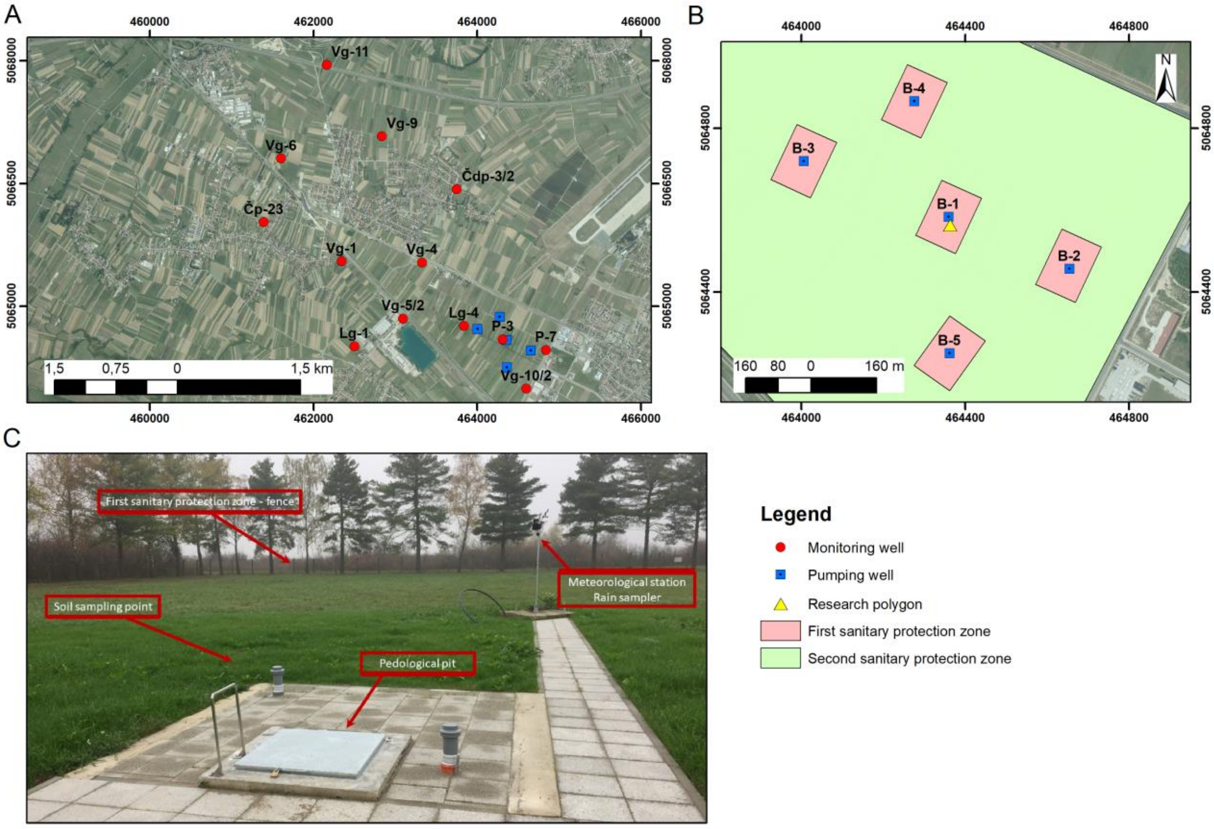

Figure 2. (A) The location of monitoring wells in the catchment area of the Velika Gorica well field; (B) the location of the scientific research polygon within the first sanitary protection zone; and (C) the location of the pedological pit, the meteorological station and the rain sampler within research polygon, as well as location of soil sampling.

1.2 Overview of previous research related to nitrates in the Zagreb aquifer system

Thus far, research related to groundwater investigations of an alluvial aquifer in the Zagreb area was focused on the calculation of ABVs of NO_3^- (Nakić et al., 2007; Kovač, 2017), relationship between natural groundwater quality indicators and NO_3^- (Kovač et al., 2017b), preliminary assessment of NO_3^- origin (Kovač, 2017; Kovač et al., 2018a), estimation of NO_3^- trends (Kovač et al., 2018b) and determination of areas with the highest risk to NO_3^- contamination (Huljek et al., 2019). Nakić et al. (2007) and Kovač (2017) determined that the upper limit of ABV of NO_3^- ranges from 4.5 to 10.7 mg/L. Correlation analysis indicated that only dissolved oxygen and electrical conductivity (EC) are positively correlated to NO_3^- (Kovač et al., 2017b). A preliminary assessment of NO_3^- contamination origin showed that NO_3^- is predominantly of organic origin, with most analyses showing an isotopic composition characteristic from wastewater (Kovač, 2017; Kovač et al., 2018a). This was unexpected due to the presence of a large proportion of agricultural areas and agricultural activity on the right bank of the Sava River. Moreover, descending NO_3^- concentration trends have been established in almost all parts of the Zagreb aquifer system (Kovač et al., 2018b) and urban part of the City of Zagreb showed the highest risk of NO_3^- contamination (Huljek et al., 2019).

Within the soil and the unsaturated part of the Zagreb aquifer system, research was primarily focused on the understanding the variability of unsaturated hydraulic conductivity and soil permeability (Ružičić et al., 2017; Ružičić et al., 2018), exploring the influence of agriculture on the unsaturated zone (Ružičić et al., 2019a), establishing the relationship between permeability and physicochemical properties (Ružičić et al., 2019b), determining the sorption characteristics of potentially toxic metals (Kovač et al., 2022b), evaluating soil-water origin (Kovač et al., 2022a) and conducting in situ monitoring of multiple soil horizons in Eutric Cambisols at the Velika Gorica site (Kovač et al., 2023). Therefore, so far, no research has been focused on investigating NO_3^- origin, distribution and related biogeochemical processes within the soil zone.

Previous studies in the Zagreb aquifer system area were focused on various aspects related to nitrate groundwater contamination, but did not focus on the following: (1) investigation of nitrogen dynamics in the soil zone and unsaturated zone, as well as defining the key processes that influence the transformations of nitrogen; (2) nitrate concentrations in the precipitation and the Sava River; (3) the biogeochemical and hydrodynamic processes affecting nitrate infiltration into the aquifer and dynamics in the aquifer; (4) evaluation of the isotopic composition of local nitrogen sources; and (5) quantification of proportional contributions from different N sources. The study area was defined based on previous findings, which identified elevated nitrate concentrations in groundwater from different NO_3^- sources and suggested a potential agricultural source of nitrate beyond wastewater, particularly on the right bank of the Sava River where catchment area of the Velika Gorica well field is located. To identify the origin and the dynamics of NO_3^- in both the unsaturated and saturated zone, it is necessary to analyse various types of water samples. If contribution from different N sources has to be evaluated, it is essential to conduct an analyses of the local nitrogen sources. It is also essential to conduct an analyses of the local soil samples. Therefore, this research involved field and laboratory investigations and analyses of groundwater, surface water, precipitation and soil water, along with determination of the isotopic composition of total nitrogen ($\delta^{15}N_{bulk}$) in the local solid samples from different potential nitrogen sources and physical and physico-chemical properties of the soil samples, that has never been previously done in the Zagreb aquifer system area. Furthermore, for the first time, a multi-method approach was used, integrating physico-chemical, hydrogeochemical and isotopic data together with analyses of molar ratios, statistical techniques and mixing models to identify the origin of nitrate contamination and the conditions influencing the N dynamics.

1.3 Methodology

The nitrate concentration datasets of past twenty years (2001–2020), from 12 monitoring wells located in the catchment area of the Velika Gorica well field and provided by the Croatian Waters through the National groundwater quality monitoring programme, were used for ambient background value estimation as the initial step in assessing the presence of nitrate groundwater contamination. The datasets were gathered from the following monitoring wells: Čdp-3/2, Čp-23, Lg-1 Lg-4, P-7, Vg-1, Vg-10/2, Vg-11, Vg-4, Vg-5/2, Vg-6 and Vg-9. For that purpose, four well-known model-based objective methods were employed: the iterative 2-σ technique (IT), the calculated distribution function (CDF), the cumulative frequency curve (CFC) and the probability plot (PP) described in detail in Buškulić et al. (2023b). The IT and CDF method include the Lilliefors test (Lilliefors, 1967) for testing the normality of data sets and significance of results (see Table 2 in Buškulić et al., 2023b). If the test statistic D is smaller than the critical value D_{crit}, the result is statistically significant. In addition, the datasets were used to evaluate the reliability of used methods in the anthropogenically influenced area and to modify the graphical interpretation of used graphical methods.

To determine the origin and dynamics of nitrate in the unsaturated and saturated zone in the study area, this research involved field and laboratory investigations as subsequent step. The groundwater, surface water, precipitation, and soil water were sampled monthly over a one-year period, from March 2021 to February 2022. Groundwater samples were taken from 10 monitoring wells: Čp-23, Lg-1 Lg-4, P-3, P-7, Vg-1, Vg-10/2, Vg-4, Vg-5/2 and Vg-6, located in the study area (Figure 2A). The location of monitoring wells, elevations, depths and screen intervals are given in Table 1. The positions of well screens were derived from the database of the EGPV project (Groundwater recording and management in the Republic of Croatia). Surface water was sampled from the Sava River at the western part of the Zagreb aquifer. These Sava River samples were taken midstream at the Podsused Bridge. Precipitation samples were collected using a Palmex rain sampler RS1 (Zagreb, Croatia) appropriate for preventing evaporation (Michelsen et al., 2018), while soil water samples were obtained from four soil horizons of the pedological pit using soil water samplers (Eijkelkamp Soil & Water, Giesbeek, The Netherlands) and an integrated automatic vacuum pump unit AVP-100 (UGT GmbH, Müncheberg, Germany). Soil water samplers are situated at -0.08, -0.33, -0.75 and -1.05 m depth. During sampling, the following in-situ parameters were measured on groundwater, Sava River and precipitation, using a WTW multi parameter 3630 IDS: pH, EC, dissolved oxygen content and temperature. In-situ parameters were recorded once the readings were stable. Each liquid sample was filtered in the field using a 0.22 μm nylon membrane filter, stored into high-density polyethylene (HDPE) bottle and transferred to the laboratory in a portable refrigerator. The sample bottles were kept frozen at –20 °C for nitrate isotope analyses.

Table 1. Groundwater sampling points location, elevation, depth and screen interval (n.a. refers to not available information).

Well	N HTRS96	E HTRS96	Elevation of well casing (m a.s.l.)	Total well depth (m)	Top of screen (m)	Bottom of screen (m)
Čp-23	5066027.50	461392.09	110.89	37.2	3.5	35.0
Lg-1	5064509.34	462502.69	109.55	41.7	17.3	40.5
Lg-4	5064758.42	463836.95	107.96	39.2	2.3	22.3
P-7	5064462.19	464842.51	107.26	10.5	n.a.	n.a.
Vg-1	5065549.08	462346.43	109.95	47.2	n.a.	43.4
Vg-4	5065533.73	463327.87	108.75	36.2	3.9	n.a.
Vg-6	5066804.24	461602.42	111.48	35.1	5.0	34.0
Vg-10/2	5063990.65	464598.59	106.97	44.9	n.a.	n.a.
Vg-5/2	5064848.39	463095.35	109.10	21.7	n.a.	n.a.
P-3	5064595.18	464310.31	107.78	10.0	n.a.	n.a.

In the laboratories of the Faculty of Mining, Geology and Petroleum Engineering, University of Zagreb, the following analyses were performed on collected liquid samples: (1) analyses of major anions and cations (Cl^-, NO_3^-, SO_4^{2-}, Na^+, Mg^{2+}, K^+ and Ca^{2+}) using Dionex ion chromatography system (ICS-90), (2) stable isotope analyses of water (δ^2H_{H2O} and $\delta^{18}O_{H2O}$) using a liquid water isotope analyzer (LWIA-45-EP, Los Gatos Research) and (3) stable isotopes analyses of nitrate ($\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$) using an ultrahigh precision advanced nitrous oxide isotopic EP analyzer (GLA451-N2OI3, ABB-LGR). The bicarbonate concentrations (HCO_3^-) in groundwater samples were determined using the titration method. The $\delta^{15}N$ and $\delta^{18}O$ analysis of NO_3^- has been made possible at the Faculty of Mining, Geology and Petroleum Engineering, University of Zagreb, through the successful implementation and application of titanium(III) reduction method to prepare the samples, first introduced and described in detail by Altabet et al. (2019). Briefly, procedure involves a few steps (Figure 3): treat Ti(III) chloride reagent with zinc metal powder, pipette sample, pipette degassed deionized water, pipette 10%

HCl, degass by rotary vacuum, inject conditioned reagent and swirl, inject N_2O free air and swirl, allow to stand 24 hours, withdraw N_2O headspace and inject into nitrous oxide isotopic analyzer. Prior to the research activities related to stable isotopes analyses of NO_3^-, several optimization steps were undertaken to optimize laboratory procedures necessary to get reliable results using nitrous oxide isotopic analyzer and Ti(III) reduction method. Optimization steps included establishing the target N_2O gas concentration inside the analyzer, determining the reagent-to-sample ratio during the sample preparation method and establishing the reaction time for optimal results.

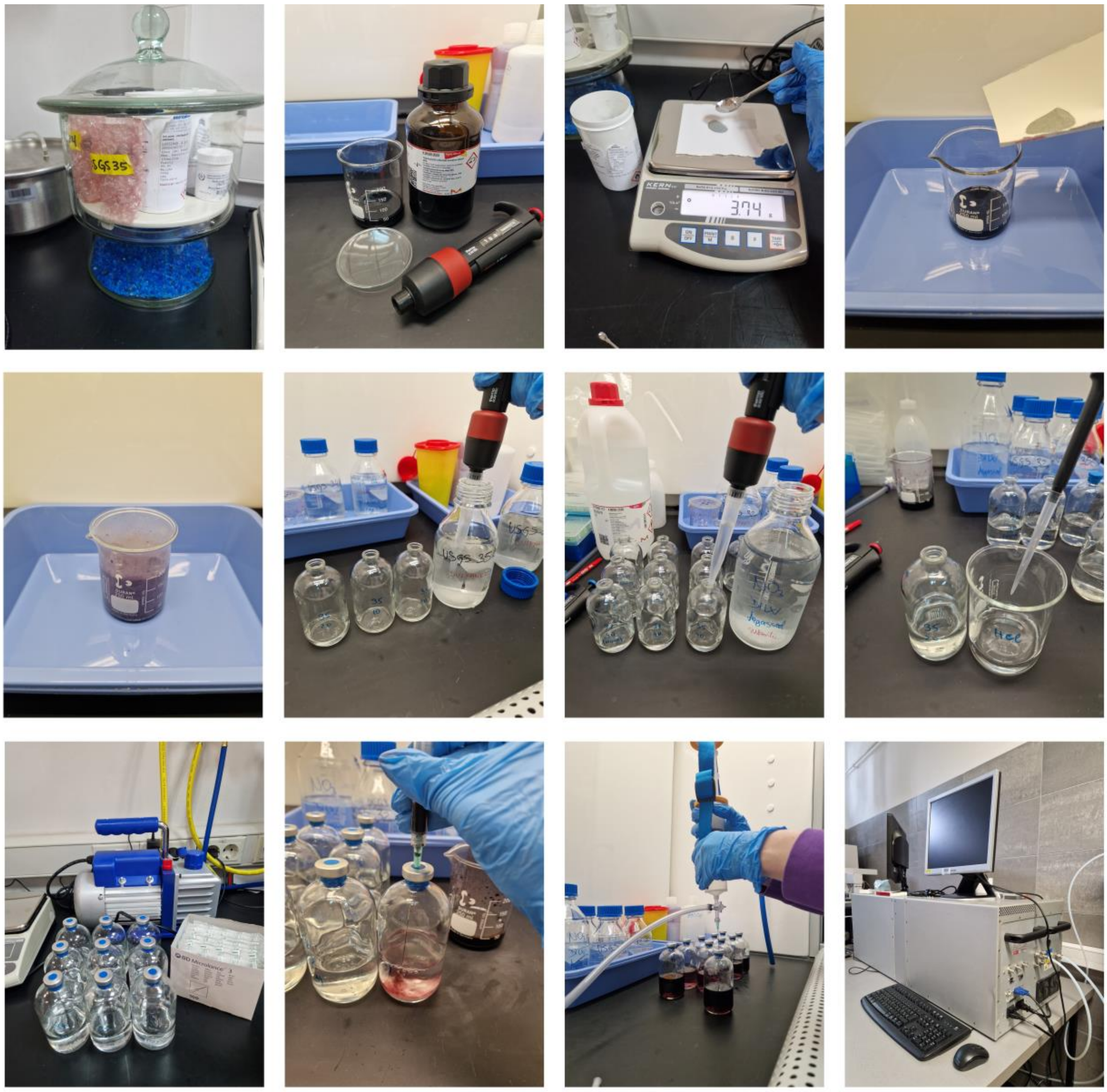

Figure 3. Sample preparation procedure steps using titanium(III) reduction method and nitrous oxide isotopic analyzer.

Hourly measurements of soil moisture and soil temperature of each soil horizon were measured using four TRIME-PICO 64 probes (IMKO Micromodultechnik GmbH, Ettlingen, Germany). Furthermore, precipitation and air temperature were measured continuously at hourly intervals over a one year by the meteorological station (Vantage Pro2, Davis Instruments). In addition to liquid samples, during September 2021, ten solid samples of nitrogen sources were collected within the area of Velika Gorica: six organic samples and four mineral fertilizers. The mineral fertilizers were NP (nitrogen-phosphorus) 20-20, NPK (nitrogen-phosphorus-potassium) 15-15-15, NPK(S) (nitrogen-phosphorus-potassium-sulphur) 15-15-15(3) and NPK(MgO) (nitrogen-phosphorus-potassium-magnesium oxide) 13-10-12(4), while the organic samples included two manure samples, two samples from septic tanks and two sewage samples. Sampled mineral fertilizers were mainly NH_4^+ based. The analyses of the isotopic composition of total nitrogen in the solid samples of N sources were carried out at The Faculty of Earth Sciences of the University of Barcelona using an Elemental Analyzer (EA) Flash IRMS with an Isotope Ratio Mass Spectrometer (IRMS) Delta V Advantage (EA IsoLink CN IRMS System, Thermo Scientific). The isotopic composition of total nitrogen ($\delta^{15}N_{bulk}$) and the nitrogen percentage (% N) in the collected solid samples of N sources are shown in Table 2. Moreover, 12 soil samples were collected near the pedological pit in July 2023 using auger set (Figure 2C). The soil samples were excavated up to a depth of 1.2 m at 10 cm intervals, stored in separate plastic bags and transported to the laboratory. Afterwards, soil samples were analyzed for pH, EC, total organic carbon (TOC), TiO_2, Fe_2O_3, MnO, heavy metals and soil texture at the Faculty of Mining, Geology and Petroleum Engineering, University of Zagreb. Soil pH and EC were measured using a WTW multi parameter 340i and WTW conductivity meter Cond 3110, respectively. Total carbon (TC) and total inorganic carbon (TIC) were evaluated with Elementary analyser multi-EA 4000 (Analytik Jena AG). TOC was calculated by subtracting TIC from TC. Hitachi XMET 8000 Expert Geo portable X-ray fluorescence (pXRF) instrument was used to determine contents of major oxides and microelements in soil samples, while the soil texture was defined with laser diffraction method using Malvern Mastersizer 3000.

Table 2. The isotopic composition of total nitrogen ($\delta^{15}N_{bulk}$), the nitrogen percentage (% N) and standard deviation (SD) of local solid samples of nitrogen sources.

Sample	$\delta^{15}N_{bulk}$ (‰)	SD ^{15}N	% N	SD % N
NP 20-20	1.2	0.2	19.3	0.3
NPK 15-15-15	1.5	0.1	13.9	0.2
NPK(S) 15-15-15(3)	1.1	0.2	14.3	0.1
NPK(MgO) 13-10-12(4)	0.5	0.5	12.2	0.2
Sewage	1.9	0.1	3.5	0.1

Sewage	2.0	0.0	2.4	0.1
Septic tank	3.6	0.2	2.5	0.0
Septic tank	3.2	0.1	3.0	0.0
Manure	8.8	0.1	2.8	0.0
Manure	2.8	0.1	1.6	0.0

After completing the field sampling, field measurements and laboratory analyses, data processing and analysis were initiated as tertiary step. The statistical techniques were applied on soil water data in order to establish significant differences within different sampling depths (Figure 4). The assumptions (dependent variable should follow a normal distribution and the variance should be constant across groups) that need to be met when performing a one-way ANOVA test (Das and Sarkar, 2020) were tested using Shapiro-Wilk test and Levene's test. The Kruskal-Wallis test was used when the assumptions of one-way ANOVA were not met (Emoyan et al., 2018). If the one-way ANOVA or Kruskal-Wallis test yielded a statistically significant difference, the post hoc tests, namely, the Tukey Honest Significant Difference (HSD) test and Mann–Whitney U test, were used to compare parameters between the groups (Benjamini and Braun, 2002). Isotopic composition and variation in time of δ^2H_{H2O} and $\delta^{18}O_{H2O}$ in soil water and precipitation, relationship between NO_3^-/Cl^- molar ratio and Cl^- and relationship between soil moisture and NO_3^-/Cl^- or NO_2^-/Cl^- molar ratios were used to evaluate the dynamics of NO_3^- through the soil profile and dominant natural form of N that infiltrates into the aquifer. Additionally, soil parameters (pH, EC, TOC, TiO_2, Fe_2O_3, MnO, Cr, Ni, Zn, Pb, As, Co, Hg, clay, silt and sand content) influencing NO_3^- distribution within the soil zone were analyzed and characterized to further support this objective. The biogeochemical processes in the soil were characterized by analyzing the relationship between $\delta^{15}N_{NO3}$ and NO_3^- concentration, along with theoretical $\delta^{18}O_{NO3}$ values characteristic for the nitrification in the soil. Given that process of nitrification utilizes two oxygens from water and one from atmospheric oxygen (Kendall, 1998; Mayer et al., 2001), characteristic nitrification $\delta^{18}O_{NO3}$ values were estimated using the following experimental equation (Andersson and Hooper, 1983; Mayer et al., 2001):

$$\delta^{18}O_{NO3} = \frac{2}{3}\delta^{18}O_{H2O} + \frac{1}{3}\delta^{18}O_{O2} \tag{1}$$

The NO_3^- origin in the soil was identified with $\delta^{18}O_{NO3}$, $\delta^{15}N_{NO3}$, δ^2H_{H2O} and $\delta^{18}O_{H2O}$ isotopic signatures. Additionally, isotopic values in the soil have been used as supporting method for verifying and confirming the biogeochemical processes and the form of nitrogen that infiltrates into the aquifer.

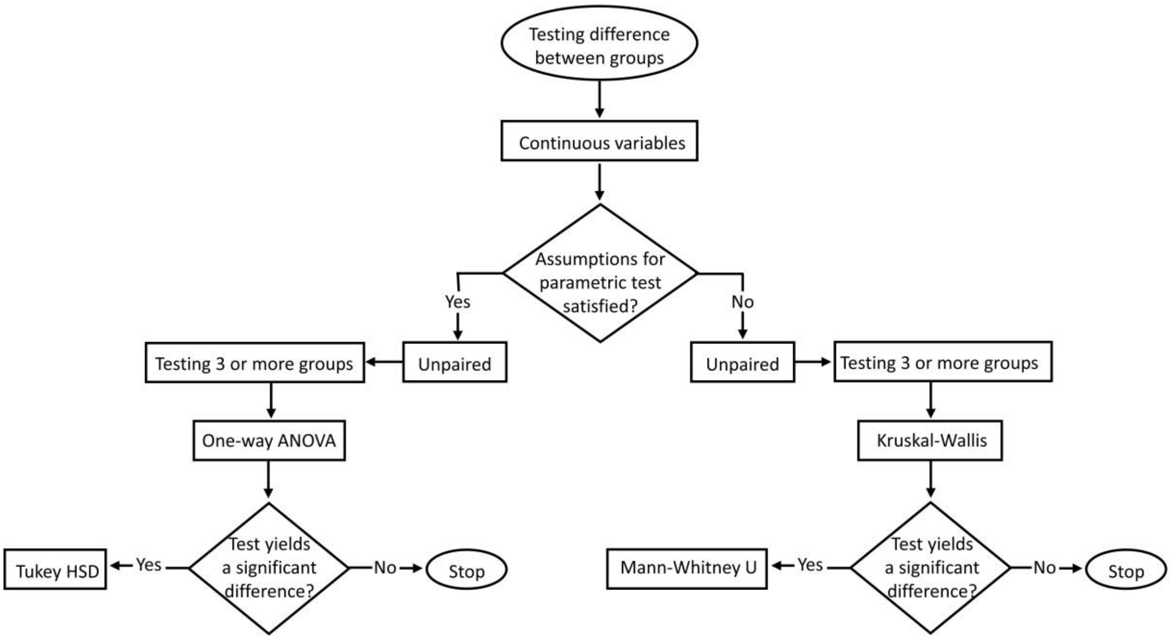

Figure 4. The statistical data analysis process on soil water data.

Before collecting the groundwater samples, groundwater levels were manually measured for 10 sampled monitoring wells to create the water table contour map of the study area during low and high groundwater levels and characterize general behavior of the aquifer system. The dynamics of NO_3^-, origin and associated biogeochemical processes in the groundwater were defined by combining various data and methods. Statistical methods included: Shapiro-Wilk test to assess if variable comes from a normal distribution; one-way ANOVA and Kruskal-Wallis test to evaluate changes over time (from March 2021 to February 2022) and changes among sampling sites; and correlation test to examine the relationship between major anions and cations in groundwater and how different variables relate to nitrate sources. Physico-chemical parameters, including pH and DO, were used to characterize biogeochemical processes and redox conditions. The hydrogeochemical parameters such as Cl^-, NO_3^-, Na^+ and K^+, as well as the relationship between TDS and $(NO_3^- + Cl^-)/HCO_3^-$, relationship between NO_3^-/Cl^- molar ratio and Cl^- concentration and between NO_3^-/K^+ molar ratio and K^+ concentration were utilized to define NO_3^- origin. The molar ratios were examined for each monitoring well separately. The biogeochemical processes in the groundwater were characterized by analyzing the relationship between $\delta^{15}N_{NO3}$ and DO, NO_3^- concentration and logarithmic concentration of NO_3^-, along with theoretical $\delta^{18}O_{NO3}$ values characteristic for the nitrification in the groundwater. Nitrate stable isotopes ($\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$) and bivariate plot of $\delta^{18}O_{NO3}$ versus $\delta^{15}N_{NO3}$ were employed to define NO_3^- origin. Moreover, quantification of proportional contributions from different N sources in the groundwater and river were calculated via the application of isotopic values of the local nitrogen sources and a Bayesian isotope mixing model using the package "simmr in R" (Parnell et al., 2010; Parnell and Parnell, 2019). The model was applied at each sampling site to identify spatial differences, as well as across different seasons for each water type (groundwater and river) to identify temporal differences. The isotopic average and standard deviation values of the three local nitrogen sources (soil, mineral fertilizers and organic wastes) were used in the analysis (see Table 1 in Buškulić et al., 2025). Precipitation was excluded from the analysis, as the N isotope precipitation signal is quickly dampened. The isotopic signature of soil was defined using measured $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ data from sampled soil water from the deepest soil horizon (C horizon) that was closest to the water table. The $\delta^{18}O_{NO3}$ value of mineral fertilizers and organic wastes was determined by considering $\delta^{18}O_{H2O}$ values of the local groundwater. In addition, in order to determine recharge sources and quantify aquifer recharge components, water stable isotopes ($\delta^{2}H_{H2O}$ and $\delta^{18}O_{H2O}$) and two-component mixing model were used, respectively. All statistical analyses used for the purposes of this research were conducted using TIBCO Software Inc. "Statistica" (Version 13.5.0.17).

Finally, the research concludes with a final step that includes development of two conceptual models: (1) the conceptual model demonstrating dynamics of N species through the unsaturated and saturated zone of the Zagreb aquifer system within the area of Eutric Cambisols on the Holocene deposits influenced by natural factors, and (2) the conceptual model demonstrating dynamics of N species through the unsaturated and saturated zone of the Zagreb aquifer system within the area of Eutric Cambisols on the Holocene deposits influenced by both natural and anthropogenic factors. The conceptual models were created using the results obtained and interpreted during this research.

1.4 Objectives and hypotheses of research

The main objectives of this research were to: (1) determine the ambient background nitrate concentration in a hydrogeologically homogeneous area within a heterogeneous aquifer system under long-term human impact; (2) implement the titanium(III) reduction method for the analysis of stable isotopes of nitrogen and oxygen in dissolved nitrate; (3) facilitate a more comprehensive understanding of nitrate dynamics in the unsaturated and saturated zone within the area of Eutric Cambisols on the Holocene deposits in the Zagreb aquifer; and (4) define the dominant natural form of nitrogen that infiltrates from the soil zone into the aquifer in the catchment area of the Velika Gorica well field.

These objectives were based on the following hypotheses:

I. Ambient background value of nitrate in the groundwater in the catchment area of the Velika Gorica well field is below 10 mg/L as NO_3^-.

II. In an area without anthropogenic activities, nitrogen is primarily introduced from the soil zone (Eutric Cambisols on the Holocene deposits) into the unsaturated zone in the form of nitrites and nitrates.

III. The source of nitrate in the groundwater in the catchment area of the Velika Gorica well field is not mineral fertilizers.

1.5 Scientific contribution of research

This research resulted with a new scientific knowledge about determination of nitrate ambient background concentrations in the hydrogeologically homogeneous area within a heterogeneous aquifer system under long-term human impact. The results of this research provide an advance understanding of the nitrogen compounds transformations in the soil zone, unsaturated zone and saturated zone. Specifically, the findings provide new insight into biogeochemical processes related to nitrogen species occurring within the Eutric Cambisols on the Holocene deposits, which are one of the most common soils developed in the area of the Zagreb aquifer system. The research includes the identification of nitrate dynamics and origin of nitrate contamination, as well as quantification of proportional contributions from different nitrogen sources, using stable isotopes ^{15}N and ^{18}O in dissolved nitrate and ^{15}N in potential sources of nitrate contamination. The development of a conceptual model of nitrogen species dynamics, through the unsaturated and saturated zone of the Zagreb aquifer system, enables sustainable management and protection of the groundwater.

1.6 Dissertation structure

The presented dissertation is divided into six chapters. Chapter 1 provides a general introduction to the research topic, a description of the study area, an overview of previous research, a description of the methodology, the listed objectives and hypotheses of research, an explanation of the original scientific contribution and a description of dissertation structure. Chapter 2 consists of three original scientific papers, presented in their original form and published in internationally peer-reviewed journals. The first paper, *“Estimation of Nitrate Background Value in Groundwater under the Long-Term Human Impact”*, estimates ambient background value of nitrate in groundwater within the catchment area of the Velika Gorica well field. The second paper, *"Determination of Nitrate Migration and Distribution through Eutric Cambisols in an Area without Anthropogenic Sources of Nitrate (Velika Gorica Well Field, Croatia)"*, presents first insights of nitrate dynamics through soil zone of the Eutric Cambisols on the Holocene deposits. The third paper, *"Tracing Nitrate Contamination Sources and Dynamics in an Unconfined Alluvial Aquifer System (Velika Gorica well field, Croatia)"*, provides better understanding of nitrate dynamics and evaluates nitrate origin in groundwater, together with the dynamics and origin of nitrate within the soil zone. Chapter 3 presents a combined discussion that integrates the results from three original scientific papers in the context of research hypotheses and objectives. Chapter 4 provides an overview of the most important results and conclusions, Chapter 5 consists of literature list, while Chapter 6 contains the biography of the author with a list of published papers.

2. ORIGINAL SCIENTIFIC PAPERS

Paper 1: Estimation of Nitrate Background Value in Groundwater under the Long-Term Human Impact

Authored by: Patricia Buškulić; Jelena Parlov; Zoran Kovač; Zoran Nakić

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Estimation of Nitrate Background Value in Groundwater under the Long-Term Human Impact

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Abstract: This study demonstrates an approach to estimate the background value of nitrate as a basis for better groundwater management and protection in areas under long-term human impact. The aim was to determine the ambient background value (ABV) of nitrate in the catchment area of the Velika Gorica well field, a hydrogeologically homogeneous area within the Zagreb aquifer. ABVs are determined using four well-known model-based objective methods (the iterative 2-σ technique, IT; the calculated distribution function, CDF; the cumulative frequency curve, CFC; and the probability plot, PP), while simultaneously testing the reliability of the results of each method. If the results are not statistically significant, data selection is performed. The results show that using data without selection can lead to statistically non-significant ABVs, but with the additional selection of data, a statistically non-significant result became a statistically significant one. In summary, all final ABVs must be statistically significant and determined using as large a data set as possible. Reducing the size of the data set is acceptable only in the case of a statistically non-significant result.

Keywords: ambient background value; nitrate; iterative 2-σ technique; calculated distribution function; cumulative frequency curve; probability plot; Zagreb aquifer

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1. Introduction

Groundwater presents the most common and valuable source of drinking and irrigation water in the world [1]. Sustainable management of water and sanitation for the world population is one of the main goals established by the United Nations under the Sustainable Development Goals [2]. A large amount of research has been focused on the evaluation of groundwater quality, which has been diminished by different pollutants [1,3–6], while others emphasize the importance of implementing measures that will slow down the deterioration of water bodies [7–9]. Nitrate pollution of groundwater is one of the main environmental issues worldwide [10–18] and nitrate's increasing trend in natural waters has made it a contaminant of concern [4,19,20]. Burkart and Kolpin [21] have shown that a nitrate concentration greater than 13 mg/L as NO_3^- in groundwater is a sign of natural groundwater quality degradation due to anthropogenic activities. In order to be sure that groundwater pollution is present, it is important to distinguish between geogenic and anthropogenic influences on groundwater quality by the estimation of geochemical background values that reflect natural processes [22]. Estimating the natural background value in groundwater enables us to recognize the concentration of the geochemical compound that is free from human influence [23]. The Groundwater Directive (2006/118/EC) introduced the term "background value" as *"the concentration of a substance or the value of an indicator in a body of groundwater corresponding to no, or only very minor, anthropogenic alterations to undisturbed conditions"*. Due to the ubiquitous human influence, which is also reflected in the chemical composition of groundwater, the natural composition of groundwater, especially in shallow aquifers, almost does not exist today. Since, in many parts of the world, human influence on the environment has been present for such a long time that it is futile to try to determine the natural background concentrations of substances in groundwater, Reiman and Garrett [24] devised the concept of the ambient background value (ABV), which describes an immeasurably disturbed and no longer intact background concentration of substances in groundwater. Because background values are location- and scale-dependent, i.e., they change regionally as well as locally [24], it is necessary to divide data sets into homogeneous groups to increase the probability of detecting an environmental problem. Sometimes, it is very difficult to clearly identify the populations related to the background processes as opposed to those reflecting contamination [25–28]. It has been shown that areas that are unaffected by human activities typically have natural background values of nitrate that do not exceed 5–10 mg/L as NO_3^- [29].

Many researchers are using approaches based on the analysis of the distribution of many measured data using different statistical methods [30–32]. It has been shown that probability density functions can be different in background and anomalous samples, but the main issue is how to recognize these differences with confidence [33]. Although model-based subjective methods of background determination that include the mean plus two standard deviations are the best for data that follow a normal distribution and were frequently used, they are far from ideal when used for regional geochemical data [33]. The problem arises from the fact that the samples represent more than one population or process, resulting in multimodal distribution, where the use of the mean can give overestimated values [34]. On the contrary, model-based objective methods of background determination differ from subjective methods in that the background values and the upper limit of background range, i.e., threshold values, are defined by the data themselves, rather than by an arbitrary decision of the researcher. Recently, model-based objective methods have been gaining more and more importance. They are based on the division of a complete set of measured data into subgroups with characteristic probability density functions that reflect relevant natural geochemical processes and/or pollution events in an area. Background populations can be approximated by normal or lognormal density functions, while different processes have produced an anomalous population that differs from the background [33]. In recent decades, various model-based objective methods (the iterative 2-σ technique, the calculated distribution function, the cumulative frequency curve, and the probability plot) and their combinations have been used worldwide to determine background concentrations and threshold values [29,35–41]. Furthermore, there are also other statistical approaches. For example, the lithology of aquifers was used to define the natural background values of different chemical parameters in six EU countries [42]. In some cases, natural background values of selected species were estimated by means of the pre-selection and the component separation method [23]. Other studies combined pre-selection methods and statistical approaches [26,43–45].

The groundwater of the Zagreb aquifer is threatened due to increasing concentrations of pollutants and decreasing water quantity. Nitrates have been recognized as one of the most common groups of contaminants in the Zagreb aquifer [22], which is designated as a vulnerable area to nitrates, due to agricultural activities, especially on the right bank of the Sava River. Thus far, the elevated nitrate concentrations have been confirmed by various studies [22,46,47], while recent research was focused on the determination of areas with the highest risk for nitrate contamination [48], the definition of nitrate origin [49], the estimation of nitrate trends [50], and the in situ monitoring of multiple soil horizons in Eutric Cambisols at the Velika Gorica site [51]. Furthermore, it has been shown that nitrate concentrations present one of the most heterogeneous pieces of data, which is due to the pronounced anthropogenic influence in different parts of the Zagreb aquifer [52]. The concept of ABVs was implemented by Nakić et al. [25] and Kovač [52] to calculate the ABVs of nitrate in the groundwater of an alluvial aquifer in the Zagreb area. The upper limit of the ABV for the Zagreb aquifer ranged from 4.5 to 10.7 mg/L. Due to the obvious human influence and heterogeneity of the Zagreb aquifer, four different methods (the iterative 2-σ technique, IT; the calculated distribution function, CDF; the cumulative frequency curve, CFC; and the probability plot, PP), which belong to the model-based objective methods, were used to estimate the ABV of nitrate in the catchment area of the Velika

Gorica well field, which is located in the southern part of the Zagreb aquifer and complies with the principle of hydrogeological homogeneity. We are referring here to the results of research by a group of authors [53] who revealed that the background concentrations of substances, determined at the level of the regional aquifer, can significantly differ from the background concentrations of the same substances, determined in homogeneous areas within the same aquifer. The importance of applying the principle of homogeneity when determining background concentrations for substances that are sensitive to changes in oxidation-reduction conditions in the subsoil is emphasized by Molinari et al. [43], while Matschullat et al. [40] emphasize that the geochemical background concentration of a chemical substance needs to be determined in spatial conditions that are characterized by homogeneous, mainly climatological, lithological, and pedological features. The objectives of this study are (a) to modify the graphical interpretation of used methods to reduce subjectivity in determining a background value, (b) to show the differences in results when estimating ABVs using all data without selection compared to selected non-normally distributed (NON-ND) data, and (c) to evaluate the reliability of used methods in the anthropogenically influenced area.

2. Study Area

The Zagreb aquifer is situated in the north-western part of the Republic of Croatia along the Sava River, between the Medvednica Mountain in the north and the hills of Vukomeričke Gorice in the south. It is an unconfined aquifer composed of unconsolidated Quaternary sediments consisting mainly of sand, gravel and silt or silty clays and covers an area of approximately 350 km^2. The research area is located in the southern part of the Zagreb aquifer (Figure 1). The average annual precipitation for the period 2001 to 2020 is 967 mm. Precipitation is evenly distributed throughout the year, with a monthly average of around 80 mm. The annual average air temperature ranges from 10.5 °C to 12.7 °C, with a minimum in January and a maximum in July (Figure 2).

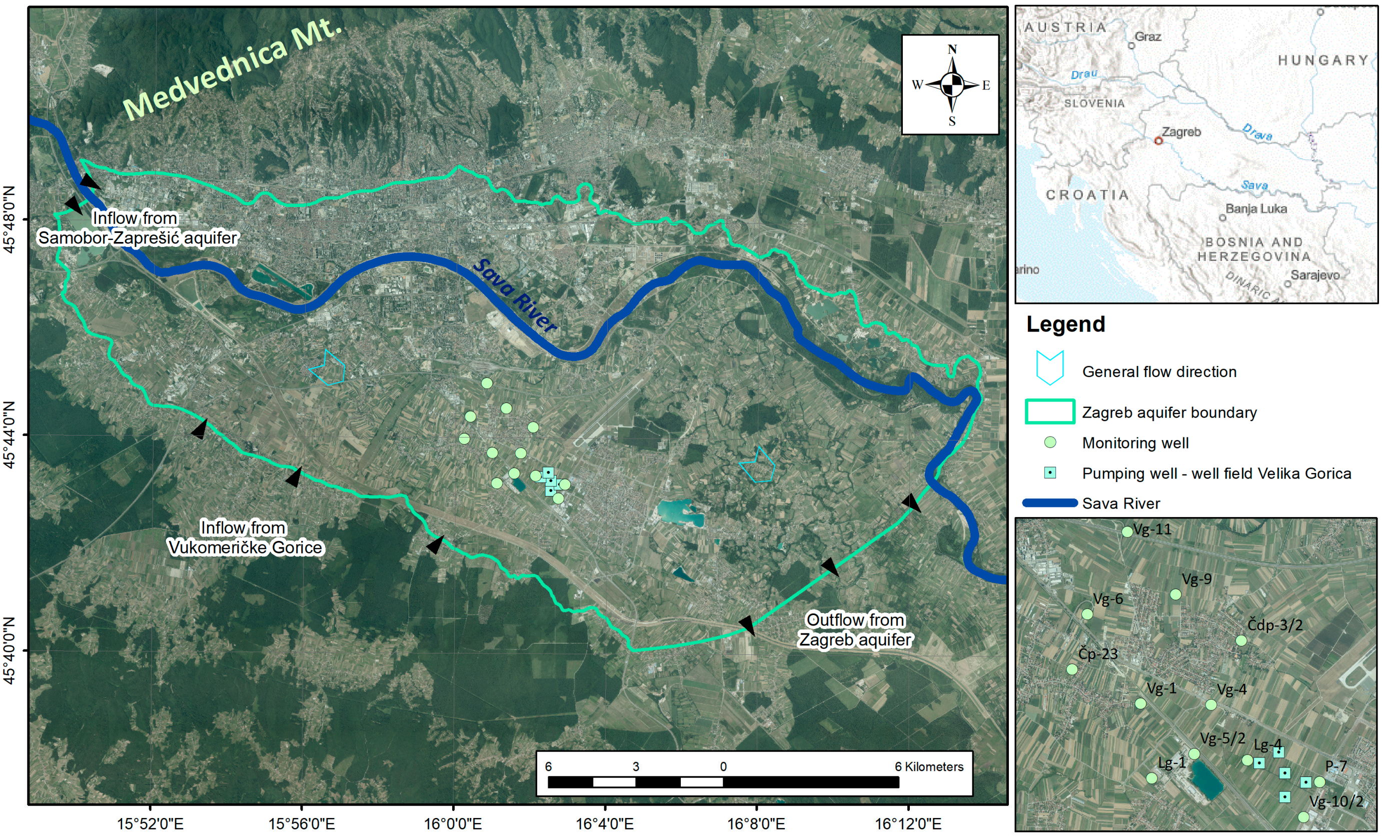

Figure 1. Research area.

Figure 2. The annual precipitation (**a**) and the average monthly precipitation (**b**) with average, minimum and maximum air temperature; depth to groundwater (**c**) and groundwater levels (**d**) for the period 2001 to 2020.

Quaternary sediments were deposited during the Middle and Upper Pleistocene and the Holocene [54]. The shallow layer contains Holocene alluvial deposits of the Sava River, while the deep layer contains Pleistocene lacustrine–marshy deposits [55,56]. The unsaturated zone consists of gravels in the lower part, while the upper part consists mainly of silty to sandy material, interbedded with clay layers in places. The thickness of the unsaturated zone varies from two to eleven meters [57].

The shallow aquifer is in direct hydraulic connection with the Sava River, which is the main source of groundwater recharge [58–61]. The groundwater level changes mainly due to water level fluctuations of the Sava River, which recharges the aquifer during high water levels, while it discharges the aquifer during low and medium water levels. Other recharge mechanisms include infiltration from precipitation, infiltration from the porous water supply system and the sewage network, but also the water inflow from the Samobor-Zaprešić aquifer in the west and the water inflow across the southern boundary in the Vukomeričke Gorice area [62]. The general groundwater flow direction concurs with the flow direction of the Sava River from west to east (south-east) (Figure 1). The Zagreb aquifer area consists of three major pedological units: Fluvisols, Stagnosols and Eutric Cambisols on the Holocene deposits [63,64].

The Velika Gorica well field is one of the most important well fields in Zagreb County, supplying drinking water to the City of Zagreb and the City of Velika Gorica. The well field consists of five wells drilled to a depth of 39 m to 50 m, drawing water from the shallow aquifer. Near the well field, there are 12 monitoring wells in an area of approximately 9 km^2, generally used to monitor groundwater quality in the shallow aquifer. In this small area, the maximum elevation difference is 7 m. The maximum thickness of the unsaturated zone is 9 to 11 m (Figure 2), and most of the land is used for agriculture, mixed with urban areas. Figure 2c,d show that the depths to groundwater and groundwater level fluctuations are similar for all 12 monitoring wells. According to the results of previous research [65–67], the groundwater in the influence area of the Velika Gorica well field belongs to the calcium–magnesium–hydrogen carbonate ($CaMgHCO_3$) water type. All this makes the catchment area of the Velika Gorica well field a hydrogeologically homogeneous area relevant for the determination of background values.

3. Materials and Methods

3.1. Available Data Set

The ABV of nitrate was estimated using groundwater chemical data from the national groundwater quality monitoring program of Croatian Waters. Nitrate concentration data from 12 monitoring wells, located in the catchment area of the Velika Gorica well field, have been used for this purpose. Nitrate data sets of the past twenty years (2001–2020) have been analyzed. The data set included three analyses with limit of quantification (LOQ) or limit of detection (LOD) values, one analysis that was not measured and written as "0", and 135 analyses with unavailable data. Since only four analyses were recorded as "0" or as below the LOQ/LOD values, it was decided that these data would not be used. After data processing, approximately 944 analyses, out of 1083 chemical analyses, were used for background estimation. For the evaluation of the nitrate concentrations in the Sava River and precipitation, 15-year time series data (2001–2015) were used. Data included 627 analyses from the samples of river water (provided by Croatian Waters) and 1225 analyses from the samples of precipitation (provided by Croatian Meteorological and Hydrological Service).

3.2. Description of Methods

In this work, four well-known model-based objective methods were used to estimate the ABV of nitrate.

The iterative 2-σ technique, IT, and the calculated distribution function method, CDF, have been implemented by the Visual Basic (VB) macro entitled BACKGROUND [25], which calculates the background values of chemical parameters and evaluates threshold values that separate background data from anomalous values. The macro integrates these two methods into a widely accessible platform (i.e., Microsoft Excel) and calculates the normal range of background values with 95% confidence. Both methods aim to define the background and threshold by approaching a normal range. The methods process the measured data (i.e., remove values) until a normal distribution is achieved. Thus, what is left in the normally distributed data are the background values and what has been removed are the non-background values [25,40]. The background values are estimated based on data derived from a population, which amounts to a large proportion of the total data set. Statistical methods include the Lilliefors test [68] for testing the normality of data sets and significance of results. If the test statistic D is smaller than the critical value D_{crit}, the result is statistically significant, i.e., the determined distribution of the background values corresponds to the normal distribution, which means that the results are acceptable. If the statistic D is greater than the critical value D_{crit}, the result is not statistically significant and the obtained background values are overestimated [25]. The advantage of using these two methods is that their application does not require normally or lognormally distributed data, they can be applied to relatively small data sets ($n > 30$), and they are applicable to unimodal and skewed distributions. Both methods work well only when the generally prevailing background data are used to define the background populations [25]. The IT is suited for the calculation of the threshold value as the outer limit of background variation and considers both the low and high values to define anomalies. The disadvantage of this method is that it cannot be applied to a polymodal distribution. The CDF method is applicable to the polymodal distributions if the data unaffected by human activities are dominant. The method is convenient for use if anthropogenic influences tend to lead to enrichments in natural systems. The lower values should thus be free from anthropogenic influences [40]. The IT and CDF methods differ in that the IT method can also be applied if it is necessary to determine the lower limit of the background value of some pollution indicator. This is the case for dissolved oxygen, which can be an indicator of extreme oxygen consumption due to groundwater pollution [25]. However, research related to the definition of the natural background values of chemical substances is usually focused on the determination of the upper limit of the background value [38,39,41,69,70].

The cumulative frequency curve, CFC, or Lepeltier's method [71], is a graphical method that analyzes the cumulative sum on a graph with a logarithmic scale. On the graph constructed in this way, a significant deviation from the lognormal distribution is visible as an abrupt change in the graph, i.e., as a point x where the appearance of the elbow is observed, and all values that are less than or equal to x are the result of the cumulative effect of natural processes that cause significant natural variability [40]. In his original work, Lepeltier suggested cumulating high to low values. The advantage of this method is that it enables the identification of meaningful ABVs for relatively small data sets with a minimum of 50 data [71]. A limitation of using this approach is that care must be taken to avoid the temptation to interpret the visual deviations between natural and anthropogenic populations in the lower part of the curve as significant [30]. This method contains an element of subjectivity due to visual detection and is highly dependent on the experience of the researcher [70].

The probability plot, PP, assumes that different processes generate data with different probability distributions and aims to identify several distributions into distinct components and points that separate them, i.e., the threshold values. If there is a partial overlap of background and non-background distributions, then a change in distribution on the PP can be considered as an inflection point [70]. Inflection points can be identified visually as changes in slope [29], or as an intersection of different trend lines [37]. The concentration at the inflection point is defined as the threshold value, below which all measured values of the substance belong to the background concentrations [33,72]. The advantage of this approach is that it enables the identification of multiple populations on the graph. The limitations of using this method are that a minimum of 100 values is needed and the determination of the threshold represents an estimation procedure, subject to random and systematic error [29,33].

In this paper, the inflection points were identified as the intersection of two "linear" segments, as observed in Rahman et al. [37]. The first inflection point separating the first two subsets is assumed to be ABV, while the first population is considered a natural event. Multiple inflection points on the plots may indicate multiple populations of natural or anthropogenic origin contributing to nitrate concentrations in groundwater [37]. To reduce the subjectivity of visual interpretation, subsets were selected to describe the population with the highest possible coefficient of determination. The minimum number of points in a subset was three and was increased by one point each time until the highest possible coefficient of determination was reached.

The first step was to estimate the ABV for data without prior selection to check the reliability of different methods. If one of the selected methods leads to a statistically non-significant result, data selection is required in the second step and re-estimation of the ABV for selected data in the third step. This is based on the fact that the values of a single normally or lognormally distributed population form a straight line on the cumulative probability plots, without the possibility of determining a background value, whereas the values of more mixed populations lead to a curved line with a pronounced inflection point [29]. Data selection was performed using the Lilliefors test [68] by testing normal distribution. TIBCO StatisticaTM (version 13.5.0.17) was utilized to perform the normality tests, while an alpha value of 0.05 was used as the statistical significance level in all data analyses. The Lilliefors test is an adaptation of the Kolmogorov–Smirnov test and uses the same calculations, but the critical value table in the Lilliefors test table is used instead of the Kolmogorov–Smirnov table, which gives the critical value D_{crit}. If the test statistic D is less than the critical value D_{crit}, the normal distribution cannot be rejected. Under the condition that the test statistic D is greater than the critical value D_{crit}, the normal distribution can be rejected.

All final ABVs must be statistically significant and determined using as large as possible a data set. A statistically significant result obtained in the first step is considered acceptable, while, in case of a statistically non-significant result, data selection is required in order to obtain a statistically significant result.

4. Results and Discussion

The descriptive statistics of nitrate concentrations (presented as mg NO_3/L) in groundwater in the catchment area of the Velika Gorica well field and Sava River and the precipitation are reported in Table 1. The nitrate concentration of groundwater ranges from 0.2 to 35.7 mg/L, with a mean value of 19.6 mg/L and a median of 19.5 mg/L. It is worth noting that the average value for each monitoring well does not exceed the maximum permissible level for drinking water (50 mg NO_3/L), stipulated by EU and Croatian regulations. Nevertheless, these higher concentrations indicate possible natural groundwater quality degradation, considering that, in areas not affected by human activities, the nitrate concentration in groundwater should be much lower [21]. The mean nitrate concentration in the Sava River, as the main source of recharge in the Zagreb aquifer, recorded in the period from 2001 to 2015, is 6.8 mg/L, with the maximum value of 14.6 mg/L. The mean nitrate concentration in precipitation from 2001 to 2015 is 0.8 mg/L, while the maximum measured concentration is 5.2 mg/L. Due to the low nitrate concentrations in precipitation, it can be assumed that precipitation has a very small effect on the nitrate content in the Zagreb aquifer [52]. Furthermore, it has been shown that the local meteoric water line of the Ljubljana area (Slovenia) better describes the stable isotopic composition of groundwater from the Zagreb aquifer than the local meteoric water line of the Zagreb area [22,49,59,73]. This confirms that the Sava River is the main source of recharge of the Zagreb aquifer, since the source of the river water is the precipitation that falls in Slovenia.

Table 1. Main statistics for nitrate in mg/L = milligram per liter as NO_3. SD is standard deviation, CV is coefficient of variation in %, N is number of samples.

	Min	Max	Mean	SD	Median	CV	N	Interval
All wells	0.2	35.7	19.6	5.7	19.5	29.0	944	2001–2020
Čdp-3/2	0.2	19.8	12.4	4.3	13.7	34.6	63	2001–2020
Čp-23	14.0	28.3	19.8	3.5	19.0	17.9	73	2001–2020
Lg-1	4.0	35.4	25.5	5.5	25.2	21.4	100	2001–2020
Lg-4	0.2	31.0	21.1	7.0	22.6	33.2	92	2001–2002; 2004; 2006–2020
P-7	6.2	33.1	17.8	6.0	18.6	33.9	99	2001–2020
Vg-1	15.1	32.0	20.3	3.0	19.5	14.6	112	2001–2020
Vg-10/2	14.6	34.7	20.5	2.9	20.5	14.4	66	2009–2020
Vg-11	10.2	24.9	15.1	2.5	15.1	16.8	63	2001–2020
Vg-4	15.5	27.4	21.3	2.5	21.2	11.7	110	2001–2020
Vg-6	12.4	27.5	17.3	3.0	17.1	17.3	73	2001–2020
Vg-9	11.5	20.2	15.1	1.8	15.1	11.9	58	2003–2020
Vg-5/2	17.7	35.7	28.9	3.3	29.2	11.5	35	2014–2020
Sava River	1.3	14.6	6.8	1.9	6.6	28.0	627	2001–2015
Precipitation	0.03	5.2	0.8	0.7	0.5	94.8	1225	2001–2015

First, the ABV of nitrate was estimated in groundwater for data without selection referring to all monitoring wells, using four different methods (Figure 3). The inflection points were determined according to the principle of the highest possible coefficient of determination using the CFC and PP methods. Estimated values vary from 7.1 to 11.8 mg/L. Usage of the IT method resulted in the highest estimate of the background value, while the CDF method gave a non-statistically significant result. As observed in Nakić et al. [25], if D > D_{crit}, the result is not statistically significant and the obtained background values are overestimated. Considering that the application of the CDF method gave a noticeable difference in terms of non-statistically significant results, the second step was data selection, performed using the Lilliefors test and re-estimation of the ABV.

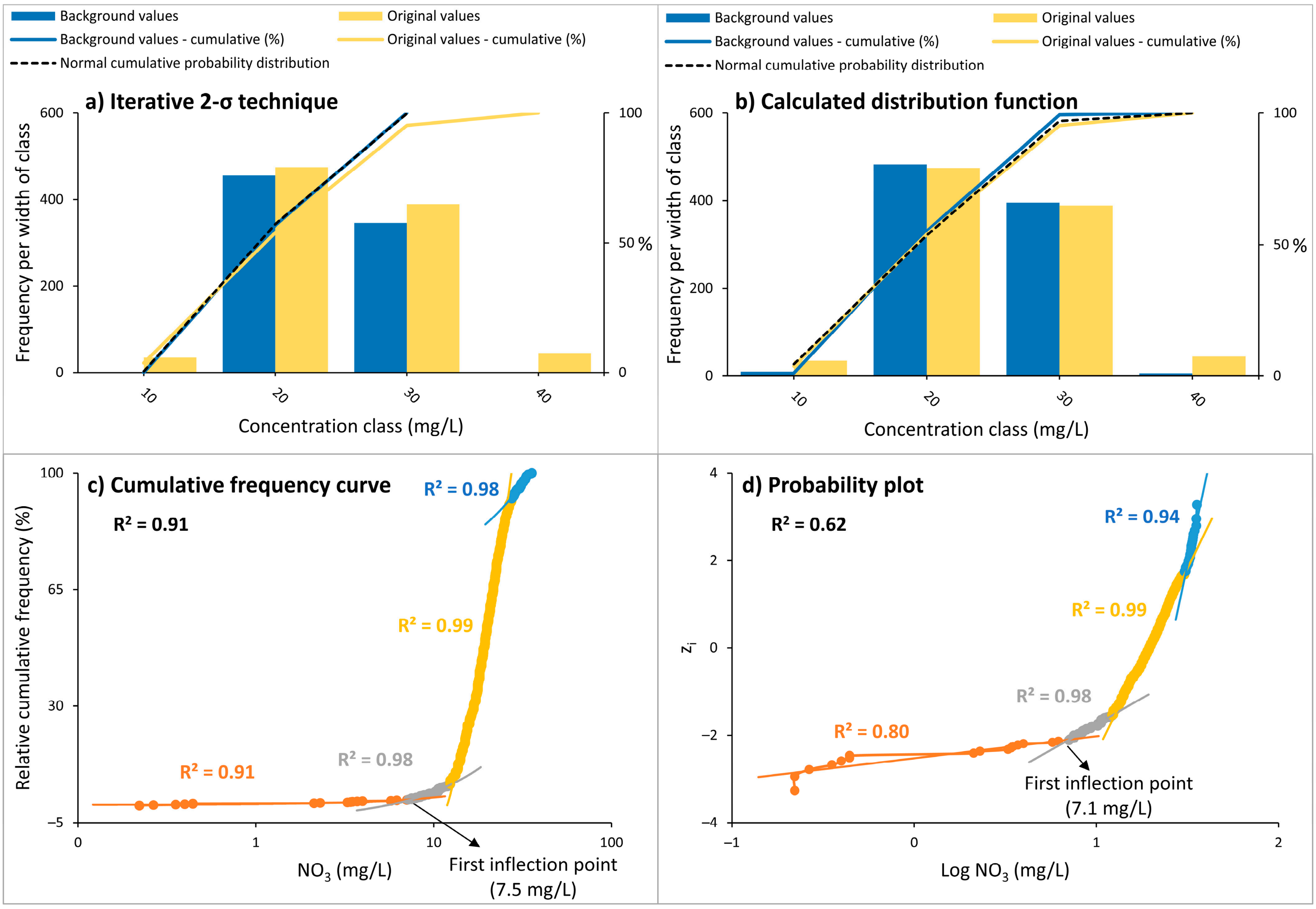

Figure 3. Four different methods to estimate ABV of nitrate for data without selection: (**a**) IT: lower background value: 11.8; D < D_{crit}, (**b**) CDF: lower background value: 8.3; D > D_{crit}, (**c**) CFC: background value: 7.5 and (**d**) PP: background value: 7.1. Arrow indicates first inflection point that separates background and non-background population.

The Lilliefors test was used to test the normality for each well separately (Table 2). This test showed normally distributed data for monitoring wells Vg-11, Vg-4, Vg-6, Vg-9, and Vg-5/2, which are not further considered for the background value estimation procedure in the third step, because, as observed in Panno et al. [29], a single normally or lognormally population forms a straight line on the cumulative probability plots, without the possibility of determining a background value. The data that were not normally distributed (wells Čdp-3/2, Čp-23, Lg-1, Lg-4, P-7, Vg-1 and Vg-10/2) were grouped for further processing as NON-ND data, because it is expected that the cumulative probability plots contain a curved line with at least one inflection point that could indicate the existence of a minimum of two different populations, natural and anthropogenically conditioned. Histograms and normal distribution curves for each well separately are shown in Figure 4.

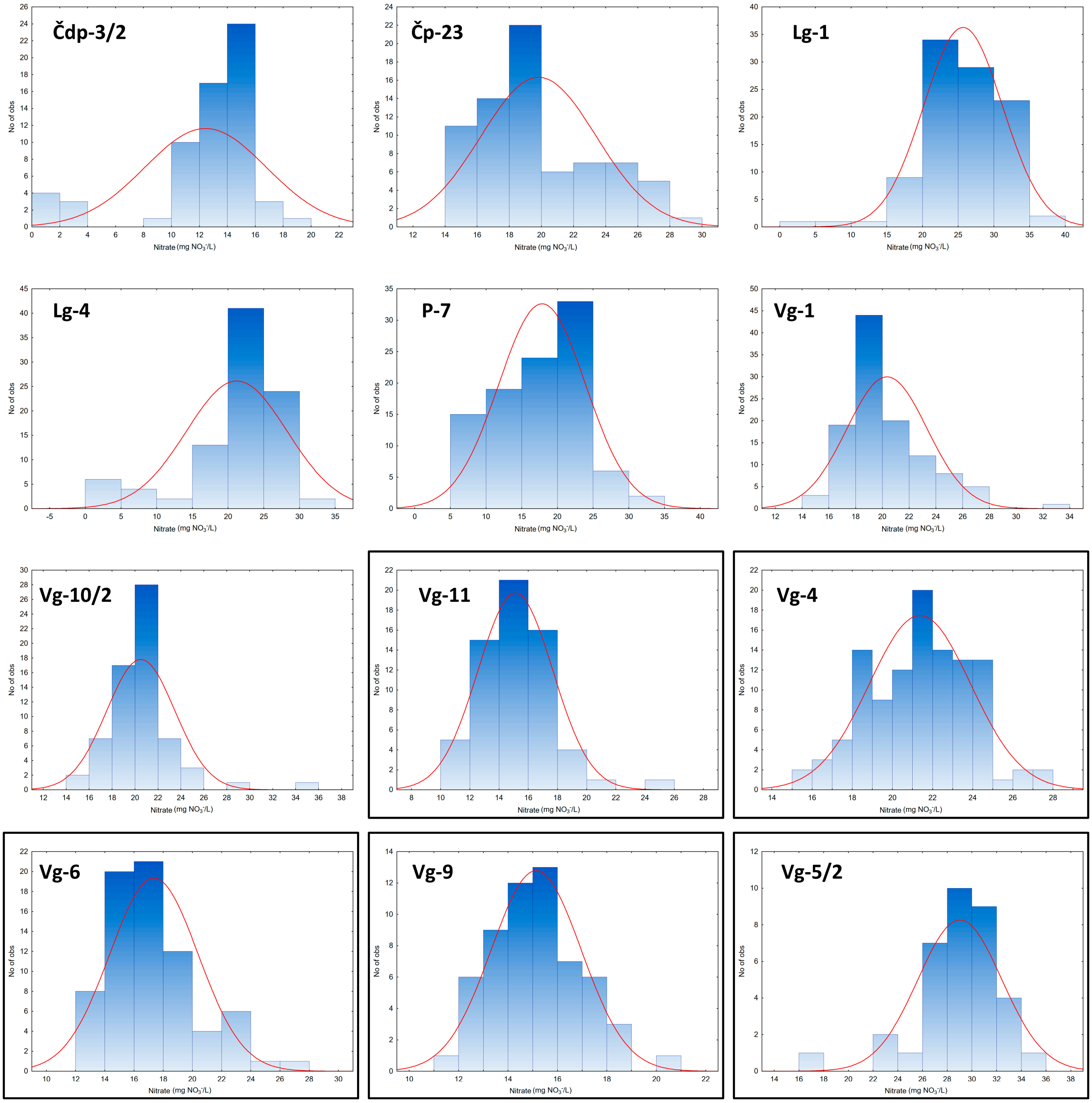

Figure 4. Histograms and normal distribution curves. Normally distributed data are bolded.

The third step was to re-estimate the ABV of nitrate in groundwater for selected data referring to NON-ND data, using the same methods. The use of selected data resulted in statistically significant results for all methods (Figure 5). It is important to emphasize that the result obtained with data without selection that was not statistically significant became significant.

Table 2. Lilliefors test of normality. Normally distributed data are bolded ($D < D_{crit}$).

Monitoring Well	Lilliefors Test	
	D	D_{crit}
Čdp-3/2	0.232	0.112
Čp-23	0.135	0.104
Lg-1	0.098	0.089
Lg-4	0.212	0.092
P-7	0.106	0.089
Vg-1	0.140	0.084
Vg-10/2	0.148	0.109
Vg-11	**0.086**	**0.112**
Vg-4	**0.070**	**0.084**
Vg-6	**0.099**	**0.104**
Vg-9	**0.087**	**0.116**
Vg-5/2	**0.149**	**0.150**

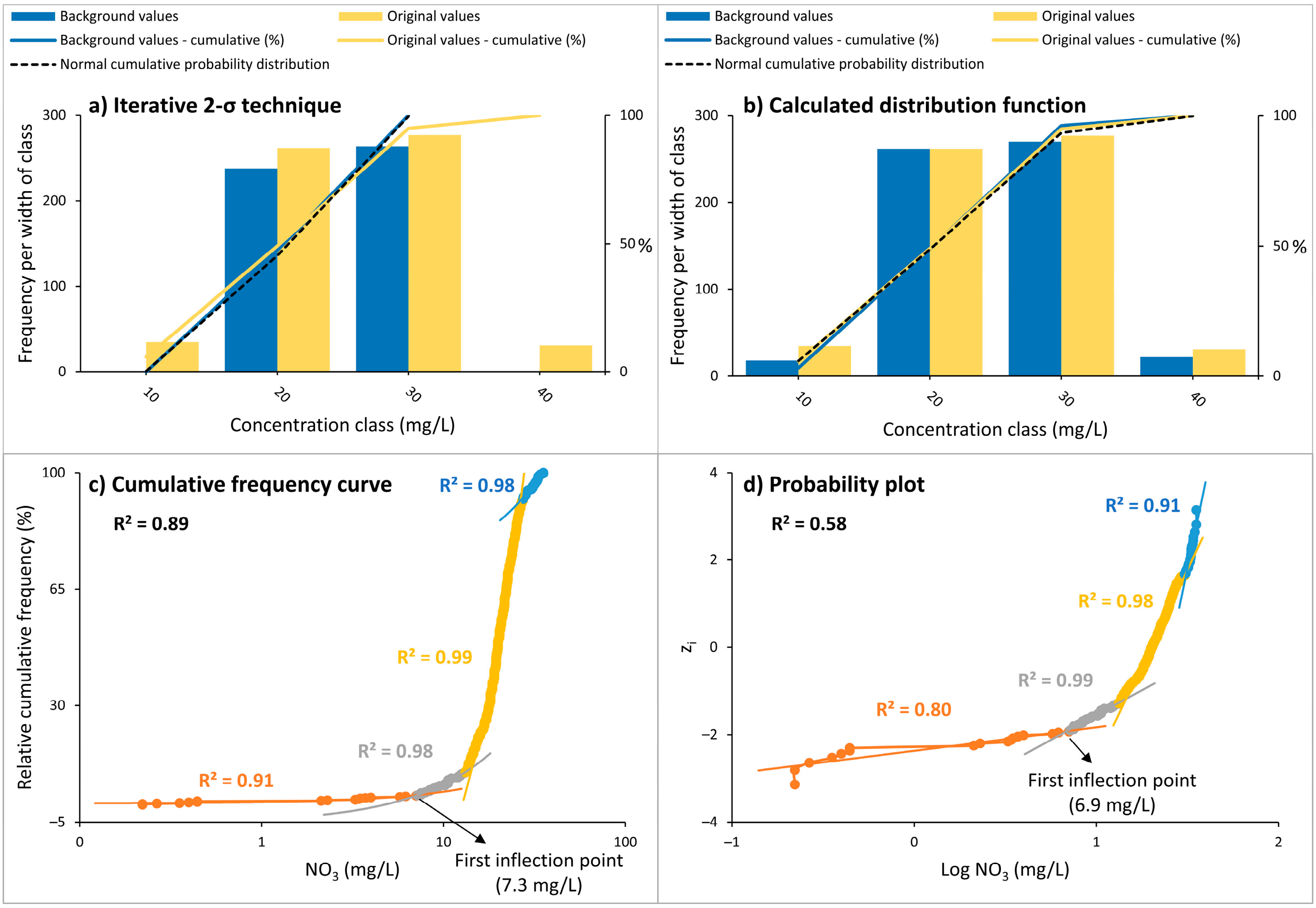

Figure 5. Four different methods to estimate ABV of nitrate for selected NON-ND data: (**a**) IT: lower background value: 13.2; $D < D_{crit}$, (**b**) CDF: lower background value: 7.2; $D < D_{crit}$, (**c**) CFC: background value: 7.3 and (**d**) PP: background value: 6.9. Arrow indicates first inflection point that separates background and non-background population.

Using the IT method again resulted in the highest background value and both results were statistically significant, while the estimated value increased from 11.8 to 13.2. Compared to other methods, IT gave a higher estimation of the nitrate background value because it considers both the low and high values to define anomalies and cannot be applied to a polymodal distribution. The CDF method gave a noticeable difference in terms of non-statistically significant results for data without selection (8.3 mg/L) and statistically significant results for a set of selected data (7.2 mg/L). Although this suggests that the CDF method depends more on which data are used for the calculation with respect to the IT, our results correspond to the results of previous research, where it was shown that it is applicable to polymodal distributions and convenient for use in conditions of anthropogenic activity [40], which are present in the catchment area of the Velika Gorica well field. Results suggest that the usage of CDF is the most applicable when using only NON-ND data, which can be confirmed in the estimation of the statistically significant background value of 7.2 mg/L, unlike the estimate calculated with the all data without selection. Furthermore, Urresti-Estalla et al. [39] suggested that the usage of both IT and CDF methods depends on the nature of the parameter to be estimated as well as the type of frequency distribution curve presented by the data series. From this perspective, the most important criterion to define is whether the low concentration of the parameter that is observed represents a possible indicator of contamination. If not, then CDF should be used instead of the IT only when aquifers are undergoing little pressure.

The usage of lower limits with the first two methods is more convenient in areas under long-term human impacts and corresponds to the usage of the first inflection points from CFCs and PPs [74], which, in the end, makes the results estimated by all methods comparable. The first inflection points observed at plots were directly considered as ABVs, because the first population of the data set was considered to be the consequence of the natural conditions that prevail in the investigated part of the aquifer. The ABV determined by the CFC method decreased from 7.5 to 7.3 mg/L, while the background value determined by the PP method decreased from 7.1 to 6.9 mg/L, for all data and for selected data, respectively. The nitrate concentration showed more than one inflection point on plots, indicating multiple local events of nitrate contamination in groundwater. This is consistent with previous research where it was defined that elevated nitrate concentrations are the result of different sources of contamination, i.e., wastewater and manure [49]. In general, both CFCs and PPs resulted in the extraction of four sets, i.e., populations. Additionally, the aim was also to increase the coefficient of determination of the chosen subset compared to the initial value that was calculated for the entire population. In all cases, the significant improvement in the coefficient of determination was present when dividing entire population into more sub-populations due to different inflection points. However, it must be emphasized that, in our case, CFCs with the entire population had very high values for the coefficient of determination of 0.91 and 0.89 (Figures 3 and 5). This was not the case with PPs, which had starting values for the coefficient of determination of 0.62 and 0.58 (Figures 3 and 5). As observed in Panno et al. [29], the CFC and PP methods can be used in the estimation of nitrate ABVs only when observation wells with non-normally distributed data are included. If these data are not present within the observed data, it is possible that the inflection point will not be recognized.

The study has shown that the CFC and PP methods for data without selection and the CDF method for selected NON-ND data gave consistent and realistic estimations of ABVs that are comparable to nitrate concentrations in the main source of recharge of the Zagreb aquifer, i.e., Sava River [58–61]. When observing all acceptable (statistically significant) results together, it is evident that all methods, except the IT, gave very similar estimations of nitrate ABVs ranging from 7.1 mg/L to 7.5 mg/L. Preziosi et al. [53] and this research confirmed that the background value of nitrate determined at the level of the Zagreb aquifer [25,52] differs from the background value of nitrate determined in the catchment area of the Velika Gorica well field, a homogeneous area within the same aquifer.

5. Conclusions

The ABVs and threshold values are crucial for identifying groundwater quality and pollution migration. The nitrate concentrations, which are a potential pressure and concern in the Zagreb aquifer, were analyzed. The new approach to estimate the background value of nitrate as a basis for better groundwater management and protection in areas under long-term human impact was applied. The study is based on statistical methodologies developed to separate the natural and anthropogenic populations in monitored concentrations from the national groundwater quality monitoring program of Croatian Waters over a 20-year period. The results show that using data without selection can lead to statistically non-significant results, but, with further data selection, a statistically non-significant result became a statistically significant one. In conclusion, all final results must be statistically significant and determined using as wide as possible a data set. Reducing the size of the data set is acceptable only in the case of statistically non-significant results.

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Paper 2: Determination of Nitrate Migration and Distribution through Eutric Cambisols in an Area without Anthropogenic Sources of Nitrate (Velika Gorica Well Field, Croatia)

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Article

Determination of Nitrate Migration and Distribution through Eutric Cambisols in an Area without Anthropogenic Sources of Nitrate (Velika Gorica Well Field, Croatia)

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Abstract: Natural potential sources of nitrate contamination involve decaying of organic matter, bacterial production, atmospheric deposition, and soil N. The study presents the first results of nitrate distribution and migration through soil horizons of the Eutric Cambisols, one of the most common soils developed in the area of the Zagreb aquifer and situated in an area without potential anthropogenic sources of nitrate (first sanitary protection zone of the Velika Gorica well field). A total of 16 parameters of soil water and 16 parameters of soil were used to conduct statistical techniques and analyse associated factors within the soil zone. The results indicate that in the deepest soil horizon, nitrogen is present mostly as nitrate due to nitrification under aerobic conditions which promote stability and the potential for nitrate transport. It was found that nitrate concentrations are the result of soil N nitrification, caused by a NO_3^-/Cl^- molar ratio higher than 1 and the absence of precipitation isotopic signature. The results also show that within the coarse-grained Eutric Cambisols N primarily migrates to deeper parts of unsaturated zone in the form of nitrate and nitrite.

Keywords: nitrogen cycle; soil zone; Eutric Cambisols; Zagreb aquifer

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1. Introduction

Nitrate ion is a main form of nitrogen (N) and one of the more common contaminants in natural environments [1,2]. Although some plants can directly use atmospheric N, ammonium (NH_4^+) and nitrate (NO_3^-) are inorganic forms of N that are usable to most plants [3]. Excluding septic systems, animal waste and commercial fertilizer, significant natural potential sources of NO_3^- contamination involve decaying of organic matter (OM), bacterial production, atmospheric deposition, and soil N [4,5]. Tracing the sources and transformations of NO_3^- is crucial for gaining insights into water quality protection and better understanding of the N cycling [2]. Furthermore, it has been shown that hydrochemistry (e.g., NO_3^-, Cl^-) and isotopes (e.g., ^{18}O-H_2O) can provide important information for differentiating between NO_3^- sources and the processes involved in N cycling [2]. The deuterium excess (d-excess) is also a valuable tool for discerning the influences of evaporation and/or mineral dissolution trends [5]. Additionally, it is an important tool in assessing the mean residence time of soil water and recharge processes [6].

NO_3^- is considered the most oxidized, stable, and mobile form of N species in solution [7]. The accumulation of NH_4^+ in soils is not common, as it undergoes rapid conversion by soil microbes [3]. The presence of NH_4^+ at some depth in the unsaturated zone indicated that reducing conditions might be present [8]. Due to oversaturation in the soil, N species are lost to groundwater through leaching, which contributes to groundwater contamination [9]. Soil conditions that enhance the retention of NH_4^+ and NO_3^- ions, i.e., a zeolite with a high exchange capacity, offers a solution by absorbing ammonium and slowing down the nitrate leaching [10]. Cl^- tends to behave in a more stable manner, with the minimum amount of chemical reactions or transformations [11–13] and minimum interactions with subsoil [5] because it is inert to physical, chemical, and microbiological processes [14]. The molar ratio of NO_3^-/Cl^- has been deemed to be a valuable tool for exploring N dynamics and sources [12,13]. Additionally, higher NO_3^-/Cl^- molar ratios suggest that some potential NO_3^- input might have been ascribed to precipitation, fertilizer application, and nitrification of soil N [2].

Soil nitrification is a two-step process performed by living soil microorganism [3,9,15–17]. Ammonia-oxidation (conversion of NH_4^+ to NO_2^-, i.e., nitritation) is carried out by ammonia oxidizers [18,19], which is widely distributed in most agricultural soils and represents the major contributor to nitrification [20]. The second step is nitrite-oxidation (transformation of NO_2^- into NO_3^-, i.e., nitratation) [3], performed by nitrite oxidizers [21,22]. When the rate of nitritation is faster than nitratation, NO_2^- accumulates. When nitratation takes place more rapidly, only a small amount of NO_2^- is produced [23,24]. The presence of NH_4^+ and NO_3^- or a significant amount of NH_4^+ at some depth in the unsaturated zone is evidence of incomplete nitrification [8]. Conversely, denitrification involves reduction of NO_3^- through the conversion of NO_3^- into N_2, N_2O, or NO, generally under anaerobic conditions [25–27].

Previous studies have shown that the nitrification process in soils depends on many factors, such as soil moisture, temperature, soil pH, organic carbon content, the presence of major oxides and heavy metals, as well as soil texture. Excess water in soils can lead to oxygen limitation, which reduces the rate of nitrification [28], while microbial activity generally increases with increasing temperature [3,9]. The highest denitrification occurs when a combination of high soil moisture and high soil temperature is present, whereas the low soil moisture appears to restrain the degree of denitrification [29]. Soil moisture closes pore spaces, which in turn impairs aeration and reduces the oxygen level. As nitrification is a biochemical oxidation process, low oxygen levels in the soil negatively affect the process of nitrification [9]. During summer months, the assimilation of NO_3^- by plants and denitrification process reduces NO_3^- concentrations [11]. On the other hand, the lowest denitrification occurs when the rainfall abruptly increases, causing enhancement of leaching. Bacterial diversity and community structure are significantly influenced by the pH of the soil [3,30,31]. In acidified soils, the intensity of nitrification is lower compared to soils with higher pH values [32–34]. The optimum activity of ammonia oxidizers and nitrite oxidizers occurs at pH 7.5 and 7.0 [9]. In general, pH values in the topsoil tends to be lower, primarily because the topsoil contains a higher concentration of OM, and the decomposition of OM lowers pH [35]. Organic carbon is another significant factor that influences the rate of nitrification in the soil [32,36,37]. The presence of organic carbon inhibits nitrification by reducing the abundance of ammonia oxidizers, whereas low organic carbon levels in soil enhance the nitrification rate, resulting in higher NO_3^- concentrations [20,38]. Elevated levels of soil organic carbon are associated with increased OM content, which in turn improves permeability and water availability [39]. On the other hand, reduced input of OM along with soil depth tends to decrease total organic carbon [40]. Additionally, other compounds in soil, such as titanium dioxide (TiO_2), can reduce the abundance of ammonia-oxidizers and nitrite-oxidizers [41]. The impact of iron (Fe) minerals should also not be ignored, especially oxides, whose influence on soil N transformation processes varies according to soil pH. In the low-pH soil, Fe oxide frequently stimulates nitrification activity, while in the high-pH soil, Fe oxide significantly decreases nitrification rate [42]. Anaerobic NH_4^+ oxidation can be linked to ferric iron reduction, resulting in the production of N_2, NO_2^- [43], or NO_3^- [44] as the end product. These reactions involve the use of ferric iron (Fe^{3+}) as an electron acceptor. Moreover, reactions of manganese (Mn) oxides in soil are similar to Fe and play significant roles in N cycling process [45]. Under oxic conditions, Mn has a toxic effect on microorganisms, whereas under oxygen-depleted conditions, Mn serves as an alternative electron acceptor. Furthermore, toxic elements such as heavy metals often negatively affect nitrification rate in soils [46,47]. Chromium (Cr) increases NH_4^+ content and decreases the accumulation of NO_3^- [48]. Nickel (Ni) [49], zinc (Zn) [50], lead

(Pb) [46], arsenic (As) [51], cobalt (Co) [52] and mercury (Hg) [53] have toxic effects on microorganisms and inhibit nitrification processes in soil, i.e., NH_4^+ oxidation to NO_2^-, leading to a reduction in NO_3^- concentration. Soil texture, which characterizes the size distribution of soil and mineral particles, is a significant factor that affects the accumulation of soil OM [54]. Clay and silt particles are small in size, however they have large specific surface areas and the ability to absorb and protect soil OM by providing stability against microbial mineralization [55,56]. Considering that levels of OM are associated with levels of soil organic carbon, the soils with higher silt and clay content tend to have higher soil organic carbon [56].

On the other hand, nitrification process (i.e., accumulation of NO_3^- ion) can lower soil pH by causing the leaching of Ca^{2+} and Mg^{2+} and reducing their concentrations [57–59]. Conversely, NH_4^+ acidifies the soil by directly exchanging base cations [60]. In an oxygen-deficient environment of soil, both nitrification and denitrification processes become more pronounced, which leads to the formation and accumulation of NO_2^- as an intermediate product [61]. In conditions of relatively low soil moisture, the oxygen content is higher, leading to stronger nitrification [61].

The scientific research polygon of the Faculty of Mining, Geology, and Petroleum Engineering, University of Zagreb [62], is located within the first sanitary protection zone of the Velika Gorica well field, situated in the southern part of the Zagreb aquifer, which presents strategic water reserves and the main source of potable water in the Zagreb area protected by the Republic of Croatia. In recent history, previous investigations were focused on various aspects related to NO_3^- contamination in the groundwater of the Zagreb aquifer [63–65]. Despite research conducted in the selected area, there has been no specific focus on investigating the distribution of nitrates through Eutric Cambisols and related geochemical processes. The primary focus of research within the soil and the unsaturated part of the Zagreb aquifer has been on establishing the relationship between permeability and physicochemical properties [66], determining the sorption characteristics of potentially toxic metals [67], evaluating soil water origin [68], and conducting soil water monitoring of multiple soil horizons in Eutric Cambisols at the Velika Gorica site [69].

The objectives of this study have been to evaluate the NO_3^- distribution and migration through pedological profile situated in an area without anthropogenic sources of nitrate. This has been tested by conducting statistical techniques to determine the significant variability within different soil horizon groups and by analysing and characterizing the factors influencing NO_3^- concentration within the soil zone. For this purpose, 16 different parameters of soil water and 16 different parameters of soil have been chosen. One of the specific goals of this research is the definition of a dominant natural N form that infiltrates into the aquifer. The preliminary findings from this research offer a new insight into geochemical processes related to N species transformation occurring within the Eutric Cambisols, which present one of the most common soils developed in the area of the Zagreb aquifer. Long-term goals also involve modelling the flow and transport of N compounds within the soil and unsaturated zone. It is expected that by achieving these goals new measures necessary for effective and sustainable management of the Zagreb aquifer will be adopted.

2. Materials and Methods

2.1. Site Description

According to Bogunović et al. [70] the research polygon is located in Eutric Cambisols on Holocene deposits (Figure 1). The unsaturated zone thickness at the study site usually ranges from 5 to 8 m and depends on the groundwater levels. At the top of the unsaturated zone, the following soil horizons were identified according to the World Reference Base classification: A (0–0.15 m), 2B (0.15–0.55 m), 3BC (0.55–0.9 m), and C (0.9–1.17 m).

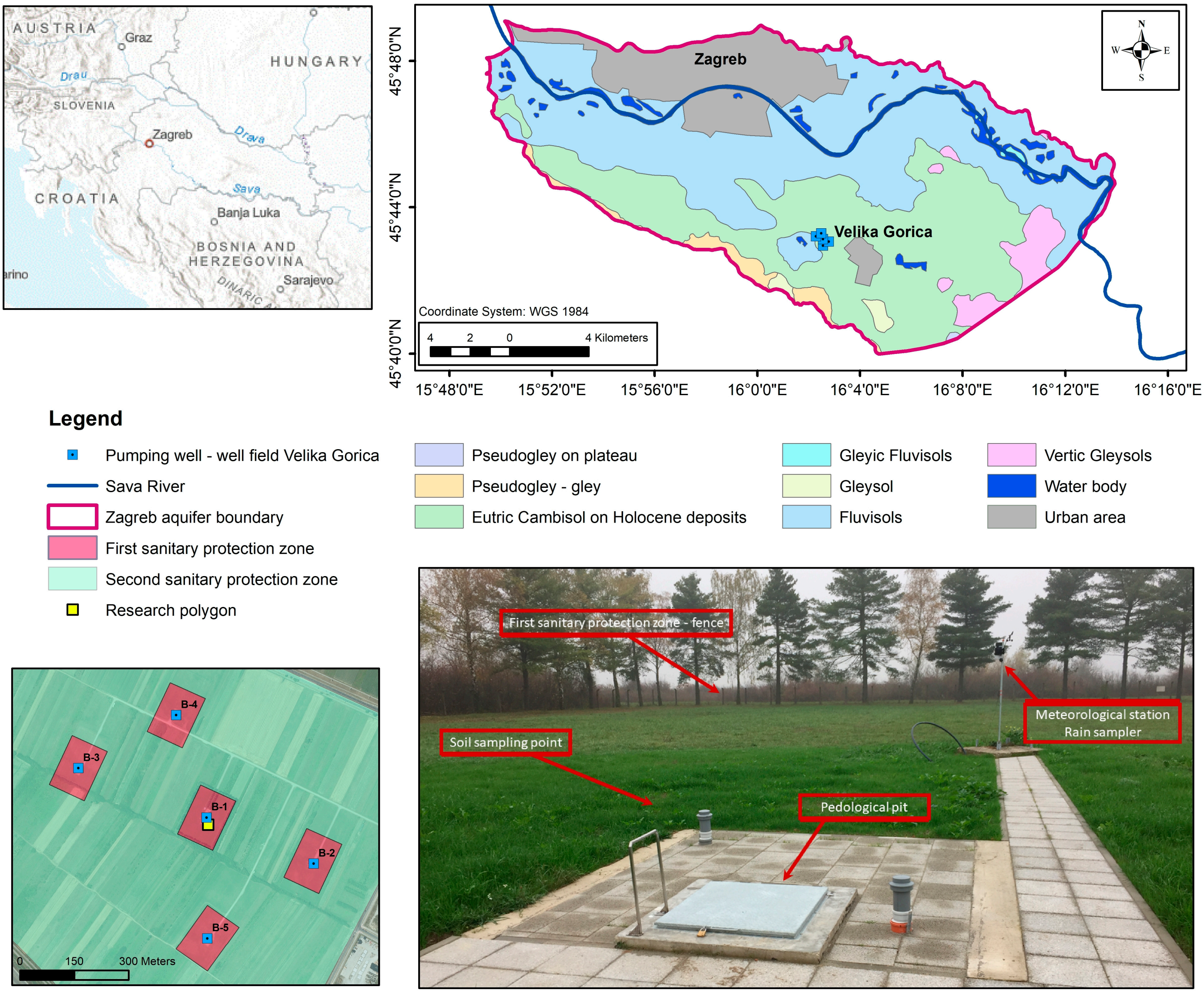


Figure 1. Location of the scientific research polygon.

In Table 1, particle size analysis and OM content are shown. OM values are determined for the first three soil horizons, where fine-grained particles are predominant. In accordance with Ružičić et al. [66], the upper 90 cm of the observed profile is predominantly composed of silty and sandy materials, with intermittent clay layers, while gravels dominate throughout the profile until reaching the water table [71]. It is important to highlight that most silt and clay particles, along with the smallest proportion of sand, can be found in the 2B horizon, while the C horizon contains a significant amount of gravel and silt. It can be seen that the A horizon has the highest OM values, while the 2B horizon has the lowest.

Table 1. Selected characteristics of the investigated soil profile.

Soil Horizon	Depth (m)	Gravel (%)	Sand (%)	Silt (%)	Clay (%)	OM (%)
A	0–0.15	-	18.30	54.83	26.87	5.35
2B	0.15–0.55	-	5.14	55.33	39.53	2.07
3BC	0.55–0.9	-	13.72	50.75	35.53	3.32
C	0.9–1.17	52.20	1.50	45.65	0.64	-

Source: Data from Refs. [66,67,69].

The climate is humid continental, with mean annual precipitation and temperature for the period 2001 to 2020 of about 967 mm and 11.9 °C, respectively, and with monthly average precipitation of around 80 mm [65].

Within the polygon, there is a weather station, rain sampler, and a pedological pit (Figure 1) equipped with various measuring instruments to observe and measure parameters in each soil horizon, as well as the unsaturated and saturated zones.

2.2. Data Collection and Sampling

Precipitation and air temperature data at hourly intervals were collected using a meteorological station (Vantage Pro2, Davis Instruments) positioned near the pedological pit. A total of 12 precipitation samples were collected using a Palmex Rain Sampler RS1 (Zagreb, Croatia) [72] in order to determine water stable isotopes and chemical compositions. Four TRIME-PICO 64 probes (IMKO Micromodultechnik GmbH, Ettlingen, Germany) are installed in soil horizons and employed to measure moisture and temperature in soil horizons. Hourly measurements of soil moisture and soil temperature were collected using dataTaker DT80 and globeLog (IMKO Micromodultechnik GmbH) loggers. Soil water samples were sampled from four soil horizons using soil water samplers (suction cups; Eijkelkamp Soil & Water, Giesbeek, The Netherlands) and an integrated automatic vacuum pump unit AVP–100 (UGT GmbH, Müncheberg, Germany) to determine isotopic and chemical compositions. Suction cups are situated at following depths: −0.08 (SC1), −0.33 (SC2), −0.75 (SC3), and −1.05 m (SC4). During certain months (July, August, and October), it was not possible to obtain soil water samples from soil water samplers SC3 and SC4, while during the most dry month, i.e., September, not a single soil water sample could be taken. This arose primarily due to low soil water content. For this reason, a total of 38 soil water samples were collected. Additionally, due to very small volume amounts available from the deepest soil water sampler SC4, the chemical composition was analyzed for 35 soil water samples. Samples and data were collected from March 2021 to February 2022. Each soil water and precipitation sample was filtered using a 0.22 µm nylon membrane filter and then moved into high-density polyethylene (HDPE) bottle.

A total of 12 soil samples were collected up to a depth of 1.2 m using Eijkelkamp auger set for soils. The soil profile was excavated near the pedological pit. Soil samples were collected at 10 cm depth intervals in order to measure the following soil parameters: pH, electric conductivity (EC), total organic carbon (TOC), TiO_2, Fe_2O_3, MnO, heavy metals, and soil texture. The samples were stored in separate plastic bags, transported to the laboratory and air-dried.

2.3. Laboratory Measurements

All laboratory measurements were performed at the Laboratory for spectroscopy of the Faculty of Mining, Geology and Petroleum Engineering, University of Zagreb. The concentrations of major anions and cations were determined using a Dionex ion chromatograph (IC). The water stable isotopes (δ^2H-H_2O and δ^{18}O-H_2O) were analysed using a Los Gatos Research laser (LWIA-45-EP, San Jose, CA, USA) by laser absorption spectroscopy. The analytical precision was 0.9 ‰ for δ^2H and 0.19 ‰ for δ^{18}O. Values are expressed in permil notation relative to Vienna Standard Mean Ocean Water (VSMOW). The data were analysed and interpreted using the Laboratory Information Management System (LIMS) for Lasers 2015 [73]. D-excess is calculated as d-excess = $\delta^2H - 8{*}\delta^{18}O$ [74].

A portion of each soil sample interval was sieved through a 2 mm sieve and homogenized in an agate grinding set. Soil pH in 1M KCl was measured using a pH meter in a 1:5 suspension of soil volume and 1M KCl solution according to ISO10390:2005. EC was measured in a suspension of 1:5 soil volume and H_2O. Total carbon (TC) and total inorganic carbon (TIC) were measured with Elementary analyser multi-EA 4000 (Analytik Jena AG, Jena, Germany). TOC was obtained by subtracting TIC from TC. Soil texture was characterized with laser diffraction method using Malvern Mastersizer 3000. The 2000-63-2-µm system was used to determine particle size fractions. Soil particle size classification was done according to the IUSS Working Group WRB [75]. Geochemical contents of major oxides and microelements were determined using Hitachi XMET 8000 Expert Geo portable X-ray fluorescence (pXRF) instrument. Soil and MiningLE (light elements) calibrations were used. The accuracy of the analysis was controlled by analysing the standard material for soil samples (NIST 2711) in the studied sample batches. Based on five measurements and the use of blanks and standards, the instrumental precision was ±5% or less.

2.4. Statistical Analysis

Statistical analyses were performed with the TIBCO software Inc. Statistica (Version 13.5.0.17). Depending on the parametric or non-parametric nature of the data, different statistical analyses are chosen. One-way ANOVA is used for parametric sample data analysis and Kruskal–Wallis (KW) test is used for non-parametric data. Sixteen soil water variables were tested and the main goal of conducting ANOVA or KW test was to establish significant differences within different sampling depths for each variable.

When performing a one-way ANOVA parametric test, there are assumptions that need to be met: dependent variable should follow a normal distribution and the variance should be constant across groups [76–78]. Shapiro–Wilk test is therefore used to check if variable comes from a normal distribution. Levene's test is utilized to test equality of variances in a dataset, i.e., to test the null hypothesis that the samples come from a population with the same variance. The KW test is used when the assumptions of one-way ANOVA are not met [79,80].

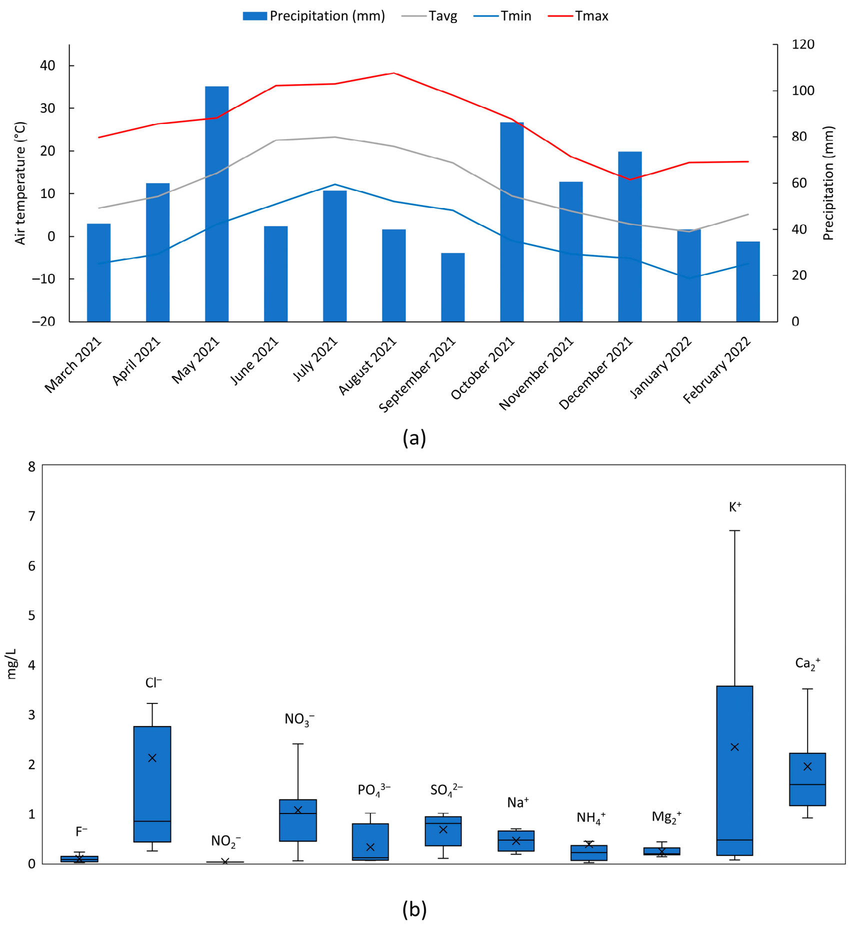
The ANOVA is a statistical technique used to assess the variability and determine the variation of the means of a group of data or variables to evaluate statistical significance [77,81]. The KW test is a non-parametric method for testing whether samples are originated from the same distribution [82].

If the ANOVA or KW test yields a statistically significant difference, the post hoc tests, namely, the Tukey Honest Significant Difference test (HSD) and Mann–Whitney U test, are employed to compare parameters between the groups. Tukey HSD test is used after one-way ANOVA test to show comparisons between each pair of groups at a significant level of 0.05 [83,84]. The Mann–Whitney U test is utilized after the KW test and it is comparable with the post hoc Tukey HSD test. The Mann–Whitney U test is used to compare the distribution among different groups of soil sample data.

3. Results

3.1. Precipitation and Air Temperature

Monthly values of precipitation and air temperature (Figure 2a), as well as precipitation chemical composition (Figure 2b) are shown. Monthly precipitation varied from 29.8 to 102 mm, and the driest months were June, August, September, January, and February. Mean air temperature ranges from 1.16 to 23.27 °C. Monthly analyses over a 12 month period yielded mean values of 0.09 mg/L for fluoride (F^-), 2.12 mg/L for chloride (Cl^-), 0.03 mg/L for nitrite (NO_2^-), 1.07 mg/L for nitrate (NO_3^-), 0.32 mg/L for phosphate (PO_4^{3-}), 0.67 mg/L for sulphate (SO_4^{2-}), 0.45 mg/L for sodium (Na^+), 0.38 mg/L for ammonium (NH_4^+), 0.23 mg/L for magnesium (Mg^{2+}), 2.34 mg/L for potassium (K^+), and 1.94 mg/L for calcium (Ca^{2+}).



(a)

(b)

Figure 2. (**a**) Monthly precipitation and air temperature at the Velika Gorica meteorological station; and (**b**) chemical composition of precipitation in mg/L.

3.2. Characteristics of Soil Water

The descriptive statistics of soil moisture, soil temperature, isotopic, and chemical characteristics for soil water samples are listed in Table S1 (Supplementary Materials), while the graphical distributions in different soil horizons are shown in Figure 3. The table involves the mean, minimum, maximum, and standard deviation (SD) of each parameter. The results show that soil moisture through pedological profile ranges from 19.15 to 45.21% with the highest mean value in the A soil horizon (36.51%) and lowest in the C horizon (23.19%). The probe at the shallowest depth shows the greatest variability (SD is 9.32), ranging from 19.69 to 45.21%, while the deepest probe ranging from 19.15 to 24.79% shows the lower variability (SD is 2.15). Soil temperature ranges from 2.88 to 25.88 °C due to a seasonal variation with highest values in summer and lowest values in winter.

The values of δ^2H and δ^{18}O for soil water range from −74.64 to −30.17‰ and from −10.68 to −4.24‰, respectively. The value of SD decreased with depth, suggesting lower variability in isotopic composition. In Figure 4a it can be clearly seen that average values of isotopic composition from all soil horizons fall on the local meteoric water line (LMWL). However, the results also suggest that in the A and 2B soil horizons, the precipitation signature can be seen, while the 3BC and C soil horizons have a different isotopic signature. Furthermore, this can be also seen in Figure 4b, which confirms similar isotopic composition in the two deepest soil horizons in the observed time interval with almost no variation and response to precipitation.

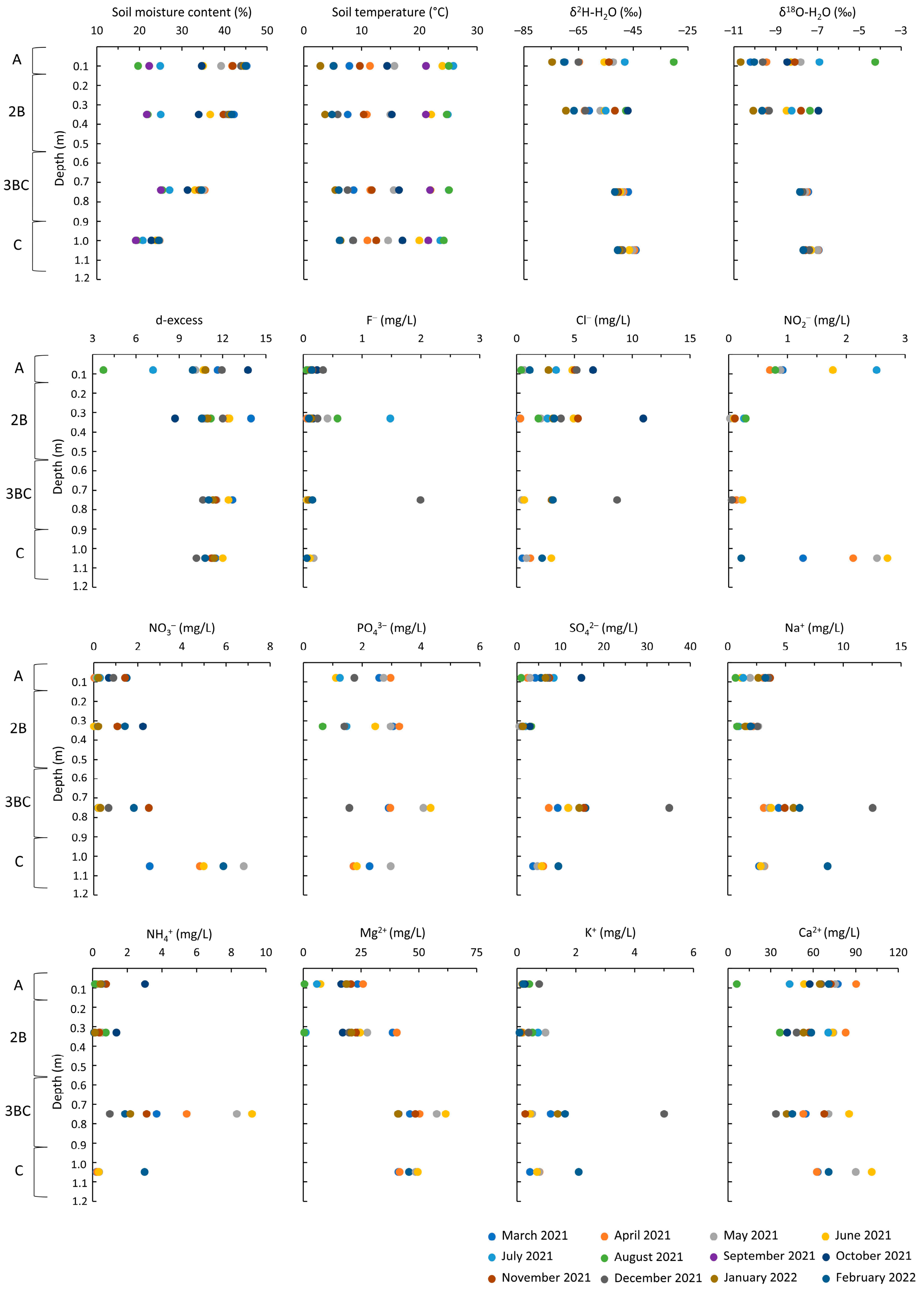
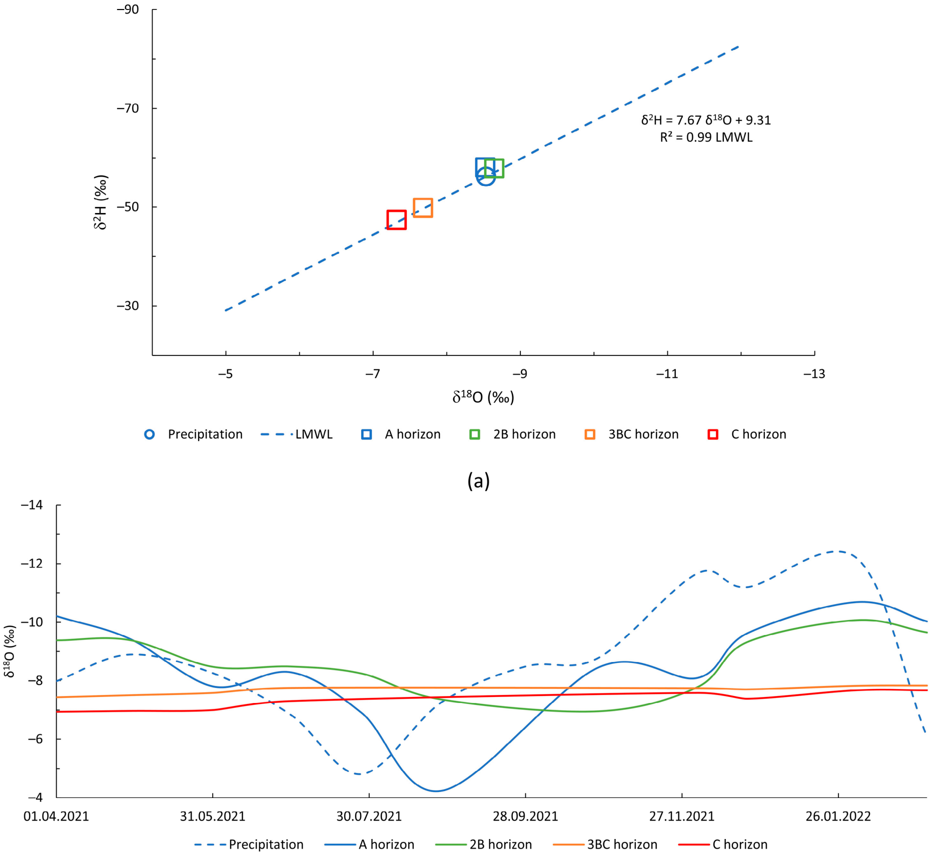


Figure 3. Distribution of soil moisture, soil temperature, isotopic, and chemical characteristics of soil water samples by depth.



(a)

(b)

Figure 4. (**a**) Isotopic composition of soil water and precipitation and LMWL of Velika Gorica; and (**b**) variation in $\delta^{18}O$ in time in sampled soil water and precipitation.

D-excess ranges from 3.75 to 13.97‰ and shows smaller variability in the 3BC and C soil horizon (Table S1). Furthermore, smaller values of d-excess in the shallowest soil horizon in the summer months of 2021 (July and August) suggest influence of evaporation, which is consistent with the previous research where it was shown that in most cases evaporation fractionation is limited to the shallowest 0.3 m of soil [85], and which has also been observed in the A soil horizon within the previous research done at the study site [69].

Concerning the chemical composition of soil water, the F^- and Cl^- mean concentrations range from 0.11 to 0.32 mg/L and from 1.55 to 3.51 mg/L, respectively. The Cl^- concentrations were higher in the colder months compared to the warmer months. A wide range of NO_2^- and NO_3^- concentrations were observed, with mean values range from 0.13 to 1.76 mg/L and from 0.52 to 5.00 mg/L, respectively. NO_2^- content is higher in the A and C soil horizon, while NO_3^- increases with depth. The nitrates stays at a low level in the first three soil horizons and then sharply rises in the C horizon (Figure 3). From Table S1, it can be observed that the SD of NO_2^- and NO_3^- at the deepest soil horizon is higher. The PO_4^{3-} and SO_4^{2-} mean concentrations range from 2.06 to 2.19 mg/L and from 1.50 to 15.15 mg/L, respectively. The PO_4^{3-} and SO_4^{2-} content in the 3BC soil horizon is higher than for the other depths. Further, a wide range of NH_4^+ concentrations were observed. The NH_4^+ mean concentrations range from 0.55 to 4.35 mg/L, with the higher content and wider range of value in the 3BC soil horizon. The Na^+ and K^+ mean concentrations range from 1.86 to 5.53 mg/L and from 0.31 to 1.36 mg/L, respectively. In comparison to other depths, the 3BC horizon exhibits higher Na^+ and K^+ concentrations. The Mg^{2+} and Ca^{2+} mean concentrations range from 16.15 to 48.47 mg/L and from 56.53 to 77.64 mg/L, respectively. Mg^{2+} increases in the first three soil horizons and then decreases in the C horizon.

Figure 5 shows the variation of the NO_3^-/Cl^- molar ratios in relation to Cl^- concentrations. NO_3^-/Cl^- molar ratios varied widely ranging from 0.03 to 0.75 with an average of 0.16 in the A soil horizon, from 0.002 to 0.33 with an average of 0.11 in the 2B horizon, from 0.04 to 0.47 with an average of 0.24 in the 3BC horizon and from 0.95 to 4.53 with an average of 2.46 in the C horizon. Therefore, compared to the first three soil horizons, the NO_3^-/Cl^- molar ratios for C horizon had higher values.

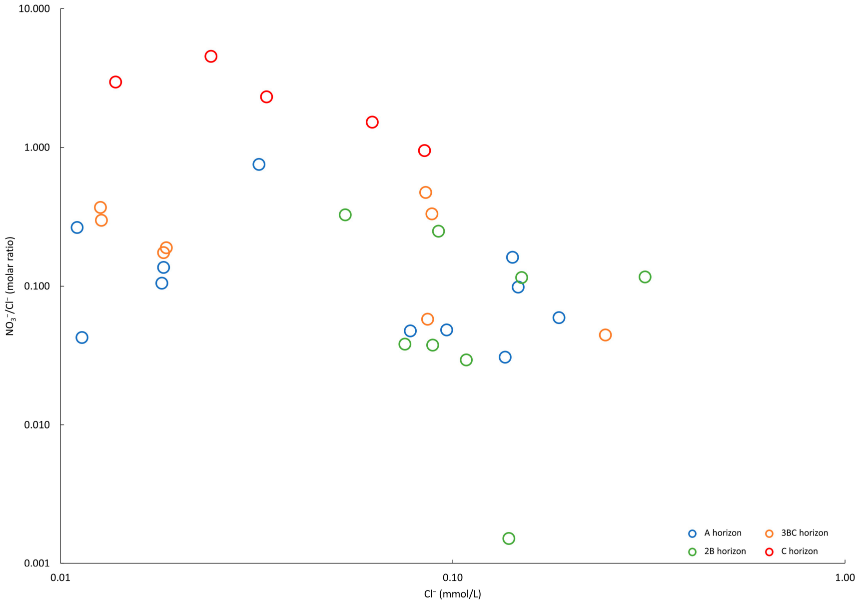


Figure 5. Relationship between NO_3^-/Cl^- molar ratio and Cl^- for A, 2B, 3BC, and C soil horizon.

In the investigated soil water samples within first soil horizon (A), the low mNO_3^-/Cl^- ratio was accompanied by the high mNO_2^-/Cl^- ratio (Figure 6). In the deepest soil horizon (C), under the relatively low soil moisture, the mNO_3^-/Cl^- values are higher than mNO_2^-/Cl^- values.

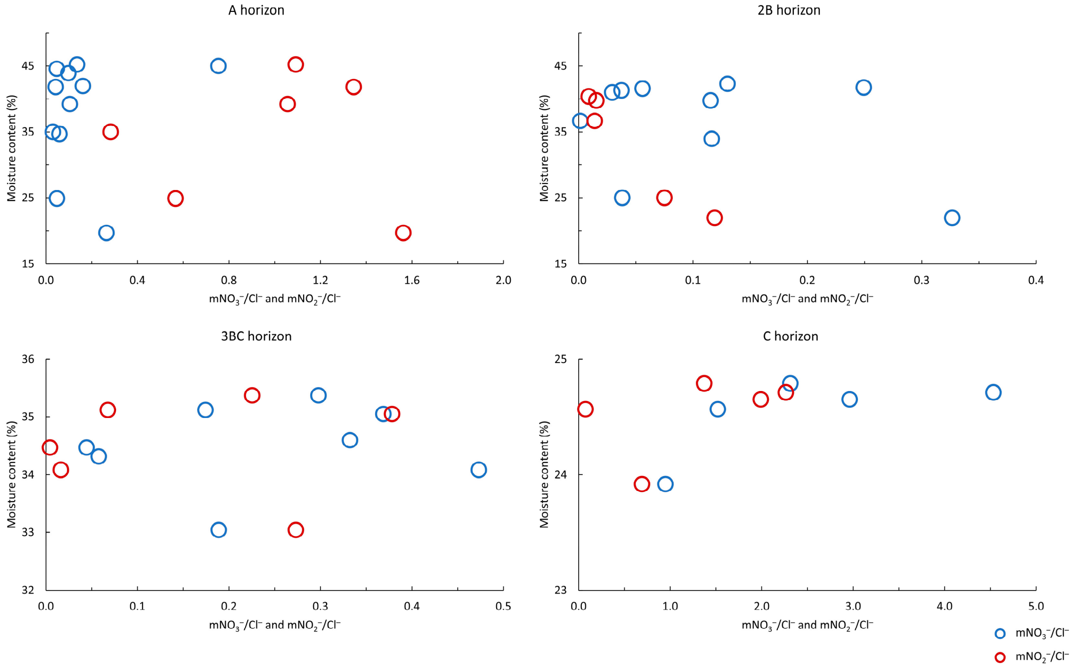


Figure 6. Relationship between soil moisture and mNO_3^-/Cl^- or NO_2^-/Cl^- for A, 2B, 3BC, and C soil horizon.

3.3. Analysis of Variance (ANOVA) and Kruskal-Wallis Test

For testing the assumptions of normality and homogeneity of variance, the Shapiro-Wilk (Table S2) and Levene's test (Table S3) are used, respectively. The test outcomes show that only four soil water parameters, i.e., temperature, PO_4^{3-}, Mg^{2+} and Ca^{2+}, follow a normal distribution and have variance constant within each group. These variables were tested by one-way ANOVA. For the remaining 12 parameters, where the assumptions are not met, testing was conducted using the KW test.

The one-way ANOVA and KW test have been conducted to compare the variability of soil water parameters from a different sampling depth. The results of ANOVA (Table S4) indicate that there are no significant differences for soil temperature, PO_4^{3-}, and Ca^{2+} within different sampling depth within the pedological pit. However, a statistically significant difference was observed for Mg^{2+} between the different sampling depths. The results of KW test indicate that there are significant differences for soil moisture, δ^2H, $\delta^{18}O$, NO_2^-, NO_3^-, SO_4^{2-}, Na^+, NH_4^+, and K^+. Conversely, d-excess, F^- and Cl^- are an insignificant difference within the four sampling groups (Table S5).

Tukey HSD test is utilized after one-way ANOVA only for the Mg^{2+} parameter (Table S6), where a significant difference is observed. The *p*-values of Mg^{2+} between A and 2B, as well as between 3BC and C, indicate an insignificant difference. Among all other groups the *p*-values indicate the significant difference. Mann–Whitney U comparison test (Table S7) is used after the KW test for nine parameters with a significant difference. The results indicate that the different soil horizon groups of moisture content (between A and C, 2B and C, 3BC and C), δ^2H (between A and 3BC, A and C, 2B and 3BC, 2B and C), $\delta^{18}O$ (between A and 3BC, A and C, 2B and 3BC, 2B and C, 3BC and C), NO_2^- (between A and 2B, A and 3BC, 2B and C, 3BC and C), NO_3^- (between A and C, 2B and C, 3BC and C), SO_4^{2-} (between A and 2B, A and 3BC, 2B and 3BC, 2B and C, 3BC and C), Na^+ (between A and 3BC, 2B and 3BC, 2B and C), NH_4^+ (between A and 3BC, 2B and 3BC, 3BC and C) and K^+ (between A and 3BC, A and C, 2B and 3BC, 2B and C) are remarkably different. There is no statistically significant difference observed among all the other independent groups.

3.4. Characteristics of Soil

The distribution of 16 soil parameters through depth is presented in Figure 7. As shown, soil pH values tend to increase with depth. Soil pH is usually below 7, ranging from 6.4 to 6.9, except for in the deepest interval (C soil horizon) where pH of 7.1 was measured. The EC values range from 80.5 to 150.6 µS/cm. TC and TIC content decreases with depth and then increases sharply at the bottom of the profile. TOC decreases with depth and ranges from 0.4 to 2.3%. Contents of TiO_2, Fe_2O_3 and MnO range from 0.3 to 0.9 wt.%, from 5.7 to 8.5 wt.% and from 0.1 to 0.2 wt.%, respectively. In comparison to other depths, the C soil horizon demonstrates the lowest TiO_2, Fe_2O_3, and MnO content. Cr ranges from 316 to 415 mg/kg and changes dramatically along the depth, with all measured values exceeding the maximum permissible limits for soil. Ni, Zn, Pb, and As concentrations range from 54 to 93 mg/kg, from 92.4 to 152.6 mg/kg, from 27.8 to 45.4 and from 13.4 to 23.6 mg/kg, respectively. Ni content in the 2B soil horizon exceeds the maximum permissible limits for soil. Ni, Zn, Pb, and As concentrations are the lowest in C soil horizon. A wide range of Co concentrations, from 35 to 89 mg/kg, was observed through pedological profile. Hg ranges from 7.8 to 11 mg/kg and all measured values exceed the maximum permissible limits. Clay content varies from 9.5 to 20.8%, and sand content varies from 0.2 to 6.3%, with the highest quantities observed in the C soil horizon. Silt content ranges from 72.9 to 89.5% and generally decreases with depth. Based on the FAO [75] soil texture classification, soil samples up to 60 cm deep are classified as silt and soil samples from 0.60 to 1.2 m are classified as silt loam, with one interval (0.90–1.0 m) being classified as silt.

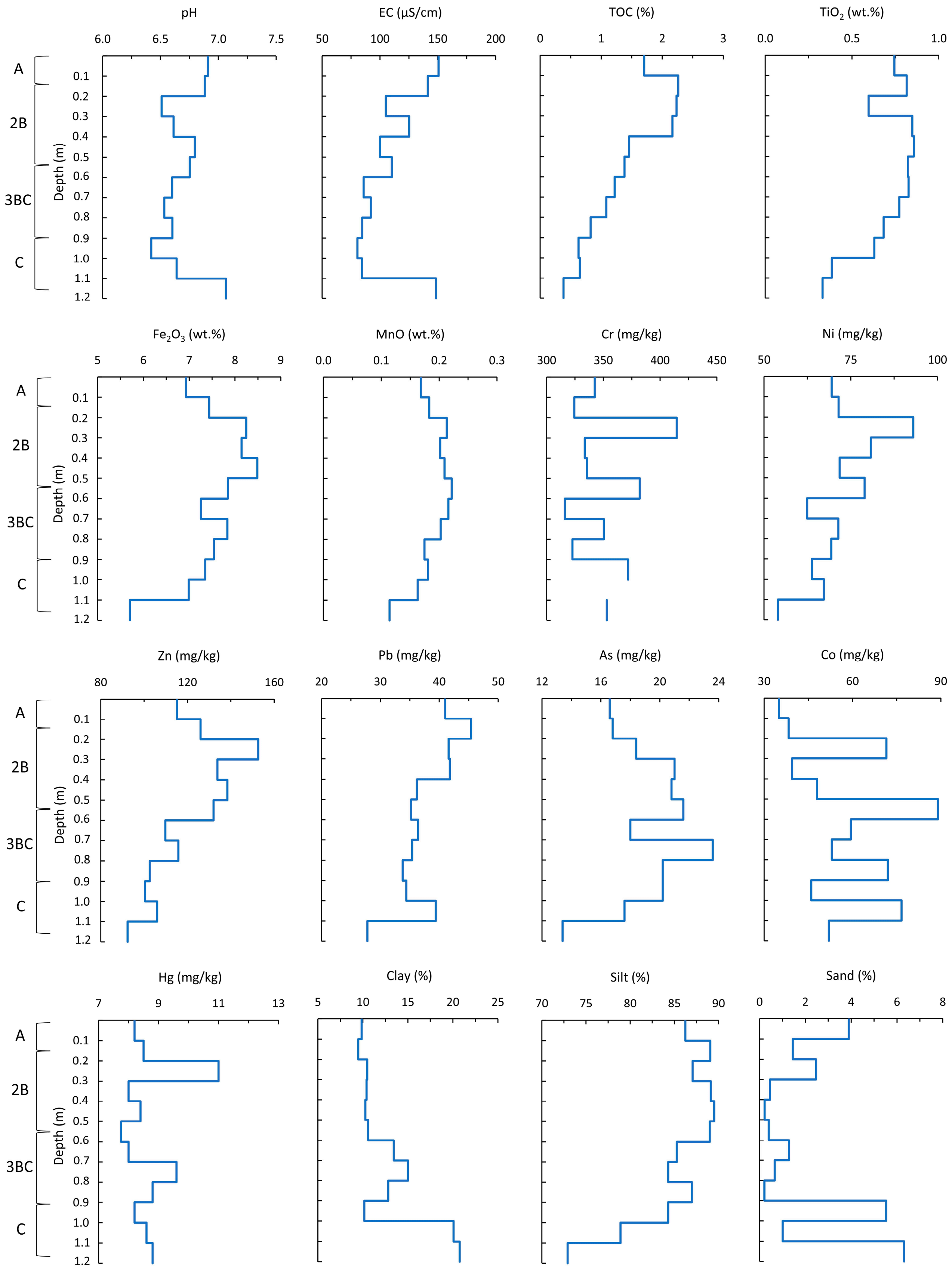


Figure 7. Distribution of pH, EC, TOC, TiO_2, Fe_2O_3, MnO, heavy metals, and soil texture of soil samples by depth.

4. Discussion

4.1. Nitrogen Species Distribution within the Soil Zone

Although the accumulation of NH_4^+ in soils is not common [3], NH_4^+ is the dominant N species in the 3BC soil horizon in the investigated soil profile. High concentrations of NO_2^- were detected in A and C soil horizon. In the first horizon, the rate of nitritation is faster than nitratation, resulting in the accumulation of NO_2^- [23,24]. NO_3^- increases with depth and, together with NO_2^-, represents the dominant species of N in the deepest soil horizon (Figure 3). Additionally, results suggest that N species distribution along soil profile depends on various factors.

Soil water content generally shows lower variability at greater soil depths [69,86], which is in line with the findings of this study. Given that soil tends to become dry and lose moisture during rainless and dry periods [87], the lowest soil moisture was observed for July, August, and September because those months were periods with precipitation below monthly average precipitation for this area. The highest concentration of NO_3^- in the C soil horizon, was observed in May, coinciding with a sudden increase in rainfall (Figure 2a) that led to enhancement of NO_3^- leaching [9]. Conversely, the lower NO_3^- concentrations observed in the first three soil horizons are a result of elevated water content, which in turn leads to oxygen limitation and reduced nitrification rate [9,28]. In contrast, the degree of denitrification in the deepest soil horizon is constrained by lower soil moisture levels, causing an increase in NO_3^- concentration [29]. Soil moisture through pedological profile has the highest mean value in the A horizon and lowest in the C horizon (Table S1). After the third soil horizon (3BC), soil moisture drops, likely due to reduced retention resulting from the presence of coarse-grained particles (Table 1).

According to Ayiti and Babalola [9], NO_2^- concentrations are higher during warmer months (June and July) when the temperature and microbial activity are higher, while there are no recorded concentrations higher than 0.2 mg/L during the coldest months (December, January, and February). NO_3^- concentrations are reduced during warmer months, particularly in the first three soil horizons, likely due to assimilation of NO_3^- through plants and denitrification process [11]. Considering that the pedological profile is situated in an area without potential anthropogenic sources of nitrate and that C soil horizon probably cannot retain the most of precipitation that infiltrates [69], which corresponds to different isotopic signature of precipitation and absence of variation in isotopic composition of soil water in C soil horizon within this research (Figure 4b), the examination of variation of the NO_3^-/Cl^- molar ratios in relation to Cl^- concentrations could be critical for the definition of nitrates produced by soil N nitrification. As shown in Figure 5, the highest values of molar ratios are observed in the C soil horizon with an average of 2.5 and minimum of 0.9 while the highest molar ratio in all other soil horizons is 0.8. This corresponds to research that has shown that higher NO_3^-/Cl^- molar ratios relative to Cl^- concentrations can suggest the occurrence of NO_3^- concentrations, which are the consequence of nitrification of soil N [2].

This corresponds to the evaluation of molar relationship of NO_3^-, NO_2^-, and Cl^- and soil water content. The observed low mNO_3^-/Cl^- ratio and high mNO_2^-/Cl^- ratio within the first soil horizon (A) could likely be attributed to an oxygen-deficient environment [61]. Under that condition, both nitrification and denitrification are relatively strong and NO_2^- is the intermediate product, resulting in a large amount NO_2^- accumulation. Within the C soil horizon, the mNO_3^-/Cl^- values are higher than mNO_2^-/Cl^- (Figure 6), under the relatively low soil moisture levels and higher oxygen content, leading to stronger nitrification.

4.2. Variability of Soil Water Parameters within the Soil Zone

The statistical results indicate that there are significant differences for soil moisture, δ^2H, $\delta^{18}O$, NO_2^-, NO_3^-, SO_4^{2-}, Na^+, NH_4^+, Mg^{2+}, and K^+, but show no significant differences across various sampling depths within the pedological profile for soil temperature, d-excess, F^-, Cl^-, PO_4^{3-}, and Ca^{2+} (Tables S4 and S5). Mg^{2+} across the first two, as well as along the third and fourth soil horizon, has a similar distribution. Soil moisture and

NO_3^- have identical distribution patterns across the first three soil horizons. The deepest soil horizon (C) stands out as the sole horizon with a distinct distribution for these two parameters. Deuterium, Na^+, and K^+ have a similar distribution within the first two and the last two soil horizons. Oxygen-18 has the same distribution pattern only within the first two soil horizons. NO_2^- across second and third, as well as within first and fourth horizons, has identical distribution. The only soil horizon within NH_4^+ that has a distinct distribution is the third one (3BC). The soil horizons with the same SO_4^{2-} distribution are the first and fourth.

4.3. Factors Influencing NO_3^- Distribution

Vertical distribution of NO_3^- content in the soil profile is influenced by pH [34], soil moisture [28], and organic carbon availability [20,38]. The distribution of soil pH and EC appears to be quite similar throughout the soil profile. Soil pH values increase with the soil depth, while on the other hand, organic carbon decreases with the soil depth [40]. This occurs mainly due to the higher OM in the topsoil (Table 1), which possibly leads to a pH reduction through the decomposition of OM [35,56]. In the first three soil horizons with lower pH values, the intensity of nitrification is reduced compared to the deepest soil horizon with a higher soil pH value [32–34]. Similar to recent research [9], the deepest soil horizon (C) with a pH above 7 has a prerequisite for optimum activity of ammonia and nitrite oxidizers, i.e., for nitrification. The NO_3^- is low in the first three soil horizons and then increases in C horizon, likely because of a decrease in the TOC content, as low organic carbon levels may increase the amount of NO_3^- [20,32,38]. The A horizon contains the highest value of OM (Table 1) which should contribute to faster water percolation and permeability [39]. Additionally, drastic reduction in soil water content can also have significant impact on the denitrification rates [88]. The same research also showed that soil nitrification can both decrease and increase depending on the soil water content. Soil nitrification increased with an increase in soil moisture when soil water content was less than approximately 27% and decreased with an increase in soil moisture if it was above 27%. Considering that clay content and the specific surface area of the soil are associated with hysteresis caused by the adsorbed water content in the soil [89], the soil horizon with the insignificant amount of clay, i.e., C soil horizon (Table 1), is characterized with the lowest soil moisture. It must be emphasized that maximum water content in C soil horizon did not exceed 25%, which also suggests more dominant occurrence of soil nitrification. Si and Kachanoski [90] and Zhang et al. [91] have shown that hysteresis effects can influence water transfer, microbial activities, as well as solute transport in soil. From that perspective it is important to investigate how and if nitrogen-related processes depend on the hysteresis effect. It was shown that the hysteresis effect can be different in multiple cycles of drying and wetting [92]. Furthermore, influence of soil shrinkage should also be investigated in future research because it is known that nitrogen-related processes depend on oxygen availability. Some research has shown that void ratio after soil shrinkage can have considerable influence on soil water characteristic curve [87]. These results suggest that both hysteresis and soil shrinkage can influence oxygen concentrations in soil, which can directly affect nitrogen transformation and the related processes. According to Six et al. [55], TOC contents are higher at sampling intervals where soil is rich in silt and poor in sand (2B and 3BC soil horizons), most likely due to silt particles which stabilize soil OM from being decomposed by microorganism. Conversely, reduced input of OM along C soil horizon, characterized by a higher presence of sand, tends to decrease TOC content. The presence of TiO_2, Fe_2O_3, and MnO [41,42,45] up to a depth of 0.9 m (Figure 7) could represent another important factor contributing to the reduction in nitrification rates. Almost all heavy metal concentrations exhibit lower concentrations in C horizon and, as mentioned, often negatively affect the nitrification rate and inhibit the activity of microorganisms [46,47], which can lead to an accumulation of NH_4^+ and reduction of NO_3^- in the soil within the first three soil horizons. Since accumulation of NH_4^+ in soils is typically uncommon [3], its presence at a certain depth indicates the existence of reducing conditions [8]. The highest accumulation of NH_4^+ is observed in the 3BC soil horizon, characterized by a notable presence of silt and clay, which also points to the existence of oxygen depleted conditions. Under such conditions, possibly both Fe and Mn act as electron acceptors resulting in the production of NO_3^- [44] and NO_2^- [43] as the end products in C soil horizon. Additionally, as noted by Varnier et al. [8], a significant amount of NH_4^+ in 3BC soil horizon could be evidence of incomplete nitrification. According to previous research, starting from a depth of 0.9 m (i.e., the C horizon), which is characterized by a significant presence of gravel, aerobic conditions may prevail and NO_3^- may accumulate.

5. Conclusions

This study used the characteristics of soil water and soil to evaluate the distribution and migration of N compounds at different soil profile depths. Statistical techniques were used to explore the significant variability of soil water parameters from different depths. It has been shown that soil moisture content, pH, TOC, and soil texture are important factors influencing the concentrations of N species within the soil zone. In addition, results reveal that all N species are present in soil water from all soil horizons. Moreover, the shallowest soil horizon has the highest NO_2^- concentrations, which suggest the dominance of nitritation. On the other hand, NH_4^+ is dominant in 3BC soil horizon, which indicates the oxygen-deficient environment of the soil zone, while in the deepest C soil horizon N is present mostly as NO_3^-, which suggests the dominance of nitrification under aerobic conditions. Considering that nitrates are very soluble and have leaching potential through soil zone, the aerobic conditions of the C soil horizon promote stability and the potential for NO_3^- transport. Additionally, it has been shown that NO_3^- concentrations are a result of nitrification of soil N, which is confirmed by the absence of precipitation isotopic signature and higher NO_3^-/Cl^- molar ratios when observing all soil horizons. Results also suggest that if NO_3^- are produced from the nitrification of the soil N within the Eutric Cambisols it should have a NO_3^-/Cl^- molar ratio higher than 1. Within the coarse-grained Eutric Cambisols, where anthropogenic sources of nitrate are not present, results suggest that soil nitrification of NH_4^+ is more common, while N primarily migrates to deeper parts of unsaturated zone in the form of NO_3^- and NO_2^-. The long-term goals include modelling of flow and N compounds transport within the soil and unsaturated zone, which is expected to enhance our comprehension of the entire aquifer system and facilitate the sustainable management of the Zagreb aquifer.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su152316529/s1, Table S1: Descriptive statistics of soil moisture content, soil temperature, isotopic, and chemical compositions for soil water samples; Table S2: The results of testing the assumptions of normality of soil moisture content, soil temperature, isotopic, and chemical compositions for soil water samples; Table S3: The results of testing the assumptions of homogeneity of soil moisture content, soil temperature, isotopic, and chemical compositions for soil water samples; Table S4: The result of the ANOVA test of soil temperature, PO_4^{3-}, Mg^{2+}, and Ca^{2+} within the four groups; Table S5: The result of the Kruskal–Wallis test of soil moisture, δ^2H, $\delta^{18}O$, d-excess, F^-, Cl^-, NO_2^-, NO_3^-, SO_4^{2-}, Na^+, NH_4^+, and K^+ within the four groups; Table S6: The result of Tukey HSD post hoc comparison between groups; Table S7: The result of the Mann–Whitney U post hoc comparison between groups.

Author Contributions: Conceptualization, P.B.; formal analysis, P.B.; investigation, P.B.; methodology, P.B.; writing—original draft, P.B.; visualization, P.B. and J.P.; supervision, J.P. and Z.K.; writing—review and editing, J.P., Z.K., T.B. and M.P. All authors have read and agreed to the published version of the manuscript.

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Paper 3: Tracing Nitrate Contamination Sources and Dynamics in an Unconfined Alluvial Aquifer System (Velika Gorica well field, Croatia)

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Tracing nitrate contamination sources and dynamics in an unconfined alluvial aquifer system (Velika Gorica well field, Croatia)†

Patricia Buškulić,[a] Zoran Kovač,*[a] Ioannis Matiatos[b] and Jelena Parlov[a]

Nitrate ions (NO_3^-) are one of the most common contaminants in the groundwater of the Zagreb alluvial aquifer, which hosts strategic groundwater reserves of the Republic of Croatia and supplies drinking water to one million inhabitants of the capital city. To better understand the origin and the dynamics of NO_3^- in the unsaturated and saturated zones, the stable isotopes of nitrogen ($\delta^{15}N$) and oxygen ($\delta^{18}O$) in dissolved nitrate, combined with physico-chemical, hydrogeochemical and water stable isotope data, were used in the current work, together with statistical tools and mixing models. The study involved monthly sampling of groundwater, surface water, precipitation and soil water samples. Additionally, the isotopic composition of total nitrogen ($\delta^{15}N_{bulk}$) was determined in solid samples representing the local nitrate sources. The combination of a nitrous oxide isotopic analyzer and the titanium(III) reduction method provides reliable measurements of $\delta^{15}N_{NO_3}$ and $\delta^{18}O_{NO_3}$, with optimal stability achieved under specific conditions. Nitrate in the study area predominantly originates from organic sources, with nitrification as the main biogeochemical process, while denitrification was identified at sampling sites under specific anaerobic conditions. Although statistical analysis can be a valuable tool, it should be applied with caution if NO_3^- originates from multiple sources. The isotopic composition of water showed that groundwater is predominantly recharged by the Sava River but its contribution varied spatially. The results also show the existence of a different recharge source in the southern part of the aquifer. Our findings highlighted the importance of employing a diverse range of analytical methods to obtain reliable and comprehensive understanding of nitrate contamination. By integrating multi-method approaches, stakeholders can better understand the complexities of groundwater contamination and implement more targeted measures to safeguard the water supplies for future generations.

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Environmental significance

Nitrate (NO_3^-) is a highly stable and mobile form of nitrogen (N) and one of the more commonly found contaminants in the environment. Tracing NO_3^- transformations and sources is essential for better understanding of N cycling and gaining insights into water quality protection. Furthermore, it is critical that we identify and understand the processes affecting N forms in the soil zone and groundwater system. We demonstrate how integration of physico-chemical, hydrogeochemical and isotopic data with statistical tools and mixing models can enhance our understanding of N cycling in the environment and improve water resource protection in regions facing similar environmental challenges.

Introduction

Dissolved nitrate (NO_3^-), which is one of the most prevalent forms of reactive nitrogen, is widespread in the environment and can originate from natural and/or anthropogenic sources.[1–5] Groundwater nitrate pollution is a global environmental problem[6–12] with increasing levels of NO_3^- in many freshwater systems.[2,13,14] The main anthropogenic sources of NO_3^- are sewage or industrial wastewaters, livestock manure and the intensive use of fertilizers.[15,16] Apart from anthropogenic NO_3^-, nitrate is also naturally produced as a result of the decay of soil organic matter, production–fixation and atmospheric deposition.[17] Tracing the sources and transformations of NO_3^- in groundwater, as well as in the soil zone, is important for an improved grasp of the nitrogen cycle, water quality protection and sustainable management of the aquifer.[18,19]

Nitrogen and oxygen stable isotopes of NO_3^- ($\delta^{15}N_{NO_3}$ and $\delta^{18}O_{NO_3}$) are powerful tracers for identifying the source(s) of nitrogen (N) contamination, as well as N transformations and

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dynamics in aquatic and atmospheric systems.[16,20–25] Although nitrate isotopes are a valuable tool for tracking NO_3^- contaminants, the nitrate sources may show overlapping isotopic ranges and biogeochemical processes like nitrification and denitrification can complicate the identification of nitrate sources.[26] Biogeochemical processes predominantly occur in shallow aquifers, influencing nitrate content and $\delta^{15}N_{NO_3}$ values. The nitrification process involves the oxidation of NH_4^+ to NO_3^-.[27] Denitrification generally occurs under anaerobic conditions and involves the reduction of NO_3^- to N_2, N_2O or NO.[28] Denitrification is a natural attenuation process that reduces NO_3^- concentrations and produces a linear relationship in the residual NO_3^- with a $\delta^{18}O_{NO_3}/\delta^{15}N_{NO_3}$ ratio which ranges from 1 : 2.1 to 1 : 1.3.[29] To improve the application of nitrate isotope techniques, the use of additional parameters, such as physico-chemical data and water stable isotopes is considered important.

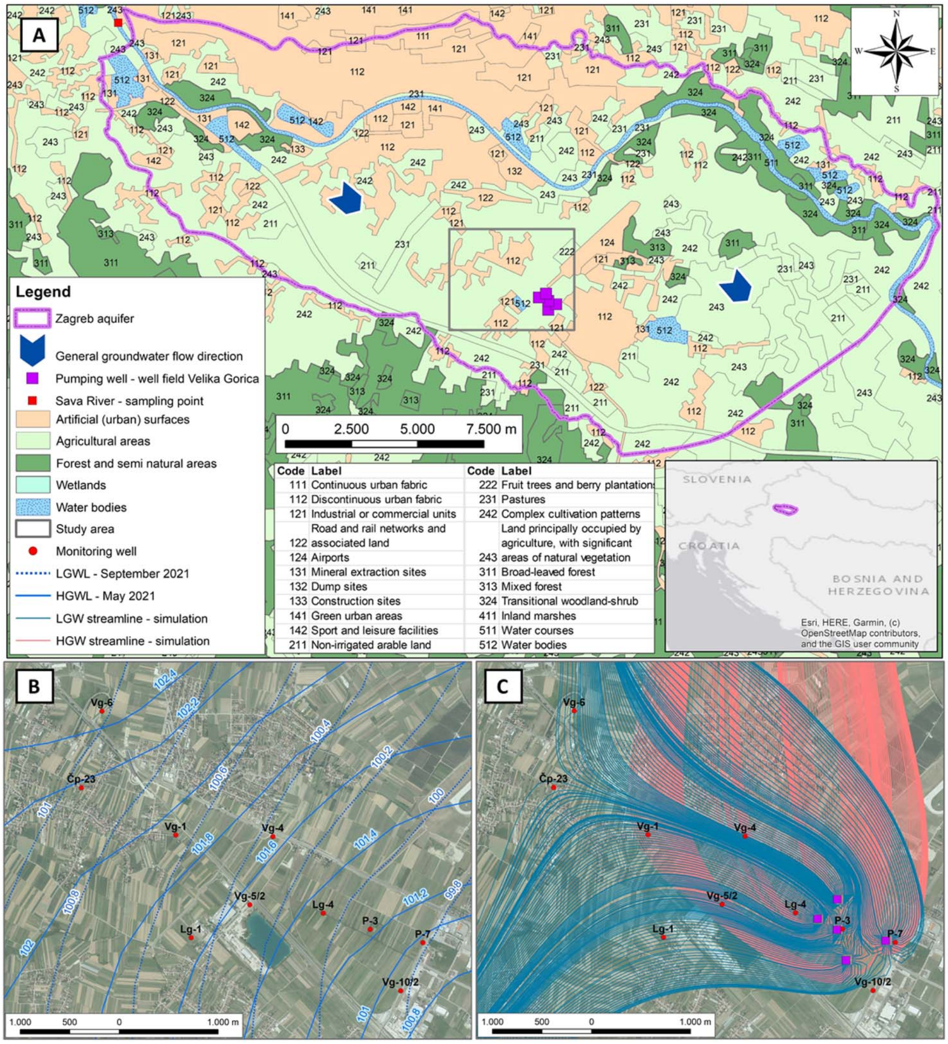
The Zagreb aquifer is one of the most important aquifer systems in Croatia with high to very high vulnerability to contamination as evidenced by the presence of five major contaminants (toxic metals, nitrates, pesticides, pharmaceuticals and chlorinated aliphatics),[30] as well as elevated NO_3^- concentrations in the groundwater, particularly near industrial and agricultural activity zones.[31] A preliminary assessment of nitrate contamination origin in the groundwater of the Zagreb alluvial aquifer showed that NO_3^- is predominantly of organic origin, particularly from wastewaters.[32]

Previous studies in the groundwater of the Zagreb area did not focus on the investigation of nitrate ion distribution and migration through the soil zone (unsaturated zone), nitrate concentrations in the precipitation and Sava River, as well as the biogeochemical and hydrodynamic processes controlling NO_3^- content and dynamics in the aquifer. To determine both the origin and the dynamics of nitrate, it is necessary to measure stable isotopes $\delta^{15}N$ and $\delta^{18}O$ in dissolved nitrate in different types of water samples, which were evaluated within this research. However, previous study related to nitrate origin in groundwater of the Zagreb aquifer[32] provided initial insights into the nitrate isotopic composition. This new research area was defined based on initial findings, which suggested an additional agricultural source beyond wastewater, primarily on the Sava River's right bank near the Velika Gorica well field. Furthermore, it is also essential to conduct an analysis of the isotopic composition of total nitrogen ($\delta^{15}N_{bulk}$) in the local solid samples of different potential nitrate sources if contribution from different nitrate sources has to be evaluated. Therefore, field and laboratory investigations included sampling and analysis of groundwater, surface water, precipitation and soil water. Here, we used physico-chemical, hydrogeochemical and isotopic data, together with statistical tools and mixing models to identify the origin of nitrate contamination and the conditions influencing the N dynamics. This was supported by the first determination of the nitrogen isotopic composition of the local sources around the Velika Gorica well field. There were four main objectives of this research: (1) to optimize laboratory procedures necessary to get reliable results using a nitrous oxide isotopic analyzer and the titanium(III) reduction method; (2) to define nitrate origin and associated biogeochemical processes as well as quantification of spatial and temporal proportional contributions from different N sources; (3) to define appropriate geochemical and statistical methods for interpretation in similar research; (4) to quantify aquifer recharge components. Our study provided new insights into the processes of nitrogen compound transformation in the soil zone, the identification of the dominant form of N entering the unsaturated zone of the aquifer from the soil, determination of the characteristic isotopic composition of N compounds appearing in the surface water, soil water and precipitation, and detailed quantification of nitrate sources which influence nitrate concentrations in the aquifer, with the aim of better understanding the nitrogen cycle to support sustainable management and protection of the Zagreb aquifer.

Methodology

Site description

The Zagreb aquifer is located in the north-western part of the Republic of Croatia within the Sava River catchment and covers an area of about 350 km^2 (Fig. 1A). It is an unconfined aquifer composed of quaternary sediments consisting mainly of sand, gravel and silt or silty clays. It comprises two hydraulically connected layers: (1) a shallow layer of Holocene alluvial deposits, primarily composed of a carbonate material, transported by the Sava River from the Alps and (2) a deep layer of Pleistocene lacustrine-marshy deposits, primarily composed of a siliciclastic material from the surrounding mountains.[33] The thickness of the aquifer varies from less than 10 meters in the western part up to 100 meters in the eastern part. The unsaturated zone comprises gravel in its lower section, with the upper part predominantly composed of silty to sandy materials, occasionally interspersed with clay layers. The thickness of the unsaturated zone varies from 2 to 11 meters.[34] In general, groundwater flows from west/northwest to east/southeast and coincides with the flow direction of the Sava River. The shallow layer is in direct hydraulic connection with the Sava River, which is the main source of groundwater recharge.[35,36] During high water levels, the Sava River recharges the aquifer along its entire course, while during medium and low water levels, it drains the aquifer in certain sections. The study area is characterized by three major pedological units, namely Fluvisols, Stagnosols and Eutric Cambisols on the Holocene deposits.[37,38] According to Ružičić *et al.*,[39] Fluvisols have higher permeability due to their lower clay content and higher sand content, which increases with depth. In contrast, Eutric Cambisols exhibit lower permeability and a slightly higher capacity for water retention. The areas where Fluvisols dominate allow for greater percolation of surface and soil water from precipitation. This is confirmed by the most recent study[40] in the zone of the Eutric Cambisols, which showed that only high average intensity precipitation events enable infiltration to the unsaturated part of the aquifer. The climate is classified as a humid continental climate, with an annual average precipitation of 967 mm and annual average air temperature of 11.9 °C, with a minimum in January and a maximum in July. The monthly average



Code	Label	Code	Label
111	Continuous urban fabric	222	Fruit trees and berry plantation
112	Discontinuous urban fabric	231	Pastures
121	Industrial or commercial units	242	Complex cultivation patterns
122	Road and rail networks and associated land	243	Land principally occupied by agriculture, with significant areas of natural vegetation
124	Airports	311	Broad-leaved forest
131	Mineral extraction sites	313	Mixed forest
132	Dump sites	324	Transitional woodland-shrub
133	Construction sites	411	Inland marshes
141	Green urban areas	511	Water courses
142	Sport and leisure facilities	512	Water bodies
211	Non-irrigated arable land		

Fig. 1 (A) The location of the study area with the land use pattern; (B) the water table contour map of the study area during low and high groundwater levels, showing equipotential lines in meters above sea level (m a.s.l.); (C) groundwater flow lines during low and high water levels assuming a maximum pumping capacity (250 L s^{-1} per well).

precipitation is around 80 mm and is evenly distributed throughout the year.[41] For a sampling period from March 2021 to February 2022, the average monthly precipitation was 55.7 mm. The driest and the wettest months were September (29.8 mm) and May (102 mm), respectively. The average air temperature ranged from 1.2 (January) to 23.3 °C (July) with an average of 11.6 °C.[42]

The Velika Gorica well field is one of the most important well fields in Zagreb County and is situated in the southern part of the Zagreb aquifer. It supplies drinking water to the capital city and the town of Velika Gorica. The well field consists of five pumping wells and observation wells generally used to monitor groundwater quality in the shallow aquifer. Within the first sanitary protection zone of the Velika Gorica well field, a pedological pit was constructed and equipped with devices for collecting soil water samples. The unsaturated zone thickness at the study site usually ranges from 5 to 8 meters and depends on the groundwater levels. At the top of the unsaturated zone, the following soil horizons are identified according to the World Reference Base classification: A (0–0.15 m), 2B (0.15–0.55 m), 3BC (0.55–0.9 m) and C (0.9–1.17 m). The upper 90 centimetres of the soil profile are predominantly composed of silty and sandy materials, with intermittent clay layers,[39] while gravels dominate in the lower part of the unsaturated zone.[43] According to Bogunović *et al.*[37] the pedological pit is located in Eutric Cambisols on Holocene deposits. The monitoring wells Vg-6, Lg-1 and Vg-5/2 are located in the area of Fluvisols, while all other monitoring wells are located on Eutric Cambisols on the Holocene deposits.

Based on previous research,[44–46] the groundwater in the wider area of the Velika Gorica well field belongs to the calcium–magnesium–hydrogen carbonate ($CaMgHCO_3$) water type. The land-uses, which constitute the potential sources of contamination, are presented in Fig. 1A.[47] The study area consists primarily of urban and agricultural land. The general direction of the groundwater flow in the study area is from north-west to south-east, with clear differences during low and high water level periods (Fig. 1B). During the high water level periods, the groundwater flows towards the south in the northern part of the study area, which is due to the increased influence from the Sava River. In addition, the pumping activities in the Velika Gorica well field also impact the groundwater flow direction near the pumping wells. By developing a groundwater flow model, Posavec[48] identified a change in the groundwater flow direction during low and high water level periods for a scenario with the maximum pumping capacity (Fig. 1C).

Sampling

Groundwater, surface water, precipitation and soil water were sampled each month from March 2021 to February 2022. Groundwater samples were collected from 10 monitoring wells, located in the catchment area of the Velika Gorica well field (Fig. 1A). Before collecting the groundwater samples, groundwater levels were manually measured while monitoring wells were pumped until pH and electrical conductivity (EC) stabilized to ensure collection of the representative groundwater sample. Surface water samples were collected from the Sava River at the western part of the Zagreb aquifer. The groundwater and Sava River samples were to be analysed for water chemistry, water stable isotopes and nitrate stable isotopes. *In situ* measurements included pH, EC, dissolved oxygen (DO) content and temperature (T) which were determined *in situ* using a WTW multi parameter 3630 IDS. Precipitation samples were collected within the first sanitary protection zone of the Velika Gorica well field using a Palmex Rain Sampler RS1 (Zagreb, Croatia) capable of preventing evaporation and appropriate for use in most hydrological studies.[49,50] For the precipitation samples, *in situ* parameters and nitrate stable isotopes were also determined. Soil water samples were collected from four soil horizons of a pedological pit using soil water samplers (suction cups; Eijkelkamp Soil & Water, Giesbeek, The Netherlands) and an integrated automatic vacuum pump unit AVP-100 (UGT GmbH, Müncheberg, Germany) to determine the isotopic composition of nitrate. The chemical and water isotopic data of soil water and precipitation were previously published.[42] Each liquid sample was filtered in the field using a 0.22 μm nylon membrane filter to remove microbes, stored in a high-density polyethylene (HDPE) bottle and transferred to the lab for later analysis. The sample bottles were kept refrigerated at 4 °C for chemical and water isotope analysis or frozen at −20 °C for nitrate isotope analysis. During September 2021, ten (10) samples of nitrogen sources were collected in the study area for nitrate isotope analysis: six organic samples and four widely used commercial inorganic fertilizers. The organic samples included two manure samples, two samples from septic tanks and two sewage samples. The selected synthetic fertilizers were NP (nitrogen–phosphorus) 20–20, NPK (nitrogen–phosphorus–potassium) 15–15–15, NPK(S) (nitrogen–phosphorus–potassium–sulphur) 15–15–15(3) and NPK(MgO) (nitrogen–phosphorus–potassium–magnesium oxide) 13–10–12(4), in which most of the nitrogen is present as ammoniacal nitrogen. The isotopic signature of soil was defined based on the sampled soil water from the deepest soil horizon. A table summarizing the type of sample, location, number of sampling points, frequency, sampling period, type of treatment and analyses can be found in the ESI (Table S1†).

Chemical analysis

The major anion and cation concentrations (Cl^-, NO_3^-, SO_4^{2-}, Na^+, Mg^{2+}, K^+ and Ca^{2+}) were determined with a Dionex ion chromatography system (ICS-90) at the LaGEMA laboratory of the Faculty of Mining, Geology and Petroleum Engineering, University of Zagreb. The ionic balance error was verified for all groundwater samples, with error values below 10% considered acceptable. The bicarbonate concentrations were determined using the titration method.

Water stable isotope analysis and mixing model

The water isotopic analyses ($\delta^2H_{H_2O}$ and $\delta^{18}O_{H_2O}$) were carried out at the Laboratory for spectroscopy of the Faculty of Mining, Geology and Petroleum Engineering, University of Zagreb, using a liquid water isotope analyzer (LWIA-45-EP, Los Gatos Research, San Jose, California) by laser absorption spectroscopy. The analytical uncertainty of duplicate samples was ±0.9‰ for δ^2H and ±0.19‰ for δ^{18}O. The results were reported using the delta notation in per mil (‰) relative to Vienna Standard Mean Ocean Water (VSMOW). The data analysis and processing were performed using the Laboratory Information Management System (LIMS) for Lasers 2015.[51]

To quantify the contribution of precipitation and the Sava River to the aquifer recharge, a two-component mixing model was utilized which has been used in the study area in previous research,[36] but also in different hydrogeology applications.[52–55] In this research it was assumed that the aquifer recharge comes from two main sources, *i.e.*, the Sava River and precipitation. The sum of the end member contributions is expressed as fractions (f) which are equal to 1. For the quantification of recharge, average values from the sampling period for each monitoring well, the Sava River and precipitation were used, based on the following equations:

$$f_{\text{river}} + f_{\text{precipitation}} = 1 \tag{1}$$

$$f_{\text{river}} \cdot \delta^{18}O_{\text{river}} + f_{\text{precipitation}} \cdot \delta^{18}O_{\text{precipitation}} = \delta^{18}O_{\text{groundwater}} \tag{2}$$

where f_{river} and $f_{\text{precipitation}}$ present Sava River and precipitation fractions respectively, $\delta^{18}O_{\text{groundwater}}$ presents isotopic composition of oxygen in the investigated monitoring well, while $\delta^{18}O_{\text{river}}$ and $\delta^{18}O_{\text{precipitation}}$ present isotopic compositions of oxygen in the Sava River and precipitation, respectively.

Nitrate stable isotope analysis

Instrumentation and data processing. The isotope analyses of $\delta^{15}N_{NO_3}$ and $\delta^{18}O_{NO_3}$ were performed using an ultrahigh precision advanced nitrous oxide isotopic EP analyzer (GLA451-N2OI3, ABB-LGR, Quebec, Canada) at the Laboratory for spectroscopy of the Faculty of Mining, Geology and Petroleum Engineering, University of Zagreb. The analyzer precision was

0.05 ppb for N_2O concentration, better than 1‰ for $\delta^{15}N$ and better than 2‰ for $\delta^{18}O$. To convert dissolved NO_3^- to N_2O gas headspace the titanium(III) reduction method was used, first introduced and described in detail by Altabet *et al.*[56] After the analysis, the data was pre-processed by using an in-house MATLAB[57] script. This MATLAB script is designed to process multiple TXT files containing raw laser measurements by iterating throughout laser output TXT files and calculating mean values for essential data. This streamlined approach facilitates effective data processing and management, enabling further analysis. Subsequently, a new CSV file is created for each input file, containing the calculated mean values. A GRG nonlinear solving method in Excel Solver™[58] was employed to correct the raw instrumental data for isotopologues of interest (^{15}N and ^{18}O) to remove any concentration dependence in the δ values.

Testing and validation. To ensure laboratory setup functionality and reliability before research activities, optimization steps were conducted, *i.e.*, target N_2O gas concentration inside the analyzer, reagent-to-sample ratio during sample preparation and reaction time. The testing of the optimal N_2O target that produced the most stable isotopic values was conducted using a control sample KNO_3 with different final NO_3–N sample concentrations injected into the analyzer at various injection volumes, ensuring that a broad range of N_2O concentrations were covered. N_2O concentrations ranging from 7 to 11 ppm N_2O exhibited the most stable isotopic values ($\delta^{15}N$ and $\delta^{18}O$) with optimal precision (Fig. 2A). Instrumental $\delta^{15}N$ and $\delta^{18}O$ values were more precise using a 1 : 30 reagent-to-sample ratio with a mean s.d. of 1.9‰ and 1.7‰, respectively (Fig. 2B). The observed calibration slopes for $\delta^{15}N$ were close to the theoretical value of 1 regardless of which reagent-to-sample ratio was used during sample preparation. However, the calibration plots generated with a reagent-to-sample ratio of 1 : 20 gave a higher slope for $\delta^{18}O$, *i.e.*, 0.9 (Fig. 2C). The $\delta^{15}N$ values were more accurate at a 1 : 40 reagent-to-sample ratio with a mean bias of −0.07‰, while a ratio of 1 : 20 resulted in a higher mean bias of 0.3‰. Conversely, the $\delta^{18}O$ normalised values were most accurate at a 1 : 20 ratio with a mean autorun bias of 0.05‰ (Fig. 2D). Therefore, a 1 : 20 Ti(III) reagent-to-sample ratio is considered practical for all further measurements. Altabet *et al.*[56] noted that a 96-hour reaction yielded similar outcomes to a 21-hour reaction, suggesting that samples can be stored for at least 3 days before analysis without adverse effects. Our study yielded slightly different results (Fig. 2E), as both the 48-h and 72-h reaction yielded isotopic results comparable to those of a 24-h reaction, while the control sample left for longer than 72 hours did not yield acceptable results.

Standards and samples. Groundwater and surface water nitrate samples were prepared with the Ti(III) reduction method to achieve a NO_3^- sample concentration of 0.55 $mg_{NO_3\text{–}N}\ L^{-1}$ in each vial in order to get the target N_2O concentration inside the analyzer using a reagent-to-sample volume ratio of 1 : 20. Due to insufficient NO_3^- levels in two samples, the stable isotope composition of the nitrate was investigated for 10 precipitation samples. Additionally, due to very small sample volume amounts available for the Ti(III) preparation method and insufficient NO_3^- levels, the stable isotope composition of the NO_3^- was analysed for eight soil water samples. Due to the lower concentration of NO_3^-, the precipitation and soil water samples were prepared to achieve a NO_3^- sample concentration of 0.1 $mg_{NO_3\text{–}N}\ L^{-1}$.

In alignment with Altabet *et al.*[56] and in order to avoid non-representative results, an overnight reaction was chosen to be convenient and practical, allowing samples to be prepared on day 1 and analysed the following morning (day 2). Nitrate reference materials (USGS32, USGS34 and USGS35) were used for data normalisation to the AIR and VSMOW scales by applying least squares regression to the measured *versus* consensus δ values. Nitrate standards have consensus $\delta^{15}N_{AIR}$ values of +180‰ (exactly), −1.8‰ (±0.1‰) and +2.7‰ (±0.1‰) and $\delta^{18}O_{VSMOW}$ values of +25.7‰ (±0.2‰), −27.9‰ (±0.3‰) and +57.5‰ (±0.3‰), respectively.[59,60] Normalized results were least squares regression mean values ±1-sigma.

Solid nitrate sources. Ten solid samples of potential nitrate sources were dried and ground to homogenize them. The isotopic composition of total nitrogen ($\delta^{15}N_{bulk}$) was investigated by an external laboratory (The Faculty of Earth Sciences of the University of Barcelona) by using an Elemental Analyzer (EA) Flash IRMS coupled in continuous flow to an Isotope Ratio Mass Spectrometer (IRMS) Delta V Advantage with a universal interphase Conflo IV (CF) (EA IsoLink CN IRMS System, Thermo Scientific). Isotope ratios are reported in parts per mil (‰) relative to the international standard V-AIR for total nitrogen ($\delta^{15}N_{bulk}$). The precision was <±0.5%.

Statistical data analysis

The normality of 17 parameters (pH, EC, DO, T, Cl^-, NO_3^-, SO_4^{2-}, HCO_3^-, Na^+, Mg^{2+}, K^+, Ca^{2+}, $\delta^{15}N_{NO_3}$, $\delta^{18}O_{NO_3}$, $\delta^2H_{H_2O}$, $\delta^{18}O_{H_2O}$ and d-excess) in the groundwater samples was assessed using the Shapiro–Wilk test. To evaluate temporal and spatial differences, normally distributed data were analysed using one-way ANOVA at a significance level of *p*-value <0.05. The test evaluated changes over time (from March 2021 to February 2022) and changes among sampling sites. If the normality assumption was not met, the non-parametric Kruskal–Wallis test was applied.

Correlation tests were conducted on the monitoring well data to examine the relationship between Cl^-, NO_3^-, SO_4^{2-}, HCO_3^-, Na^+, Mg^{2+}, K^+ and Ca^{2+} in groundwater, using a significance level of *p*-value <0.05. All statistical analyses were performed using the TIBCO Software Inc. Statistica (Version 13.5.0.17).

Bayesian mixing model and uncertainly analysis

The proportional contribution of 3 nitrogen sources in the groundwater and the river was estimated *via* the application of a Bayesian isotope mixing model using the package *simmr* in R (Stable Isotope Mixing Model in R).[61,62] In brief, the model is expressed by using the following equations (eqn (1)–(4);[63]):

$$X_{ij} = \sum_{k=1}^{K} p_k \left(S_{jk} + C_{jk}\right) + \varepsilon_{ij} \tag{3}$$

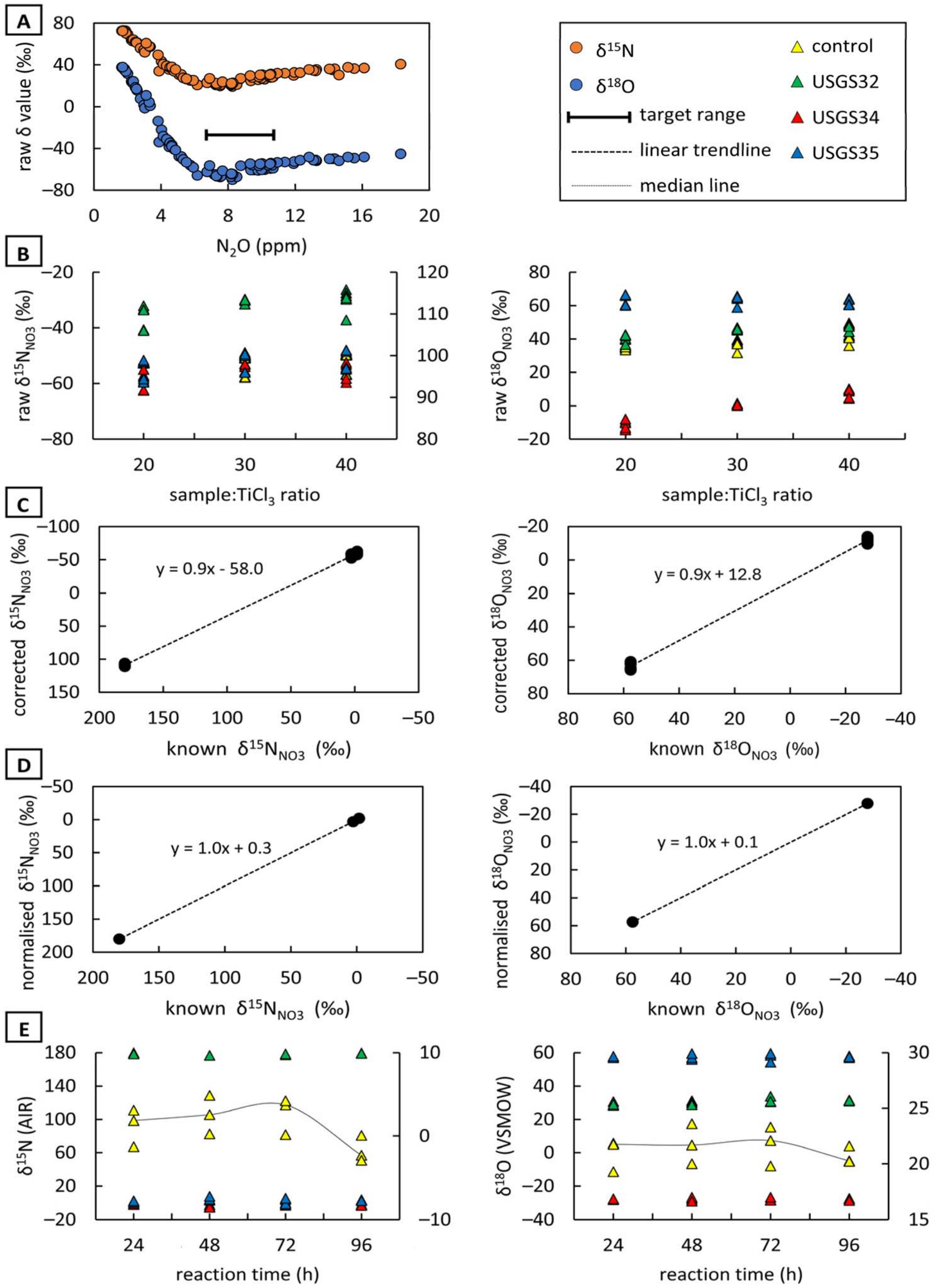


Fig. 2 Optimization steps taken to ensure laboratory setup functionality and reliability before initiating research activities include: (A) establishing the target N$_2$O gas concentration; (B)–(D) determining the reagent-to-sample ratio during the preparation method; (E) establishing the reaction time for optimal results. Note: USGS32 in (B) and the control sample in (E) are presented using the secondary axis.

$$S_{ij} \sim N(\mu_{jk}, \omega_{jk}^{2}) \tag{4}$$

$$C_{jk} \sim N(\lambda_{jk}, \tau_{jk}^{2}) \tag{5}$$

$$\varepsilon_{jk} \sim N(0, \sigma_{j}^{2}) \tag{6}$$

where X_{ij} is the isotope value j of the mixture i, in which $i = 1, 2, 3, \ldots, N$ and $j = 1, 2, 3, \ldots, J$; p_k is the proportion of source k ($k = 1, 2, 3, \ldots, K$) estimated by the model; S_{jk} is the source value k on isotope j normally distributed with mean μ_{jk} and standard deviation ω_{jk}; c_{jk} is the isotope fractionation factor for isotope j on source k normally distributed with mean λ_{jk} and standard deviation τ_{jk}; and ε_{ij} is the residual error of the additional unquantified variation between individual mixtures normally distributed with mean 0 and standard deviation σ_j.

The model was applied at each sampling site to identify spatial differences in proportional contributions, as well as across different seasons for each water type (groundwater and river). The isotopic average and standard deviation values of the 3 local nitrogen sources in the study area (soil, synthetic fertilizers and organic wastes) were used in the analysis and are shown in Table 1, while isotopic ranges are shown in Fig. 6 and were the following: soil (δ^{15}N from −7.1 to −4.9‰, δ^{18}O from −9.1 to −4.4‰); synthetic fertilizers (δ^{15}N from 0.5 to 1.5‰, δ^{18}O from −9.6 to −8.6‰); organic wastes from sewage, septic waste and manure (δ^{15}N from 1.9 to 8.8‰, δ^{18}O from −9.6 to −8.6‰); and precipitation (δ^{15}N from −15.1 to 2.2‰, δ^{18}O from 62.1 to 94.7‰). The δ^{15}N values of the local solid sources were within expected ranges.[64]

We excluded precipitation from the analysis, as the N isotope precipitation signal is quickly dampened.[64] For the sampling sites (*i.e.*, Lg-1 and Lg-4) where denitrification was identified, since they fall within the optimal denitrification zone due to low DO levels[3] and have characteristic slopes associated with denitrification,[29] an enrichment factor was calculated using the following equation:[27]

$$\delta \sim \delta_o + \varepsilon \ln(f) \tag{7}$$

where δ_o is the initial isotopic composition of the substrate, δ is the isotopic composition of the substrate, ε is the enrichment factor and f is the remaining fraction of the substrate. The average $\delta^{15}N_{NO_3}$ value of the local sources (+1.5‰) was considered to be the initial isotopic composition.

Synthetic fertilizers were mainly NH_4^+ based, which allowed determining only the δ^{15}N value, whereas the δ^{18}O value of this endmember was determined by considering the $\delta^{18}O_{H_2O}$ values of the local groundwater *via* nitrification. In general, the nitrification of NH_4^+ in soil, precipitation or fertilizers utilizes two oxygens from water and one from atmospheric oxygen.[27,65] Thus, nitrification values may be estimated using the following experimental equation:[65,66]

$$\delta^{18}O_{NO_3} = \frac{2}{3}\delta^{18}O_{H_2O} + \frac{1}{3}\delta^{18}O_{O_2} \tag{8}$$

The soil signature was defined based on measured $\delta^{15}N_{NO_3}$ and $\delta^{18}O_{NO_3}$ data from soil water samples collected from the deepest soil horizon (C horizon) that was closest to the water table.

An uncertainty analysis of isotopic composition for each nitrogen source was conducted using the probability statistical method introduced by Ji *et al.*[67] In summary, the proportional contribution values from the *simmr* model for each potential nitrogen source were assigned frequency values based on the number of iterations. A plot of the cumulative frequency distribution *versus* the proportional contributions of the potential nitrogen sources was then created. The uncertainty index (UI90) for each nitrogen source was calculated by finding the difference between the proportional contributions at the 0.95 and 0.05 cumulative frequency distributions and then dividing this difference by 0.9.[68]

Results and discussion

Evaluation of nitrate origin and the processes affecting nitrogen species concentrations is complex and requires comprehensive analysis. Effective data interpretation necessitates the integration of various analytical approaches and perspectives. This approach is particularly crucial in areas where nitrate concentrations arise from a combination of anthropogenic sources and natural environmental conditions.[1–4] Diverse data and methods enhance reliability, with isotopic signatures and the composition of main nitrate sources being critical. The results are discussed through hydrogeochemistry, statistical analysis, isotopic composition and Bayesian mixing models, contextualized for both urban and agricultural environments.

Hydrogeochemical aspects

The pH values ranged from 6.9 to 7.8 for groundwater, from 7.8 to 8.7 for Sava River water and from 6.5 to 8.1 for precipitation. The groundwater EC varies spatially from 675 to 938 μs cm^{-1} with the lowest EC values observed at Lg-1 and Vg-10/2 and the highest at Vg-4. Surface water EC varies between 353 and 461 μs cm^{-1}, while precipitation EC ranged from 12.1 to 54.5 μs cm^{-1}. The DO content of the groundwater ranged from 0.6 to 9.0 mg_{O_2} L^{-1}; in the Sava River it varied between 7.1 and 14.1 mg_{O_2} L^{-1}, while in the precipitation it varied between 7.2 and 13.9 mg_{O_2} L^{-1}. The lowest groundwater DO values were observed at Lg-1 and Lg-4 sites with a mean value of 3.4 mg_{O_2} L^{-1} and 3.6 mg_{O_2} L^{-1}, respectively. Groundwater temperatures varied between 12.4 and 15.0 °C, while river water temperatures ranged from 7.2 to 25.0 °C. Higher temperatures were recorded at Vg-4 and Vg-6 sites, while the lowest temperatures were recorded at the Vg-10/2 location.

The NO_3^- concentrations in groundwater ranged from 7.4 to 31.9 mg_{NO_3} L^{-1} (Table S2†) with an average value of 17.1 mg_{NO_3} L^{-1}. None of the analysis exceeded the maximum permissible level for drinking water (50 mg_{NO_3} L^{-1}) as stipulated by EU and Croatian regulations. Elevated mean NO_3^- concentrations were found at Vg-10/2 (20.3 mg_{NO_3} L^{-1}) and Vg-5/2 monitoring wells (25.1 mg_{NO_3} L^{-1}), which was linked to fertilizers and wastewater, respectively. The NO_3^- concentrations in the Sava River ranged from 3.5 to 6.5 mg_{NO_3} L^{-1}. The lower NO_3^- concentrations in the Sava River compared to groundwater could be because of biogeochemical processes, such as assimilation (NO_3^- consumption by plants) and dilution with rain water. Given that the ambient background value

Table 1 Isotopic average values of the local nitrogen sources used in the mixing model

	δ^{15}N (‰)	δ^{18}O (‰)
Soil ($N = 3$)	−5.8 ± 0.9	−6.3 ± 2.0
Synthetic fertilizers ($N = 4$)	1.1 ± 0.4	−9.0 ± 0.3
Organic wastes (sewage, septic waste and manure) ($N = 6$)	3.7 ± 2.3	−9.0 ± 0.3

of NO_3^- of the study area is around 7 mg_{NO_3} L^{-1} (ref. 41) and the expected NO_3^- content in the soil zone is similar,[42] the observed nitrate concentrations in the groundwater cannot be attributed only to natural conditions, *i.e.*, sources. This is evident as the lowest recorded concentrations were around 9 mg_{NO_3} L^{-1} in all groundwater samples apart from the Lg-4 site in August, where the NO_3^- concentration was 7.4 mg_{NO_3} L^{-1}. Most NO_3^- concentrations generally exceeded natural background levels.

Considering that monitoring wells Lg-1 and Lg-4 generally had DO levels lower than 4 mg_{O_2} L^{-1}, they were in the zone optimal for the denitrification process (Fig. 3A). Conversely, all other groundwater sampling sites had DO levels greater than 4 mg_{O_2} L^{-1} and a pH range between 6.5 and 8, indicating that the measured NO_3^- concentrations could be a result of nitrification under aerobic conditions.[3] The Sava River has a slightly higher pH values, with a maximum recorded value of 8.7 in January 2022.

The lowest mean SO_4^{2-} concentrations were observed in Lg-1 (13.5 mg L^{-1}), P-7 (13.1 mg L^{-1}) and Vg-10/2 (14.0 mg L^{-1}) sites. Conversely, the Lg-4 and Vg-1 sites showed the highest content of sulphate with an average value of 23.1 and 23.3 mg L^{-1}, respectively. The content of SO_4^{2-} in the Sava River ranged from 6.3 to 17.0 mg L^{-1}.

The Cl^- concentrations in groundwater varied from 7.6 to 71.6 mg L^{-1}, with an average of 26.9 mg L^{-1}. The levels of Cl^- in the Sava River were significantly lower, from 5.9 to 12.4 mg L^{-1}. The highest Cl^- levels were observed at the monitoring wells Vg-4 and Vg-6, with average values of 55.6 mg L^{-1} and 37.2 mg L^{-1}, respectively. In contrast, the lowest average Cl^- concentrations were found at Lg-1 (11.1 mg L^{-1}) and Vg-10/2 (10.1 mg L^{-1}) sites.

The concentration of K^+ in groundwater ranged from 0.9 to 6.0 mg L^{-1} with an average of 2.2 mg L^{-1}. The highest concentrations of K^+ were detected at the monitoring wells Vg-5/2 and Vg-6 with average values of 4.8 and 3.7 mg L^{-1},

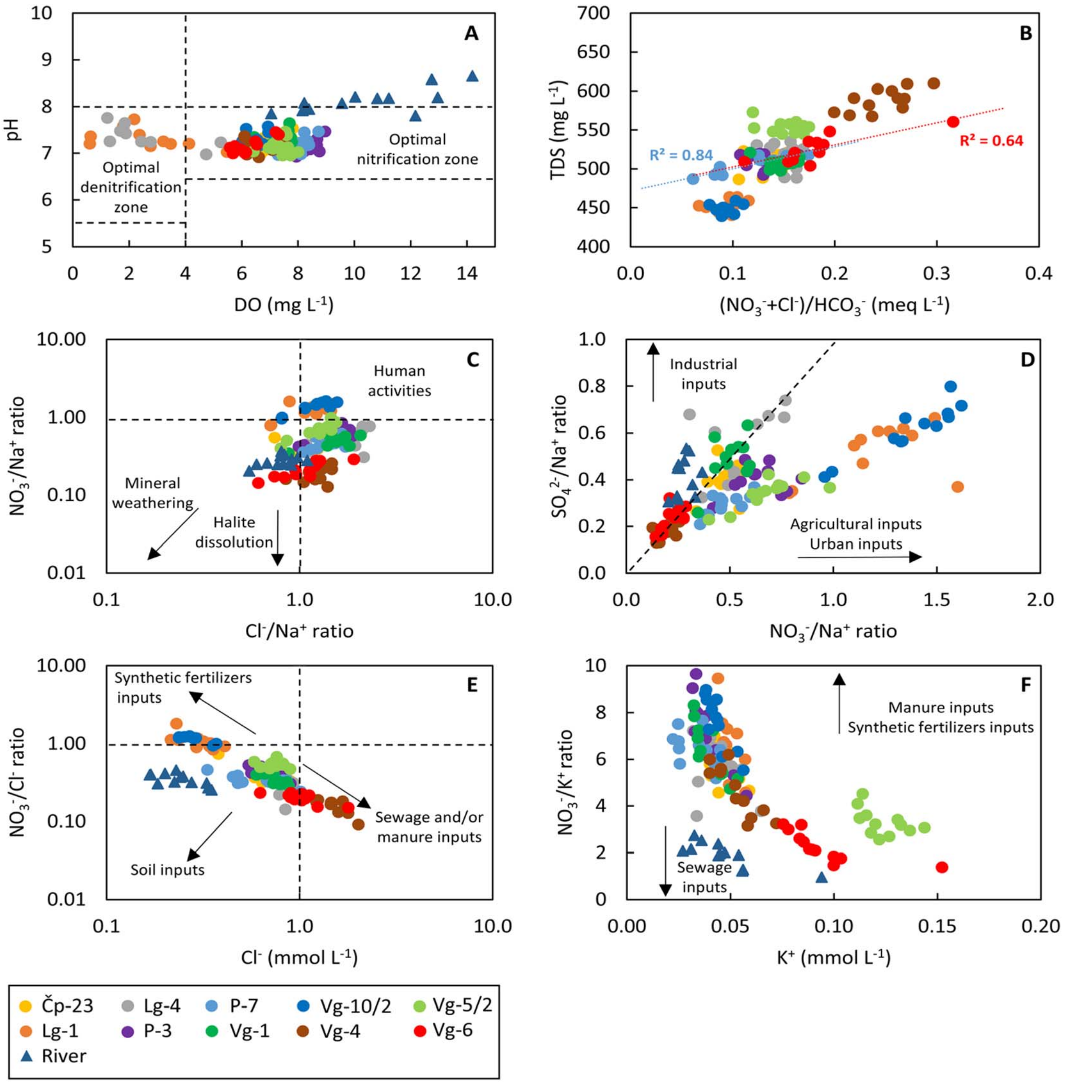


Fig. 3 (A) Scatterplot of pH *versus* DO, with a dashed line separating the zone of nitrification and zone of denitrification; (B) TDS *versus* (NO_3^- + Cl^-)/HCO_3^-; (C) relationship between NO_3^-/Na^+ and the Cl^-/Na^+ molar ratio; (D) SO_4^{2-}/Na^+ *versus* the NO_3^-/Na^+ molar ratio; (E) relationship of the NO_3^-/Cl^- molar ratio and Cl^- molar concentration; (F) relationship between the NO_3^-/K^+ ratio and K^+.

respectively. The K^+ concentration in the Sava River varied between 1.1 and 3.7 mg L^{-1}.

The concentration of Na^+ in groundwater ranged from 4.8 to 37.2 mg L^{-1} with an average of 13.4 mg L^{-1}. The levels of Na^+ in the Sava River were lower, from 4.6 to 10.1 mg L^{-1}. The highest concentrations of Na^+ were observed at the sampling sites Vg-4 and Vg-6 with average values of 29.1 and 22.2 mg L^{-1}, respectively. The increase in Na^+ concentration is due to sewage infiltration, as Vg-4 and Vg-6 are wells located in urban areas. In contrast, the lowest Na^+ contents were recorded at Lg-1 and Vg-10/2 sites. Considering that these two locations had higher concentrations of NO_3^-, the excess NO_3^- is related to agricultural or urban inputs (Fig. 3D).

As depicted in Fig. 3B the positive strong correlation between TDS and $(NO_3^- + Cl^-)/HCO_3^-$, $R^2 = 0.64$ and $R^2 = 0.84$ for the monitoring wells Vg-6 and P-7 respectively, indicated that the water chemistry at these locations is influenced by human activities, such as sewage infiltration or agricultural activities.[69] When considering the molar ratios of NO_3^-/Na^+ and Cl^-/Na^+ (Fig. 3C), the anthropogenic influence on the groundwater quality is evident in almost all groundwater samples.

The Cl^- content and NO_3^-/Cl^- ratios have been used to evaluate the sources of NO_3^-.[70] Here (Fig. 3E), the Vg-4 and Vg-6 sites exhibited high Cl^- content and low NO_3^-/Cl^- ratios, indicating a contribution from sewage and/or manure, *i.e.*, from organic contamination. Vg-6 is the only monitoring well that contains elevated Cl^- concentrations suggesting contamination from sewage[71] and at the same time shows a correlation between Cl^- and NO_3^- suggesting contamination from domestic wastewater discharge.[72] No monitoring well showed low Cl^- concentrations and a low NO_3^-/Cl^- ratio. The samples from Lg-1 and Vg-10/2 sites, which showed low Cl^- content and high NO_3^-/Cl^- ratios, could be associated with agricultural inputs.[73] The remaining six sampling sites did not fall within the range of any specific potential NO_3^- input, suggesting that NO_3^- was derived from a mixing between different sources.

As shown in Fig. 3F, a low NO_3^-/K^+ molar ratio for samples from sites Vg-6 and Vg-5/2, as well as for Sava River samples, confirms possible contamination by sewage effluent[32,74] from the urban area. Synthetic fertilizers and manure often have higher NO_3^- concentrations relative to K^+. Thus, the high NO_3^-/K^+ molar ratios observed at the remaining sampling locations (Čp-23, Lg-4, P-7, Vg-10/2, Lg-1, P-3, Vg-1 and Vg-4) indicated multiple contamination inputs affecting NO_3^- and K^+ contents.

The normality test showed significant differences between groundwater sites for all tested parameters ($p < 0.05$). However, no significant ($p > 0.05$) seasonal differences were observed for DO, EC, T, Cl^-, NO_3^-, Na^+, $\delta^2H_{H_2O}$ and $\delta^{18}O_{H_2O}$. The correlation coefficients provided a preliminary understanding of how different variables relate to nitrate sources. Detailed correlation matrices illustrating these relationships can be found in Tables S3 to S12.† For all monitoring wells, except for Vg-6, a significant and very strong positive correlation between Cl^- and SO_4^{2-} was observed indicating that these concentrations may be related to sewage disposal,[71,75] or agricultural activities, excretions of migrating livestock and domestic wastewater discharge.[72] A significant and strong positive correlation between NO_3^- and Cl^- was observed for the sampling sites Vg-6 ($r = 0.80$) and Vg-5/2 ($r = 0.69$) but not between NO_3^- and other variables. This is probably due to contamination from septic tanks or wastewater discharge.[72] Moreover, the sampling site P-7 showed a significant and strong positive correlation between NO_3^- and Cl^-, K^+, Na^+ and SO_4^{2-} ($r = 0.89$, $r = 0.89$, $r = 0.89$ and $r = 0.91$, respectively) suggesting that most of these ions come from the same source of contamination, although this method does not allow the precise identification of the source. All other locations showed insignificant correlation between NO_3^- and K^+ indicating that nitrate concentrations are related to the different non-point sources, for example manure, sewage or synthetic fertilizers.[76,77] Sampling site Vg-10/2 showed a significant and strong positive correlation between NO_3^- and Cl^- ($r = 0.79$) and SO_4^{2-} ($r = 0.87$) suggesting that these ions are likely influenced by similar sources. No significant correlation between NO_3^- and other ions was detected for the sampling sites Čp-23, Lg-1, Lg-4, Vg-1 and Vg-4. The monitoring well P-7 showed significant and strong positive correlation between K^+ and Cl^- ($r = 0.89$), as well as between Na^+ and Cl^- ($r = 0.85$), which could be related to salinization due to the application of KCl synthetic fertilizers.[78] Furthermore, the positive correlation between Na^+ and Cl^- at P-7 is indicative of contamination from wastewater, while no correlation for all other sampling sites indicates the existence of natural sodium concentrations, probably due to rock weathering and cation exchange with Ca^{2+}.[79] The strong positive correlation between NO_3^- and SO_4^2 for P-7 and Vg-10/2 sites agrees with the findings of Spoelstra *et al.*[80] who suggest that a significant proportion of the groundwater SO_4^{2-} originates from fertilizers. In general, SO_4^{2-} ions can originate from industrial products, fertilizers, and precipitation, but also from natural sulphide mineral dissolution.[81] The correlation coefficients for the P-7 location are attributed to the intensified application of synthetic fertilizers.

Isotopic composition of water

The $\delta^2H_{H_2O}$ and $\delta^{18}O_{H_2O}$ values for the groundwater samples ranged from −65.0 to −57.5‰ and from −9.6 to −8.6‰, respectively. For the surface waters, the $\delta^2H_{H_2O}$ and $\delta^{18}O_{H_2O}$ values ranged from −61.8 to −59.2‰ and from −9.4 to −8.9‰, respectively. As documented earlier,[42] the local meteoric water line (LMWL) of Velika Gorica is characterized by a linear evaporation trend described by $\delta^2H_{H_2O} = 7.7\ \delta^{18}O_{H_2O} + 9.3$ and the average $\delta^{18}O_{H_2O}$ value of precipitation is −8.54‰ obtained from the Velika Gorica meteorological station. Additionally, the article also notes that the two shallowest soil horizons share isotopic signatures similar to precipitation, whereas the two deepest soil horizons have comparable isotopic compositions to each other but differ from those of precipitation. For both groundwater and surface water samples, the $\delta^{18}O_{H_2O}$ *versus* $\delta^2H_{H_2O}$ average values were plotted close to the LMWL of Velika Gorica (Fig. 4) indicating their meteoric origin. The current work showed that the groundwater from seven sampling sites, namely, Čp-23, Lg-4, P-3, P-7, Vg-1, Vg-4 and Vg-5/2 and the Sava River exhibited similar isotopic compositions. Conversely, the monitoring well Vg-6 showed an isotopic composition similar to

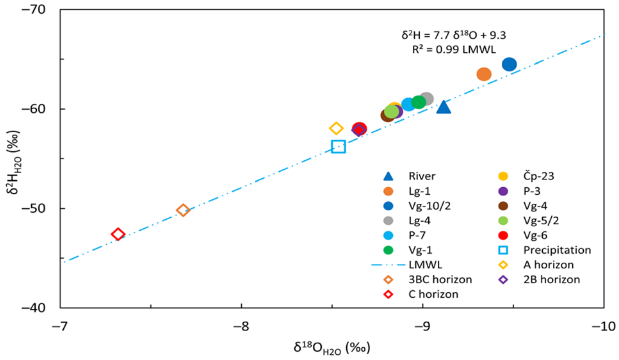


Fig. 4 The relationship between $\delta^{18}O_{H_2O}$ and $\delta^{2}H_{H_2O}$ average values in groundwater, surface water, precipitation and soil water. LMWL is the local meteoric water line of Velika Gorica. The water isotopic composition of soil water and precipitation was published earlier in Buškulić *et al.*[42]

precipitation and the two shallowest soil horizons, while the Lg-1 and Vg-10/2 sites suggest the existence of a different recharge source.

Similar concentrations between sites Lg-1, Vg-10/2 and the Sava River do not imply a direct connection or common origin, as the $\delta^{18}O_{H_2O}$ and $\delta^{2}H_{H_20}$ values of the Lg-1 and Vg-10/2 sites differed from those of the Sava River, indicating that these wells are primarily recharged by water inflow from the southwest part of the aquifer system (Fig. 1C). The average $\delta^{18}O_{H_2O}$ values for Lg-1 and Vg-10/2 were −9.3‰ and −9.5‰, respectively. For all other sampling sites, the values ranged from −9.0 to −8.7‰.

The results of the mixing model showed that the contribution of the Sava River to aquifer recharge spatially varied from 19.9% to 83.4%, with an average of 58.5%, while the recharge from precipitation varied from 16.6% to 80.1%, with an average of 41.5%. Additionally, the monitoring wells can be grouped as follows: Vg-6 showed the smallest fraction of the Sava River (19.9%); in Čp-23, P-3, Vg-4 and Vg-5/2, the Sava River fraction ranged from 47.1% to 54.2%; and in Lg-4, P-7 and Vg-1 group, the Sava River fraction ranged from 66.7% to 83.3%. The monitoring well Lg-4 showed the highest fraction of Sava River influence (83.4%). Conversely, for the sampling site Vg-6, precipitation represents the most important source of recharge, evidenced by the highest fraction of precipitation of 80.1‰. This was expected, given that Vg-6 is located in an area characterized by Fluvisols, which are characterized by a higher infiltration rate.[39]

Isotopic composition of nitrate

The $\delta^{15}N_{NO_3}$ values in the groundwater ranged from 2.6 to 18.3‰, while $\delta^{18}O_{NO_3}$ values ranged between −6.7 and 9.7‰. The highest $\delta^{15}N_{NO_3}$ and $\delta^{18}O_{NO_3}$ values were recorded in the Lg-4 site in August 2021, with values of 38.9 and 17.6‰, respectively, which could be an outlier. Overall, the $\delta^{15}N_{NO_3}$ values ranged from 4.4 to 13.1‰ for the Sava River, from −15.1 to 2.2‰ for precipitation and from −7.1 to 9.0‰ for soil water. The $\delta^{18}O_{NO_3}$ values for the Sava River ranged from −0.8 to 3.9‰, between 62.1 and 94.7‰ for precipitation and between −12.7 and 10.1‰ for soil water.

The DO levels in eight monitoring wells (Čp-23, P-3, P-7, Vg-1, Vg-10/2, Vg-4, Vg-5/2 and Vg-6) ranged from 5.6 to 9.0 mg_{O_2} L^{-1} indicating aerobic conditions that would favor nitrification, while denitrification is less likely to occur (Fig. 5A). In the Sava River, during the sampling period, the DO was measured in the range from 7.1 to 14.2 mg_{O_2} L^{-1}, which favors the nitrification process. Denitrification was detected in sampling site Lg-4 since it fell in the optimal zone for denitrification (Fig. 3A) and exhibited reductive conditions favourable for denitrification.[3] Additionally, the site exhibited an increasing DO trend as $\delta^{15}N_{NO_3}$ values decrease (Fig. 5B).

For almost all groundwater sampling locations and the Sava River, the $\delta^{15}N_{NO_3}$ values were relatively constant with only minor variations as the NO_3^- concentration changes, which is characteristic of the dilution effect (Fig. 5C), suggesting mixing of nitrate from different sources with similar $\delta^{15}N_{NO_3}$ signatures. Conversely, based on specific trends in $\delta^{15}N_{NO_3}$ isotopes during NO_3^- attenuation, denitrification processes were identified in the monitoring wells Lg-4 and P-3 (Fig. 5D). This is indicated by an increase in $\delta^{15}N_{NO_3}$ values along with a decrease in NO_3^- concentrations. Furthermore, when examining the relationship between $\delta^{15}N_{NO_3}$ and the logarithmic concentration of NO_3^- (Fig. 5E), samples from Lg-4 and P-3 exhibit a negative slope with moderate correlations ($R^2 = 0.66$ and $R^2 = 0.54$, respectively) also suggesting natural attenuation, *i.e.*, denitrification.[73] According to Böttcher *et al.*,[29] the slopes of $\delta^{15}N_{NO_3}$ *versus* $\delta^{18}O_{NO_3}$ for Lg-4 and P-3 were characteristic of denitrification, *i.e.*, 0.6 and 0.4, respectively. However, the sampling site P-3 showed high DO levels (7.6 to 8.3 mg_{O_2} L^{-1}) suggesting that denitrification is unlikely to occur and cannot be explained under such conditions. Conversely, the low DO levels in the monitoring well Lg-1 and a slope of 0.8 confirmed the occurrence of the denitrification process.[29] When observing the relationship between $\delta^{15}N_{NO_3}$ and $\ln(NO_3^-)$, sampling sites Čp-23, P-7, Vg-1, Vg-4 and Vg-6 exhibit a negative slope with low correlations ($R^2 = 0.004$, $R^2 = 0.03$, $R^2 = 0.19$, $R^2 = 0.15$ and $R^2 = 0.29$, respectively) (Fig. 5E) indicating a mixing process.[73]

Nitrate consumption, *i.e.* the assimilation process, can be observed in the soil zone (Fig. 5D) through changes in the isotope signal. The deepest (C) horizon contains the highest NO_3^- concentrations and the lowest measured $\delta^{15}N_{NO_3}$ values, whereas the shallowest (A) horizon shows the lowest NO_3^- concentrations and the highest $\delta^{15}N_{NO_3}$ content. This suggests NO_3^- uptake by plants as the water infiltrates the deeper horizons.

The nitrification process was identified by using the local $\delta^{18}O_{H_2O}$ values to calculate the theoretical $\delta^{18}O_{NO_3}$ values after applying eqn (8) and comparing them with the observed $\delta^{18}O_{NO_3}$ values (Fig. 5F). The estimation of $\delta^{18}O_{NO_3}$ values characteristic for the nitrification in the groundwater was performed using the $\delta^{18}O_{O_2}$ value of atmospheric O_2 (23.5‰)[20] and the minimum and maximum $\delta^{18}O_{H_2O}$ values of groundwater (−9.6‰ and −8.6‰).

The calculated $\delta^{18}O_{NO_3}$ values ranged from 1.5 to 2.1‰ (grey area shown in Fig. 6). Almost all groundwater samples fell between the line of the nitrification process and the water exchange line (Fig. 5F) confirming the occurrence of nitrification, except for monitoring well Lg-4, where all the isotopic

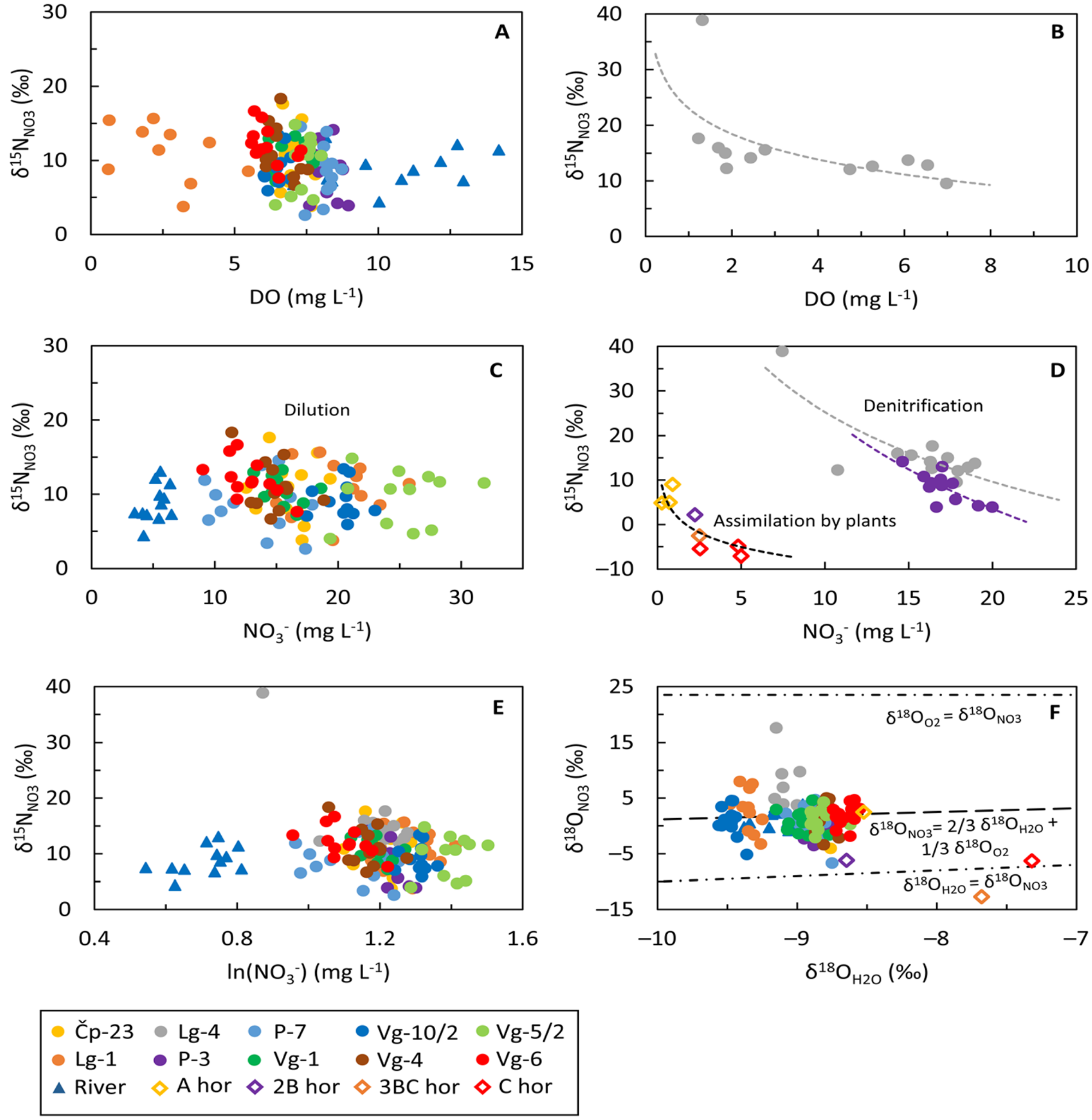


Fig. 5 (A) Scatterplot showing the $\delta^{15}N_{NO_3}$ and DO values of groundwater and surface water samples; (B) the $\delta^{15}N_{NO_3}$ *versus* DO values and trendline for the Lg-4 sampling site; (C) the $\delta^{15}N_{NO_3}$ and NO_3^- values of groundwater and surface water samples; (D) the $\delta^{15}N_{NO_3}$ *versus* NO_3^- values and trendlines for Lg-4, P-3 and soil zone samples; (E) the $\delta^{15}N_{NO_3}$ *versus* $\ln(NO_3^-)$ values of groundwater and surface water samples; (F) the $\delta^{18}O_{NO_3}$ *versus* $\delta^{18}O_{H_2O}$ where the uppermost line indicates the limit of exchange with O_2, the middle line represents the limit of the nitrification process and the bottom line shows the limit of exchange with H_2O.

signatures were higher than those theoretically calculated, confirming the denitrification process, *i.e.*, nitrate attenuation.[82] Similar to groundwater, the estimation of $\delta^{18}O_{NO_3}$ values characteristic of nitrification in the river was conducted using the minimum and maximum $\delta^{18}O_{H_2O}$ values of −9.4‰ and −8.9‰, respectively. The resulting $\delta^{18}O_{NO_3}$ values ranged from 1.6 to 1.9‰, which were quite similar to $\delta^{18}O_{NO_3}$ content observed in the Sava River, where $\delta^{18}O_{NO_3}$ values range from −0.8 to 3.9‰. As shown in Fig. 5F, the Sava River samples fell along the nitrification line. The estimation of $\delta^{18}O_{NO_3}$ content in the soil zone was carried out using $\delta^{18}O_{H_2O}$ values of −10.7‰ and −4.2‰, *i.e.* minimum and maximum observed values. The calculated $\delta^{18}O_{NO_3}$ values ranged from 0.7 to 5.0‰, while the observed $\delta^{18}O_{NO_3}$ values ranged from −12.7 to 10.1‰. As depicted in Fig. 5F, soil samples from the first two horizons (A and 2B horizons) and the deepest horizon (C horizon) fell between the nitrification line and the water exchange line, thereby confirming the occurrence of nitrification. In contrast, the sample from the third soil horizon, *i.e.*, the 3BC horizon, deviates from this range, precluding confirmation of nitrification for that particular sample. Considering that the investigated soil profile is situated within the first sanitary protection zone of the Velika Gorica well field, nitrification of NH_4^+ from soil and/or precipitation could occur in the two upper soil horizons. Conversely, in the deepest horizon, nitrification is restricted to NH_4^+ present in the soil due to the lack of isotopic signatures associated with precipitation.

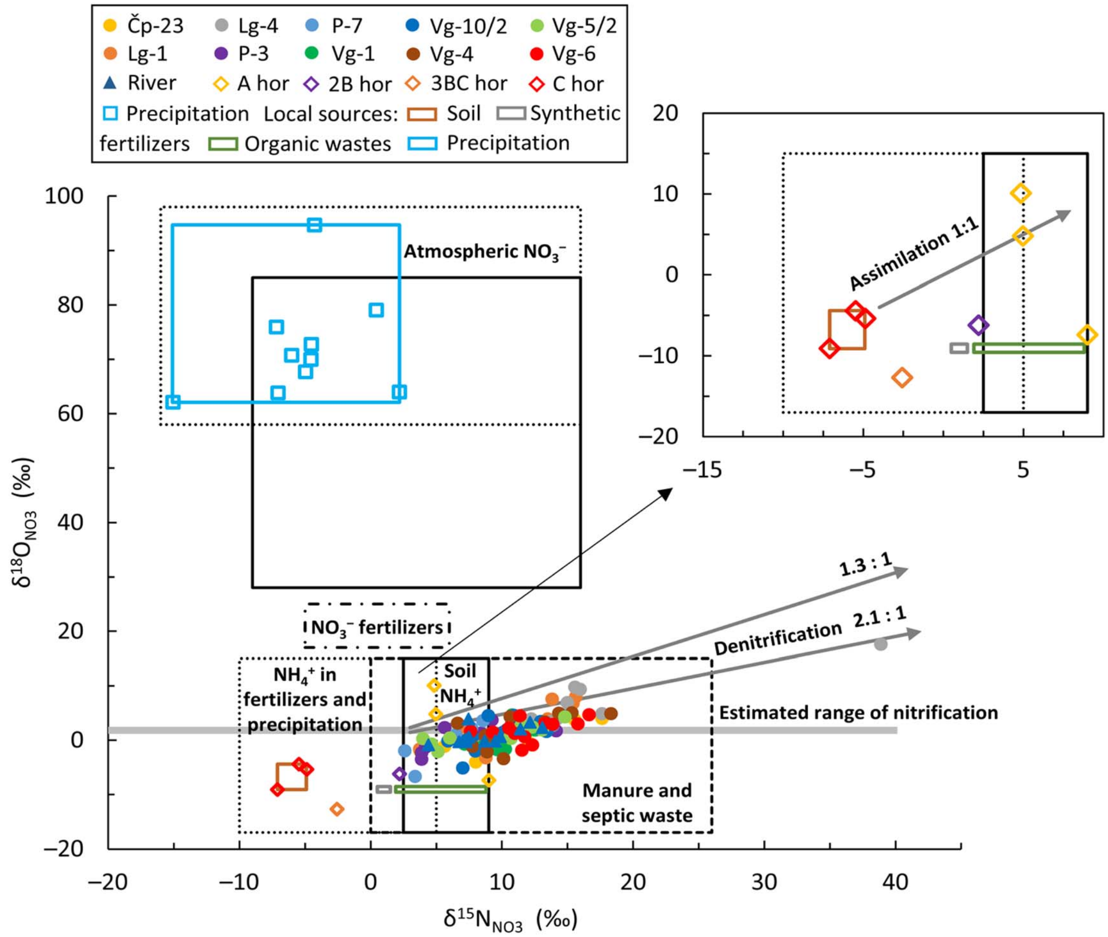


Fig. 6 Bivariate plot of $\delta^{18}O_{NO_3}$ *versus* $\delta^{15}N_{NO_3}$ values of groundwater, surface water, precipitation and soil water in the study area. The range of isotope compositions for five major N sources is derived from Kendall *et al.*[64] and indicated by boxes: (1) atmospheric NO_3^-; (2) NO_3^- based fertilizers; (3) NH_4^+ in fertilizers and precipitation; (4) soil NH_4^+; and (5) manure and septic waste. Note: the brown, grey, green and blue boxes represent local N sources: soil, synthetic fertilizers, organic wastes and precipitation, respectively.

Specifically, the samples taken from sampling site Lg-4 indicated both anaerobic (March to September 2021) and aerobic conditions (from October 2021 to February 2022). During the anaerobic period, the average values of groundwater depth, DO, EC, NO_3^-, $\delta^{15}N_{NO_3}$, $\delta^{18}O_{NO_3}$, $\delta^2H_{H_2O}$ and $\delta^{18}O_{H_2O}$ were 7.1 m, 1.9 mg_{O_2} L^{-1}, 777.6 µS cm^{-1}, 14.0 mg L^{-1}, 18.5‰, 8.0‰, −61.6‰ and −9.1‰, respectively. Nitrite (NO_2^-) values during April to September ranged from 0.04 to 3.6 mg L^{-1} but were below the detection limit in March. The phosphate ions ranged from 1.0 to 2.4 mg L^{-1} and were below the detection limit in September. The ammonium ions were detected in May and June with an average value of 0.4 mg L^{-1}. Under aerobic conditions, the average values of groundwater depth, DO, EC, NO_3^-, $\delta^2H_{H_2O}$ and $\delta^{18}O_{H_2O}$ were 7.8 m, 5.9 mg_{O_2} L^{-1}, 815.2 µS cm^{-1}, 17.9 mg L^{-1}, −60.3‰ and −8.9‰, respectively, significantly higher than those in the anaerobic period. The average values of $\delta^{15}N_{NO_3}$ and $\delta^{18}O_{NO_3}$ were lower, at 12.2‰ and 1.9‰, respectively. In summary, during high water periods, decreased concentrations of DO and NO_3^- were observed, whereas the concentrations of NO_2^-, PO_4^{3-}, NH_4^+, $\delta^{15}N_{NO_3}$ and $\delta^{18}O_{NO_3}$ were higher. Conversely, during low water periods, elevated concentrations of DO and NO_3^- were recorded, alongside decreased levels of $\delta^{15}N_{NO_3}$ and $\delta^{18}O_{NO_3}$, and nearly undetectable levels of NO_2^-, PO_4^{3-} and NH_4^+ contents.

Based on the dual isotopic approach, typical $\delta^{15}N_{NO_3}$ and $\delta^{18}O_{NO_3}$ values from various N sources[64] and possible biogeochemical processes are shown in Fig. 6 together with the groundwater, surface water, precipitation and soil water samples. The $\delta^{18}O_{NO_3}$ values observed in the groundwater and river samples were lower than the values reported for NO_3^- based fertilizers (ranging from 17 to 25‰) and atmospheric deposition of NO_3^- (ranging from 28 to 98‰) suggesting no contribution from these two sources. The lack of atmospheric NO_3^- deposition supports the statement that nitrate concentrations in the groundwater cannot be solely attributed to natural sources. Moreover, the $\delta^{18}O_{NO_3}$ values were within the range for NH_4^+ in fertilizers and precipitation, soil NH_4^+ and manure/septic waste (ranging from −17 to 15‰), as well as around the estimated theoretical range of nitrification. Among the total number of groundwater and Sava River samples, 66% of groundwater samples and 42% of Sava River samples exhibited $\delta^{15}N_{NO_3}$ isotope signatures higher than 9‰, indicating contamination predominantly originating from manure and/or septic waste. The remaining samples fell within the overlapping range reported for NH_4^+ in fertilizers and precipitation and soil NH_4^+.

Given that the investigated soil profile is located within the sanitary protection zone of the well field, where anthropogenic activities are forbidden and strictly controlled, the only possible sources of nitrates are soil NH_4^+, NH_4^+ in rain and atmospheric NO_3^-. According to Kendall *et al.*,[64] the nitrate sources in the first two shallowest horizons (A and 2B) could be soil NH_4^+ and/or NH_4^+ in precipitation (Fig. 6). Since previous studies show that the two deepest soil horizons (3BC and C) are less influenced by precipitation,[40,42] the negative $\delta^{15}N_{NO_3}$ isotopic signature is probably the result of nitrification, *i.e.*, soil NH_4^+ nitrification. As depicted in Fig. 5D, NO_3^- concentrations increase with depth while $\delta^{15}N_{NO_3}$ decreases, which could be due to the nitrification process. The isotopic values in the deepest soil horizon confirmed the NH_4^+ nitrification and supported a previous finding documented in Buškulić *et al.*[42] that N primarily migrates to deeper parts of the unsaturated zone in the form of NO_3^-.

Bayesian mixing model results and uncertainly analysis

A Bayesian isotope mixing model was employed to estimate spatial and seasonal variations in the proportional contributions of three local nitrogen sources to both the groundwater and river water.

Regarding spatial variations, the model outputs showed a similar pattern across all sampling sites (Fig. 7). The results for the Sava River are similar to those for groundwater, due to their continuous mixing. Across the entire study area, soil contributions ranged from 10 to 17%, making it the least significant source which is consistent with the results obtained using other approaches. In contrast, organic wastes showed the highest contribution, ranging from 56 to 68%, in agreement with Fig. 6 which illustrates that more than half of the groundwater samples indicate contamination due to leakage from the sewage system in urban areas, septic tank leakage in rural areas and/or the application of manure. The proportional contribution of synthetic fertilizers was obtained for each location and partly aligned with the results from the hydrogeochemical approach that detected the influence of synthetic fertilizers only on individual sampling sites (*e.g.*, P-7). This is not a surprise given that nitrate concentrations represent the result of mixing of individual nitrate pollution sources, which makes it difficult to disentangle by using only hydrogeochemical parameters. The synthetic fertilizers contributed moderately, with a range of 21 to 27%. Thus, the contributions of NO_3^- sources can be ranked as follows: organic wastes > synthetic fertilizers > soil.

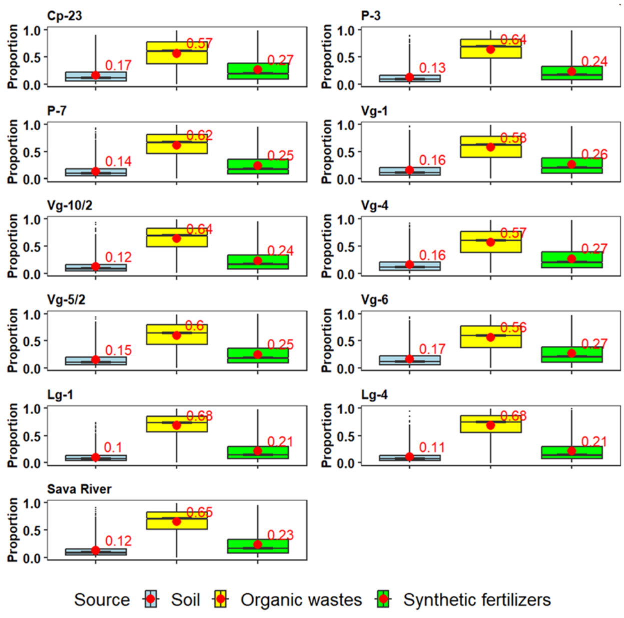


Fig. 7 Spatial variations in the proportional contributions of different local N sources per sampling site based on Bayesian isotope mixing model results. Note: the different local N sources are shown in different colours; the average proportional contributions are shown in red; boxplots illustrate the 25th, 50th and 75th percentiles.

When the model results are examined in the context of land use (urban and agricultural areas) and the average values from the corresponding sampling sites, a clear difference of the proportional contribution of organic waste between these two areas becomes apparent, but the presence of synthetic fertilizers in both areas is not very different. The average value of the organic waste in the urban area is 57%, while in the agricultural area it is 64%.

Regarding seasonal changes, there are noticeable variations in proportions for groundwater, as well as between groundwater and surface water (Fig. 8). The highest contributions of soil and synthetic fertilizers in groundwater were observed in the summer period (9% and 16%, respectively), while the lowest were in the spring (5% and 11%, respectively). Conversely, the proportional contribution of organic wastes is the lowest during summer (75%) and highest during spring (84%). For surface water, the model outputs showed a significant difference compared to groundwater, but a consistent pattern: soil contributions ranged from 26 to 30%, synthetic fertilizers ranged from 32 to 33% and organic wastes ranged from 38 to 41%. Such a significant difference compared to groundwater is attributed to the fact that only three values per season were available for Sava River water.

The results of the uncertainty analysis, *i.e.*, the plots of the cumulative frequency distribution *vs.* the proportional contribution, of source apportionment per site are given in Fig. S1.† When all sites were considered, the average UI90 index per nitrate source followed the order: organic wastes (0.71) > NH_4^+ in synthetic fertilizers and rain (0.63) > soil (0.36). Overall, the uncertainty analysis of the *simmr* model showed UI90 values comparable with those calculated in other case studies (*e.g.*, ref. 24). However, the higher uncertainty in the first sources (organic wastes and NH_4^+ in synthetic fertilizers and rain) is probably due to the fact that their isotopic signatures were relatively close in terms of $\delta^{15}N_{NO_3}$ and $\delta^{18}O_{NO_3}$. The average UI90 index per season (Fig. S2A†) was lower for the groundwaters (<0.5) showing the following order: summer (0.40) > winter

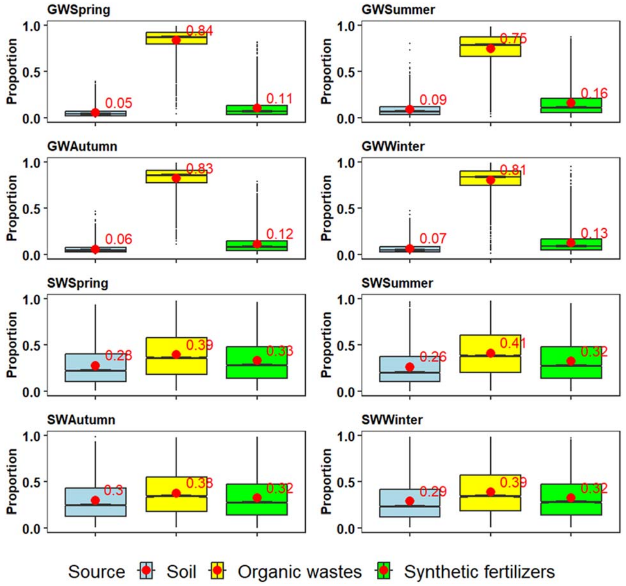


Fig. 8 Seasonal variations in the proportional contributions of different local N sources per water type based on Bayesian isotope mixing model results. Note: the different local N sources are depicted in distinct colours; the average proportional contributions are shown in red; boxplots illustrate the 25th, 50th and 75th percentile; gw stands for groundwater and sw stands for surface water (Sava River).

(0.31) > autumn (0.28) > spring (0.18) but higher for the Sava River (>0.7) for all seasons (Fig. S2B†). This is because the amount of data for the Sava River used in the model was smaller ($n = 12$) than the groundwater ($n = 119$), which increased the uncertainty of the analysis for the surface waters.

Overall remarks

To summarize, the physico-chemical and hydrogeochemical data showed that groundwater and surface water quality is deteriorated by human activities, primarily due to urban or/and agricultural inputs. The concentrations of major anions and cations in the Sava River are lower compared to groundwater. However, certain sites (Lg-1 and Vg-10/2) contain Na^+, SO_4^{2-} and Cl^- concentrations similar to those in the Sava River. Although these sites have similar concentrations, they are located furthest from the Sava River, suggesting that their primary recharge source may differ.

The statistical analysis revealed significant spatial differences in nitrate content, as well as strong correlations between NO_3^- and various ions, suggesting multiple contamination sources.

Furthermore, the nitrate isotopic composition indicated that NO_3^- contamination of groundwater and the Sava River during the study period mainly originated from three distinct nitrate sources: soil NH_4^+, NH_4^+ in fertilizers and manure/septic waste. This is especially important because previous research[32] indicated that the nitrate isotopic composition suggested that nitrates originated solely from manure and wastewater sources. This was unusual due to the presence of a large proportion of agricultural areas, particularly on the right bank of the Sava River. The sites Čp-23, Lg-1, P-3, P-7, Vg-5/2 and the Sava River had $\delta^{15}N_{NO_3}$ and $\delta^{18}O_{NO_3}$ values which indicated contamination from NH_4^+ based fertilizers, soil and manure/septic wastes, while the data from the other four locations (Vg-1, Vg-10/2, Vg-4 and Vg-6) had typical values that indicated contamination from soil and manure/septic waste. Furthermore, the $\delta^{15}N_{NO_3}$ and $\delta^{18}O_{NO_3}$ values from Lg-4 suggested contamination solely from manure and septic waste, as indicated by $\delta^{15}N_{NO_3}$ values higher than 9%. The statistical analyses confirmed significant variations in $\delta^{15}N_{NO_3}$ and $\delta^{18}O_{NO_3}$ across groundwater samples from different locations ($p < 0.05$).

The results of the Bayesian model agree with findings from a previous study[32] which stated that nitrate origin in the Zagreb aquifer area is predominantly organic. However, findings from this research for the first time cannot exclude synthetic fertilizers as the N source and possible cause of elevated NO_3^- concentrations. Given that synthetic fertilizers could not be identified as a potential nitrogen source at each sampling site using the hydrogeochemistry and $\delta^{15}N_{NO_3}$ *vs.* $\delta^{18}O_{NO_3}$ bivariate plot, the mixing model results suggest that the biogeochemical process, most likely nitrification, masked the nitrogen isotopic signature typically associated with synthetic fertilizers and indicated the importance of employing multi-method approaches for more accurate N source identification.

Our results showed that separate usage of each method cannot give a clear understanding. In dynamic systems such as unconfined aquifers, where there are various sources of contamination, it is necessary to use and combine numerous types of data and methods, as well as perceive results from different aspects, if more reliable conclusions about nitrate origin and processes that affect them need to be achieved.

Additionally, it was not possible to consider other aspects, such as the inherent delay of N transport through the unsaturated and saturated zone,[83] in this work. This means that nitrate ions in groundwater may still be present even after years of the reduction or cease of fertilization rates.[84] This delay in the transport of water and nutrients in groundwater highly depends on the local hydraulic properties and heterogeneity of the unsaturated zone and the rates of biogeochemical processes.[85]

Conclusions

This study presents a comprehensive integration of physico-chemical and hydrogeochemical data, isotopic analysis, statistical correlations and mixing models to elucidate the complex sources and processes affecting NO_3^- contamination in groundwater of an unconfined aquifer. The use of a combination of water stable isotopes alongside stable isotopes from NO_3^- dissolved in water, provides a unique way of identifying both natural and anthropogenic influences on groundwater quality, particularly in relation to agricultural and wastewater inputs. With respect to the main objectives of this research, the following can be concluded:

(1) A nitrous oxide isotopic analyser and the titanium(III) reduction method present a reliable combination for the determination of $\delta^{15}N$ and $\delta^{18}O$ from nitrate. It was shown that the results can vary based on the target N_2O gas concentration inside the analyser, reagent-to-sample ratio during sample preparation

and reaction time. N_2O concentrations ranging from 7 to 11 ppm N_2O, 1 : 20 Ti(III) reagent-to-sample ratio and maximum 72-h reaction are found to generate the most stable isotopic values.

(2) The origin of nitrate in the research area is mainly related to the organic sources and the process of nitrification prevails in the most observed locations. Additionally, for the first time, the isotopic composition of local nitrate sources has been determined, which showed that the contribution from soil and synthetic fertilizer exists and varies from both a spatial and temporal point of view. From this perspective, a more detailed inspection of NO_3^- sources in future research must be performed in the study area with a focus on the evaluation of the isotopic composition of synthetic fertilizers, especially those which contain both ammonium and nitrate nitrogen in their formula. It must be noted that determination of the isotopic composition of NO_3^- sources is a crucial part from an interpretation point of view because it shows the range and variability of N which is introduced into the investigated system. The results of this research also showed that it is extremely important to monitor isotopic values in the soil horizons, especially in the deepest one, in order to get information about dominant nitrogen species which can enter the aquifer.

(3) Multitude of analysis is necessary when determination of nitrate origin and related processes presents the main subject of the research. Although correlation analysis was found to be useful in numerous previous research studies, in the areas where NO_3^- concentrations are the consequence of different sources it must be considered with great caution. Even though they can be indicative and helpful within the interpretation procedure, their usage without the isotopic composition should be avoided. Similar insights can be obtained from the hydrogeochemical data when observing ion ratios, but again not unambiguous. The initial insight into the nitrate isotopic composition clearly suggested that most of the nitrate concentrations were associated with manure and organic waste, which turned out to be correct, but at the same time did not recognize the influence of synthetic fertilizer. This is probably associated with the process of nitrification, which masked the nitrogen isotopic signature typically associated with synthetic fertilizers.

(4) The Sava River is the main source of recharge of the Zagreb aquifer, but the results also show that in the southern part of the aquifer groundwater flows from the south-western part of the aquifer and is recharged from the surrounding hills.

The significance of this work is underlined by its ability to distinguish between different sources of contamination and processes such as nitrification and denitrification in a specific hydrogeological context. This approach not only advances the understanding of nitrate dynamics in groundwater but also provides valuable insights for the management and protection of water resources in regions facing similar environmental challenges.

Data availability

Data will be made available on reasonable request. The MATLAB script used to preprocess data is available at the GITHUB repository: **https://github.com/hrvojelukacic**.

Author contributions

Patricia Buškulić: conceptualization, methodology, formal analysis, investigation, data curation, writing – original draft, writing – review & editing, visualization. Zoran Kovač: formal analysis, investigation, writing – review & editing. Ioannis Matiatos: formal analysis, investigation, writing – review & editing, visualization. Jelena Parlov: writing – review & editing, visualization, supervision. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

There are no conflicts to declare.

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3. DISCUSSION

One of the scientific contributions was to create a conceptual model of nitrogen species dynamics through the unsaturated and saturated zone of the Zagreb aquifer system within the area of Eutric Cambisols on the Holocene deposits. To accomplish that scientific contribution, following objectives were defined: (1) determine the ambient background nitrate concentration in a hydrogeologically homogeneous area within a heterogeneous aquifer system under long-term human impact; (2) implement the titanium(III) reduction method for the analysis of stable isotopes of nitrogen and oxygen in dissolved nitrate; (3) facilitate a more comprehensive understanding of nitrate dynamics in the unsaturated and saturated zone within the area of Eutric Cambisols on the Holocene deposits in the Zagreb aquifer; and (4) define the dominant natural form of nitrogen that infiltrates from the soil zone into the aquifer in the catchment area of the Velika Gorica well field. To address these objectives, three hypotheses were tested using all relevant parameters and methods described in the methodology section. The results are summarized and discussed below.

Hypothesis #1 Ambient background value of nitrate in the groundwater in the catchment area of the Velika Gorica well field is below 10 mg/L as NO_3^-.

The ambient background values are crucial for assessing the presence of groundwater contamination as a basis for better groundwater management and protection in areas under long-term human impact. It is often very difficult to clearly identify the populations related to the background processes as opposed to those reflecting contamination (Nakić et al., 2007; Cruz and Andrade, 2015; Biddau et al., 2017; Sellerino et al., 2019). The main issue is how to recognize these differences with confidence (Sinclair, 1991).

In the catchment area of the Velika Gorica well field, the nitrate concentration in the groundwater over a period of 20 years, from 2001 to 2020, was between 0.2 and 35.7 mg/L, with an average of 19.6 mg/L (Buškulić et al., 2023b). Although the average concentration at each individual monitoring well remained below the MCL for drinking water (50 mg/L as NO_3), stipulated by EU and Croatian regulations, these elevated nitrate concentrations indicate natural groundwater quality degradation caused by anthropogenic influence such as agriculture, industry and urbanization. Considering that in areas unaffected by human activities the nitrate concentrations are much lower (Burkart and Kolpin, 1993), distinguishing anomalies or outliers from background nitrate concentrations within the study area is essential.

This research introduces a novel approach for estimating the ABV of nitrate, based on statistical methodologies developed to separate the natural and anthropogenic contributions in monitored nitrate levels (Buškulić et al., 2023b). The first step during the research involved estimation of ABV of nitrate in groundwater using original data from 12 monitoring wells, located in the catchment area of the Velika Gorica well field, without any preprocessing, such as data filtering or selection. In the second step, after examining the data structure, a data was selected and the ABV of nitrate in groundwater was re-estimated using the same methods. Separating or reducing the large datasets into homogeneous groups enhances the ability to detect an environmental problem (Nakić et al., 2007). The selection focused on grouping non-normally distributed (NON-ND) data, as cumulative probability plots for such data contain a curved line with at least one inflection point that could indicate the presence of at least two different populations (Sinclair, 1991; Panno et al., 2006). Normally distributed data were excluded from the second step, because normal or lognormal density function approximates the background population (Sinclair, 1991) which form a straight line on the cumulative probability plots, without the possibility of determining an inflection point, i.e., background value (Panno et al., 2006). The goal was to compare the results of ABV estimation using all data with those derived from selected NON-ND data and to evaluate the reliability of used methods. Additionally, the research aimed to modify the graphical interpretation of two methods (the cumulative frequency curve – CFC and the probability plot – PP) to reduce subjectivity in determining the background value.

As stated by Nakić et al. (2007), the use of iterative 2-σ technique (IT) and calculated distribution function (CDF) does not require normally or lognormally distributed data, which was confirmed with this research considering that the methods yielded statistically significant results using only the NON-ND data. Furthermore, these methods have the limitation that they only work effectively if the predominant background data are used to define the ABV (the number of contaminated samples must be less than the number of background samples) (Nakić et al., 2007). Higher nitrate concentrations in groundwater in the catchment area of the Velika Gorica well field indicated possible natural groundwater quality degradation, considering that in areas not affected by human activities, the nitrate concentration in groundwater should be much lower. Given that background data did not prevail in the original dataset used in this research, the results confirmed the limitation of the methods, as the IT method yielded a higher and unrealistic value, and the CDF method produced a statistically non-significant result (Buškulić et al., 2023b). Therefore, under these conditions, where the original dataset was used and the background data did not prevail, both methods found to be ineffective in estimating the

ABV. In contrast to the IT method, which yielded high and unrealistic results both before and after selection, the CDF method was applicable when using NON-ND data, as it enabled the estimation of a statistically significant background value. The CDF method's effectiveness appears to depend heavily on the dataset used for calculation, making it most suitable when using a selected subset of data that is not normally distributed. It is important to emphasize that the result obtained with data without selection, which was not statistically significant, became significant when using the selected dataset (Buškulić et al., 2023b).

The last two methods (CFC and PP) were graphical methods, which contain an element of subjectivity due to the visual detection, dependent heavily on the experience of the researcher (Nakić et al., 2020) and subject to random and systematic error (Sinclair, 1991; Panno et al., 2006). In order to minimize the subjectivity of the visual interpretation, subsets were selected to describe the population with the highest possible coefficient of determination (Buškulić et al., 2023b). Starting with a minimum of three points in a subset, the subset size was increased by one point at a time until the highest possible coefficient of determination was reached. The coefficient of determination of each subset increased compared to the initial value of entire population. The advantage of this approach is the identification of multiple populations on the diagram that correspond to a relevant process of natural or anthropogenic origin that contributes to nitrate concentrations in groundwater. The nitrate concentrations used in this study led to the identification of three inflection points and the extraction of four subsets, indicating four populations of natural or anthropogenic origin contributing to nitrate concentrations in groundwater (Buškulić et al., 2023b). The first inflection point, which separate the first two subsets, were directly considered as ABV, as the first population of the dataset was considered to be the result of natural conditions. This is partly in line with previous studies, which found that elevated nitrate concentrations are the result of various sources of contamination such as wastewater and manure (Kovač, 2017; Kovač et al., 2018a), and consistent with the analysis of stable isotopic composition of nitrate in this study, which suggests that nitrate contamination of groundwater during the sampling period originated from following nitrate sources: soil and precipitation NH_4^+, NH_4^+ mineral fertilizers, manure and septic waste (Buškulić et al., 2025).

The study has shown that in area under long-term human impact, such as the catchment area of the Velika Gorica well field, CFC and PP methods for data without selection and CDF method for selected NON-ND data give consistent and realistic estimation of ABV ranging from 7.1 to 7.5 mg/L (Table 3). Conversely, the IT method is not convenient for use when anthropogenic activities tend to lead to enrichments in natural systems, as it can lead to an overestimation of the ABV (Buškulić et al., 2023b). The estimation of nitrate ABV in the catchment area of the Velika Gorica well field is comparable to the maximum measured NO_3^- content in the pedological profile, as well as in the main recharge source of the Zagreb aquifer system, i.e., the Sava River. Nitrate concentrations free from human influence are considered to be below 7.5 mg/L, while values above this threshold are linked to anthropogenic sources. The usage of lower limits from first two methods (IT and CDF) is more convenient in the anthropogenically influenced area and corresponds to the usage of the first inflection points from CFCs and PPs, which in the end makes the results estimated by all methods comparable (Buškulić et al., 2023b). The ABV of nitrate in the groundwater in the catchment area of the Velika Gorica well field is below 10 mg/L as NO_3^-, hence the 1st hypothesis is confirmed.

Table 3. Results of four different methods to estimate ABV of nitrate for data without selection and selected NON-ND data. For the IT and CDF method, the lower limits of normal background concentrations are presented.

	IT	CDF	CFC	PP
data without selection	11.8	8.3 ($D > D_{crit}$)	7.5	7.1
selected NON-ND data	13.2	7.2	7.3	6.9

Another justification for determining the ABV specifically for the catchment area of the Velika Gorica well field, rather than relying on regional background levels from previous research, is the potential variability in environmental conditions and anthropogenic influences across different parts of the Zagreb area. These variations can significantly affect background concentrations, making it essential to establish ABV at a local scale for accurate assessment and interpretation. Based on the findings of Preziosi et al. (2010), the background concentrations of substances, determined at the level of the regional aquifer system, can differ from the background concentrations of the same substances, determined in homogeneous areas within the aquifer system. The importance of applying the principle of homogeneity when determining background concentrations for substances that are sensitive to changes in oxidation-reduction conditions was emphasized by Molinari et al. (2012). Additionally, using regional background levels of some elements could result in underestimating contamination in affected areas and overestimating it elsewhere (Nakić et al., 2007). This research showed that background value of nitrate determined at the level of the Zagreb aquifer system (Nakić et al., 2007; Kovač, 2017) differs from the background value of nitrate determined for the Velika Gorica well field catchment area, a hydrogeologically homogeneous area within a heterogeneous aquifer system (Buškulić et al., 2023b). If regional background values determined in previous research were used, instead of the established ABV, the anthropogenically conditioned concentrations would be underestimated. This arises from the fact that the upper limit of the ABV for the Zagreb aquifer system ranges from 4.5 to 10.7 mg/L (Nakić et al., 2007; Kovač, 2017), whereas the lower limit of the ABV for the study area ranged from 7.1 to 7.5 mg/L (Buškulić et al., 2023b). In the context of this hypothesis, objective 1 was fully achieved.

Hypothesis #2 In an area without anthropogenic activities, nitrogen is primarily introduced from the soil zone (Eutric Cambisols on the Holocene deposits) into the unsaturated zone in the form of nitrites and nitrates.

The second objective of this research was to implement the titanium(III) reduction method for the analysis of stable isotopic composition of nitrogen and oxygen in dissolved nitrate. Successful implementation was preceded with laboratory procedures optimization necessary to get reliable and stable results using nitrous oxide isotopic analyzer. Several optimization steps were conducted, i.e., target N_2O gas concentration inside the analyzer, reagent-to-sample ratio during sample preparation and reaction time, to ensure laboratory setup functionality and reliability before research activities. N_2O concentrations ranging from 7 to 11 ppm N_2O, 1:20 Ti(III) reagent-to-sample ratio and maximum 72-h reaction were found to generate the most stable isotopic values (see Figure 2 in Buškulić et al., 2025). Therefore, the objective 2 was fully achieved.

Characteristics of soil water samples and soil samples collected during this research were used to provide new insights into geochemical processes associated with N species transformation within the Eutric Cambisols, one of the most common soil types in the Zagreb aquifer region (Buškulić et al., 2023a). The specific goals included evaluating the dynamics of NO_3^- within the soil zone and identifying the dominant natural form of N that infiltrates into the aquifer. The investigated soil profile is located within the first sanitary protection zone of the well field Velika Gorica, where anthropogenic activities are forbidden and strictly controlled, therefore the only potential sources of nitrates are soil NH_4^+, NH_4^+ in rain and/or atmospheric NO_3^-.

Following the analyses of major anions and cations, all N species were detected in the soil water samples across all soil horizons. A wide range of NO_2^- and NO_3^- concentrations were observed, with mean values ranging from 0.1 to 1.8 mg/L and from 0.5 to 5.0 mg/L, respectively. Similarly, NH_4^+ concentrations showed wide variability, with mean values ranging from 0.6 to 4.4 mg/L (Buškulić et al., 2023a).

Statistical techniques (Figure 4) have been conducted to compare the significant variability of soil water parameters from different depths within the soil profile. The statistical tests showed that key variables in this research, including soil moisture, δ^2H, δ^{18}O, NO_2^-, NO_3^- and NH_4^+, did not follow a normal distribution and had not constant variance across groups. Further statistical analyses (the post hoc tests) revealed significant differences across various sampling depths within the pedological profile.

Average values of water isotopic composition from all soil horizons fell on the Local meteoric water line (LMWL) indicating their meteoric origin (see Figure 4a in Buškulić et al., 2023a). However, two shallowest soil horizons (A and 2B) shared isotopic signatures similar to precipitation, while the two deepest soil horizons (3BC and C) had comparable isotopic compositions to each other but with no response to precipitation events. This study, along with previous research (Kovač et al., 2023), indicated that the two deepest soil horizons (3BC and C) are less influenced by precipitation. Additionally, as reported by Bačani (2024), boundary between fine-grained and coarse-grained sediments (boundary between 3BC and C horizon) prevents infiltration of precipitation into deeper layers. Infiltration occurs only when a sufficient amount of water is accumulated at this boundary (Bačani, 2024). Furthermore, lower values of d-excess in the A soil horizon during the summer months of 2021 (July and August) suggested influence of evaporation (see Figure 3 in Buškulić et al., 2023a). This aligns with the previous research, which demonstrated that evaporation fractionation is generally limited to the top 0.3 m of soil (Sprenger et al., 2016) and was similarly observed in the A soil horizon during earlier research conducted at this site (Kovač et al., 2023).

Within the A soil horizon, a low NO_3^-/Cl^- molar ratio and high NO_2^-/Cl^- molar ratio was observed, alongside the highest mean soil moisture content (36.5%). The elevated soil moisture negatively affected oxygen levels, which in turn reduced the nitrification rate (i.e., accumulation of NO_3^-) (Buškulić et al., 2023a). Under such conditions, NO_2^- serves as the intermediate product (Qi et al., 2019), due to the faster rate of nitritation compared to nitratation, resulting to significant NO_2^- accumulation in the system (Buday et al., 1999; Philips et al., 2002). As a result, due to the faster nitritation rate, the A horizon exhibited higher NO_2^- concentrations, with a mean value of 1.3 mg/L and lower NO_3^- concentrations, with a mean value of 0.5 mg/L (Buškulić et al., 2023a). NO_2^- concentrations were higher during the warmer months (June and July), when the temperature and microbial activity were higher. Additionally, the seasonal variability in NO_2^- concentrations could be explained by the presence of low permeability soil which tends to accumulate N species during the dry season between infiltration events. Nitrification was also confirmed through estimation of $\delta^{18}O_{NO3}$ values characteristic for the nitrification, as soil samples from the first two horizons (A and 2B) fell between the nitrification line and the water exchange line (see Figure 5F in Buškulić et al., 2025). Additionally, based on the range of isotopic composition for major N sources derived from Kendall et al. (2007) and the bivariate plot of $\delta^{18}O_{NO3}$ versus $\delta^{15}N_{NO3}$ values (see Figure 6 in Buškulić et al., 2025), the nitrate sources in the A and 2B horizons were likely derived from soil NH_4^+ and/or NH_4^+ in precipitation.

Statistical analyses (Figure 4) showed that the 3BC soil horizon was the only horizon where NH_4^+ had a distinct distribution (Buškulić et al., 2023a). Although the accumulation of NH_4^+ in soils is uncommon, as it undergoes rapid conversion by soil microbes (Amoo and Babalola, 2017), NH_4^+ was the dominant N species in the 3BC soil horizon within the investigated soil profile, with a mean concentration value of 4.4 mg/L (Buškulić et al., 2023a). The presence of NH_4^+ may indicate an oxygen-deficient environment and reducing conditions (Varnier et al., 2017). The 3BC horizon is characterized by a notable presence of silt and clay, which further points to existence of oxygen depleted conditions from 0.55 to 0.9 m soil depth. Under such conditions in soil, reactions of iron (Fe) and manganese (Mn) oxides could play a significant role in N cycling (Clément et al., 2005; Yang et al., 2012; Xin et al., 2017). NH_4^+ may serves as electron donor, while Fe and Mn may serve as electron acceptors. Elevated iron and manganese concentrations in soils deposited in alluvial sediments have a geogenic origin, as iron is likely bound to ferromagnesian minerals and the fine sediment fraction. The ferric iron content exceeds manganese concentrations, wherefore manganese likely serves as an alternative electron acceptor. Under oxygen-depleted conditions in the 3BC horizon, the reaction of anaerobic NH_4^+ oxidation and ferric iron (Fe^{3+}) reduction indirectly lead to the production of NO_3^-, given that the NO_3^- concentration has slightly increased compared to the upper horizons (Buškulić et al., 2023a). Moreover, nitrification was not even confirmed using isotopic analyses, as the sample from the 3BC horizon, falls outside the range characteristic of the nitrification process, thereby precluding confirmation of nitrification for that soil horizon (see Figure 5F in Buškulić et al., 2025). A significant concentration of NH_4^+ (8.3 mg/L) was recorded in May 2021, when monthly precipitation reached 102 mm and exceeding the monthly average precipitation for this area (see Figure 2a in Buškulić et al., 2023a). Although the results of water isotopic composition showed that 3BC horizon is less influenced by precipitation, such sudden increase in rainfall could have enhanced the NH_4^+ leaching from the upper soil horizons. Given that positively charged ammonium tends to adsorb to negatively charged soil particles (e.g., clay minerals), the adsorption process within the 3BC horizon helps retain NH_4^+ and prevents its downward movement into deeper soil horizon.

Across the first three soil horizons, soil moisture and NO_3^- showed identical distribution patterns. First three soil horizons mainly consist of silt, clay and sand, while gravel dominate below 0.9 m depth. Lower NO_3^- concentrations were observed in the first three soil horizons (with mean values ranging from 0.5 to 0.8 mg/L) as a result of elevated water content (with mean values ranging from 32.1 to 36.5%), which in turn leaded to oxygen limitation and reduced nitrification rate (Norton and Stark, 2011; Ayiti and Babalola, 2022). NO_3^- concentrations were even more reduced during warmer months, likely due to assimilation of NO_3^- through plants. Nitrate consumption, i.e. assimilation process, was observed through changes in the isotope signal along the soil profile (see Figure 5D in Buškulić et al., 2025) and likely occurs up to a depth of 40 cm, corresponding to the depth of plant root penetration. The deepest horizon showed the highest NO_3^- concentrations and the lowest $\delta^{15}N_{NO3}$ values, whereas the shallowest horizon contained the lowest NO_3^- concentrations and the highest $\delta^{15}N_{NO3}$ content. This confirmed NO_3^- uptake by plants in the upper soil layers and leaching potential. Soil pH values increased with depth, while TOC content decreased with depth (see Figure 7 in Buškulić et al., 2023a). According to previous research (Cui et al., 2022), nitrification intensity was reduced in the first three soil horizons where soil pH was below 7. TOC contents are higher within the first three horizons where soil is rich in silt and clay, due to fine-grained particles which stabilize soil OM (organic matter) from being decomposed by microorganism. The higher content of TiO_2 up to a depth of 0.9 m reduces the abundance of ammonia and nitrite oxidizers, representing another important factor contributing to the reduction in nitrification rates. Additionally, higher heavy metal concentrations observed in the soil within the first three soil horizons (see Figure 7 in Buškulić et al., 2023a), negatively affect nitrification rate and inhibit microorganisms' activity, which can lead to reduction of NO_3^- levels (Buškulić et al., 2023a).

The deepest soil horizon stood out as the sole horizon with a distinct distribution for soil moisture and NO_3^-. Given that pedological profile is situated in an area without potential anthropogenic sources of nitrate and considering that C soil horizon cannot retain the most of precipitation that infiltrates, which corresponds to different isotopic signature of precipitation and absence of variation in isotopic composition of soil water in C soil horizon observed in this research, the examination of the NO_3^-/Cl^- molar ratio in relation to Cl^- concentrations could be crucial for identifying nitrates produced by soil N nitrification. The highest values of NO_3^-/Cl^- molar ratios were observed in C soil horizon with average of 2.5 and minimum of 0.9 while the highest molar ratio in all other soil horizons was 0.8 (see Figure 5 in Buškulić et al., 2023a). Hence, if nitrates are produced from the nitrification of the soil N within the Eutric Cambisols, a NO_3^-/Cl^- molar ratio higher than 1 should be present. This is consistent with previous research, which demonstrated that elevated NO_3^-/Cl^- molar ratios relative to Cl^- concentrations can suggest the nitrate production driven by the nitrification of soil N (Zhao et al., 2020). In the C soil horizon, the NO_3^-/Cl^- molar ratio values were higher than NO_2^-/Cl^- molar ratio values, under the relatively low soil moisture levels, leading to higher oxygen content (see Figure 6 in Buškulić et al., 2023a). The lowest mean value of soil moisture (23.2%) was observed in the C horizon which contains the insignificant amount of clay. After the third soil horizon (3BC), soil moisture dropped, likely due to reduced water retention resulting from the presence of coarse-grained particles. It is important to highlight that maximum water content in C soil horizon did not exceed 25% which also suggest more dominant occurrence of soil nitrification. Since nitrification is a biochemical oxidation process, lower moisture content in the C horizon had a positive effect on oxygen levels and nitrification process. The degree of denitrification in the deepest soil horizon was constrained by lower soil moisture levels (Chen et al., 2009). NO_3^- remained at low levels in the first three soil horizons and then increased sharply in the C horizon (see Figure 3 in Buškulić et al., 2023a). Higher NO_2^- concentrations were also detected. NO_3^- together with NO_2^-, were the dominant species of N in the deepest soil horizon, with mean values of 5.0 mg/L and 1.8 mg/L, respectively. The higher concentrations of NO_3^- (6.8 mg/L) and NO_2^- (2.5 mg/L) in the C soil horizon were observed in rainfall-intensive month (May 2021). The sudden increase in precipitation led to enhanced leaching of oxidized forms of N from the upper soil horizons (Buškulić et al., 2023a). Similar to recent research (Ayiti and Babalola, 2022), C soil horizon with a pH above 7 have a prerequisite for optimum activity of ammonia and nitrite oxidizers, i.e., for nitrification. The higher presence of coarse-grained particles along C soil horizon reduces input of OM and decreases TOC content. The NO_3^- content increased in C horizon, likely because of a decrease in TOC content, as low organic carbon levels may increase the amount of NO_3^- (Xiao et al., 2021; Xing et al., 2021). TOC content represents another significant factor whose partial presence in the deepest studied soil horizon promote nitrification (Buškulić et al., 2023a). In comparison to other depths, the C soil horizon demonstrated the lowest TiO_2 content, as well as Ni, Zn, Pb and As concentrations (see Figure 7 in Buškulić et al., 2023a) which positively affected nitrification rate. Starting from a depth of 0.9 meters (i.e., the C horizon), which is characterized by a significant presence of gravel, aerobic conditions prevail and NO_3^- accumulate. Regarding estimation of $\delta^{18}O_{NO3}$ values characteristic for the nitrification, the deepest horizon fell between the nitrification line and the water exchange line, thereby confirming the occurrence of nitrification (see Figure 5F in Buškulić et al., 2025). NO_3^- concentrations increased with depth while $\delta^{15}N_{NO3}$ decreased, which is characteristic of the nitrification process. However, nitrification within this horizon is restricted to NH_4^+ present in the soil, due to the lack of isotopic signature associated with precipitation which does not confirm possible NH_4^+ leaching from the upper horizons due to water movement. Within C horizon, soil OM and other organic sources decompose over time through process of mineralization and contribute to NH_4^+ production. The rate of decomposition is slower at this depth due to reduced microbial activity, but it still occurs and contributes to availability of NH_4^+. The isotopic values in the deepest soil horizon further confirmed the occurrence of soil NH_4^+ nitrification and supported a notion that N primarily migrates from C horizon to deeper parts of the unsaturated zone in the form of NO_2^- and NO_3^- (Buškulić et al., 2025). Therefore, the 2nd hypothesis is confirmed and in the context of this hypothesis, objective 3 was achieved in terms of a more comprehensive understanding of nitrate dynamics in the unsaturated zone within the area of Eutric Cambisols on the Holocene deposits was achieved. Additionally, objective 4 was also successfully achieved.

Hypothesis #3 The source of nitrate in the groundwater in the catchment area of the Velika Gorica well field is not mineral fertilizers.

The evaluation of the processes influencing N species and NO_3^- origin is complex and requires comprehensive analysis. An accurate interpretation of the data requires the integration of multiple analytical methods. This approach is especially important in areas where nitrate concentrations arise from a mixture of anthropogenic activities and natural environmental conditions (Xue et al., 2009; Fenech et al., 2012; Nikolenko et al., 2018; Linhoff, 2022). The use of diverse data and methods improves reliability, whereby isotope signatures and the composition of main nitrogen sources are decisive. Therefore, physico-chemical, hydrogeochemical and isotopic data (nitrate stable isotopes and water stable isotopes), together with isotopic values of the local N sources, molar ratios, statistical tools and mixing models were used to identify the biogeochemical processes, dynamics and origin of NO_3^- in the groundwater (Buškulić et al., 2025). The specific goals included defining appropriate geochemical and statistical methods for interpretation, quantification of spatial and temporal proportional contributions from different N sources and determination of aquifer recharge sources.

During the study period, from March 2021 to February 2022, the NO_3^- concentrations in groundwater ranged from 7.4 to 31.9 mg/L, with an average of 17.1 mg/L (Buškulić et al., 2025), which is quite similar to historical datasets of past twenty years. None of the analyses exceeded the MCL for drinking water. Elevated mean NO_3^- concentrations were found at Lg-1 (20.1 mg/L), Vg-10/2 (20.3 mg/L) and Vg-5/2 (25.1 mg/L) monitoring wells. In the Sava River, the NO_3^- concentrations ranged from 3.5 to 6.5 mg/L, which makes them lower compared to groundwater, and the concentrations of other major anions and cations were also lower compared to groundwater. Considering that the ambient background value of NO_3^- of study area was around 7 mg/L (Buškulić et al., 2023b), the maximum measured NO_3^- content in the soil where anthropogenic influence are not present was 6.8 mg/L (Buškulić et al., 2023a) and the maximum NO_3^- content in the Sava River was 6.5 mg/L (Buškulić et al., 2025), the observed NO_3^- concentrations in the groundwater cannot be attributed only to natural environmental conditions and sources. This is apparent as the lowest recorded concentrations were around 9 mg/L in almost all groundwater samples. An exception is a single analysis from the Lg-4 site, where the NO_3^- concentration was 7.4 mg/L in August. Therefore, NO_3^- concentrations in the groundwater exceeded ABV, indicating natural groundwater quality degradation caused by anthropogenic activities, which might be linked to urban or/and agricultural inputs. Nitrate concentrations of precipitation during sampling period ranged from 0.1 to 2.5 mg/L.

Biogeochemical processes such as nitrification, denitrification and assimilation by plants are key drivers of nitrate cycling in the environment, while physical processes like dilution and mixing influence the distribution and concentration of nitrate in aquatic systems. By analysing parameters such as DO, pH, NO_3^- content, nitrate stable isotopes, their trends and relationship, along with theoretical $\delta^{18}O_{NO3}$ range for nitrification, a more comprehensive understanding of the underlying processes was achieved (Buškulić et al., 2025). Monitoring wells Lg-1 and Lg-4 generally had DO levels lower than 4 mgO_2/L, placing them within the optimal zone for the denitrification process (see Figure 3A in Buškulić et al., 2025). Conversely, all other groundwater sampling sites had DO levels greater than 4 mgO_2/L (ranged from 5.6 to 9.0 mgO_2/L) and a pH range between 6.5 and 8, indicating aerobic conditions that favour nitrification, while denitrification is less likely to occur (Nikolenko et al., 2018). Considering that the sampling site Lg-4 is within the optimal zone for denitrification, the well exhibited one of the key conditions necessary for reductive conditions favourable to this biogeochemical process. Additionally, denitrification process was identified for Lg-4 site based on specific trends in $\delta^{15}N_{NO3}$ isotopes during NO_3^- attenuation. This is evidenced by an increase in $\delta^{15}N_{NO3}$ values along with a decrease in NO_3^- concentrations, which is typical for denitrification (see Figure 5D in Buškulić et al., 2025). When examining the relationship between $\delta^{15}N_{NO3}$ and logarithmic concentration of NO_3^-, samples from Lg-4 exhibited a negative slope with a moderate correlation ($R^2 = 0.7$) (Buškulić et al., 2025), further supporting natural attenuation, i.e., denitrification (Torres-Martínez et al., 2021). Moreover, according to Liu et al. (2006), the slope of the $\delta^{15}N_{NO3}$ versus $\delta^{18}O_{NO3}$ for Lg-4 was typical for denitrification, with a value of 0.6. At that specific location, denitrification was also confirmed through estimation of $\delta^{18}O_{NO3}$ values characteristic for the nitrification, as all the isotopic signatures from Lg-4 were higher than theoretically calculated values (see Figure 5F in Buškulić et al., 2025). Moreover, the samples taken from sampling site Lg-4 indicated anaerobic conditions only from March 2021 to September 2021. That period coincides with a high water periods, decreased concentrations of DO and NO_3^-, while the content of NO_2^-, NH_4^+, $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ were elevated. Regarding estimation of $\delta^{18}O_{NO3}$ interval characteristic for the nitrification process, all other groundwater samples fell between the line of nitrification process and the water exchange line confirming the occurrence of nitrification (see Figure 5F in Buškulić et al., 2025). During the sampling period, the DO levels in the Sava River ranged from 7.1 to 14.2 mgO_2/L, indicating that the measured NO_3^- concentrations could result from nitrification under aerobic conditions. Additionally, the samples from the Sava River were also located along the nitrification line. Compared to groundwater, however, NO_3^- concentrations in the Sava River were lower, which is probably due to a combination of assimilation (NO_3^- consumption by aquatic organisms) (Kendall et al., 2007) and dilution by precipitation. For almost all groundwater and the Sava River samples, the $\delta^{15}N_{NO3}$ values remained relatively constant with only minor variations as NO_3^- concentration changed (see Figure 5C in Buškulić et al., 2025). This is characteristic of a dilution effect, suggesting the mixing of NO_3^- from different sources with similar $\delta^{15}N_{NO3}$ signatures. Since a more comprehensive understanding of nitrate dynamics in the saturated zone within the area of Eutric Cambisols on the Holocene deposits was gained, along with a more comprehensive understanding of nitrate dynamics in the unsaturated zone described earlier, objective 3 has now been fully achieved.

The physico-chemical and hydrogeochemical parameters, along with molar ratios and statistical methods, described below, provided an initial understanding of nitrate origin in groundwater and how different variables relate to nitrate sources. The statistical analyses revealed significant spatial differences for NO_3^- content ($p < 0.05$), while no significant ($p > 0.05$) seasonal differences were observed (Buškulić et al., 2025). The molar ratio analyses revealed differences in the values, which are most likely the result of different sources of contamination during the sampling period, as well as the result of natural variability in the ion concentrations. Following conclusions from Xiao et al. (2017), the positive strong correlation between TDS and $(NO_3^- + Cl^-)/HCO_3^-$ for the monitoring well Vg-6 ($r = 0.8$) indicates that this site is influenced by human activities, such as sewage infiltration or agricultural activities.

Moreover, the highest concentrations of Cl^- and Na^+ were observed at the sampling sites Vg-4 and Vg-6, with an average of 55.6 and 37.2 mg/L for Cl^-, and 29.1 and 22.2 mg/L for Na^+, respectively. Since there are no significant natural sources of NaCl in the study area, it can be assumed that the elevated concentrations of Cl^- and Na^+ are a result of organic contamination sources, rather than inorganic ones. Additionally, the Vg-4 and Vg-6 sites exhibited high Cl^- content and low NO_3^-/Cl^- ratios, characteristic for organic wastes (see Figure 3E in Buškulić et al., 2025). Furthermore, unlike Vg-4, monitoring well Vg-6 showed significant and strong positive correlation between NO_3^- and Cl^- ($r = 0.8$), further implying contamination from organic sources, most likely from urban pollution. In addition, at the monitoring well Vg-6, elevated concentrations of K^+ with an average of 3.7 mg/L and lower concentrations of NO_3^- with an average of 12.7 mg/L were detected. This resulted in a low NO_3^-/K^+ molar ratio, further confirming contamination by sewage effluent from the urban area (see Figure 3F in Buškulić et al., 2025). In contrast, the lowest Cl^- and Na^+ concentrations were recorded at Lg-1 and Vg-10/2 sites, with an average of 11.1 and 10.1 mg/L for Cl^-, and 6.2 and 5.6 mg/L for Na^+, respectively. Considering that these two locations showed the highest concentrations of NO_3^-, the excess of NO_3^- were likely related to agricultural inputs. The samples from Lg-1 and Vg-10/2 sites showed low Cl^- content and high NO_3^-/Cl^- ratios, further indicating the influence of agricultural sources. Additionally, a higher NO_3^-/K^+ molar ratios confirming contamination by agricultural activities. To summarise, an initial evaluation of the nitrate origin has shown that the quality of groundwater is degraded by human activities, primarily due to urban and/or agricultural inputs (Buškulić et al., 2025). Moreover, the monitoring wells can be grouped as follows: monitoring wells Vg-1 and Vg-10/2 with higher molar ratios ($NO_3^-/Cl^- > 0.8$, $NO_3^-/Na^+ > 0.8$, $NO_3^-/K^+ > 5$) indicating dominant inorganic contamination associated with agricultural activities; monitoring well Vg-6 with lower molar ratios ($NO_3^-/Cl^- \leq 0.2$, $NO_3^-/Na^+ \leq 0.3$, $NO_3^-/K^+ \leq 3$,) indicating dominant organic contamination associated with urban areas; and the remaining monitoring wells (Čp-23, Lg-4, P-3, P-7, Vg-1, Vg-4 and Vg-5/2) which molar ratios did not point to any specific potential NO_3^- source, indicating multiple contamination inputs and suggesting that NO_3^- was derived from a mixing between different sources, i.e., manure, sewage or mineral fertilizers (Buškulić et al., 2025).

The general direction of the groundwater flow in the study area is from north-west to south-east, with clear differences during low and high water level periods. During the high groundwater levels, the groundwater flows towards the south in the northern part of the study area, which is due to the increased influence from the Sava River for that hydrological condition (see Figure 1B in Buškulić et al., 2025). Moreover, water stable isotopes (δ^2H_{H2O} and $\delta^{18}O_{H2O}$)

were used to determine sources of recharge, whereas two-component mixing model was performed to quantify aquifer recharge components. Mixing model has been used in the study area through the previous research (Parlov et al., 2019), but also in different hydrogeology applications (Jean-Baptiste et al., 2020; Behrouj-Peelya et al., 2020; Tian et al., 2021; Jian et al., 2022). Within this research, it was assumed that the aquifer recharge comes from two main sources, i.e., Sava River and precipitation. Groundwater from seven sampling sites (Čp-23, Lg-4, P-3, P-7, Vg-1, Vg-4 and Vg-5/2) shared average values of water isotopic compositions similar to the Sava River. Conversely, the monitoring well Vg-6 showed a water isotopic composition similar to precipitation, while the Lg-1 and Vg-10/2 monitoring sites did not show isotopic signatures similar to either of the two main sources (see Figure 4 in Buškulić et al., 2025). $\delta^{18}O_{H2O}$ and $\delta^{2}H_{H20}$ average values of the Lg-1 and Vg-10/2 diverged from those of the Sava River and precipitation, indicating existence of the different recharge source. These two wells are located the farthest from the Sava River and were likely recharged by water inflow from the southwest part of the aquifer. These are the same two monitoring wells whose hydrogeochemical parameters and molar ratios, in contrast to all other sampling locations, indicated that the nitrate concentrations are likely related to inorganic contamination due to agricultural activities. Two-component mixing model results revealed spatial variability in the contribution of the Sava River and precipitation to aquifer recharge, with values ranging from 19.9% to 83.4% for the Sava River and from 16.6% to 80.1% for precipitation (Buškulić et al., 2025). Vg-6 demonstrated the lowest fraction of the Sava River (19.9%) and the highest fraction of precipitation (80.1%). As expected, precipitation was the primary source of recharge for monitoring well Vg-6, given that well is located in the area of Fluvisols, which are characterized by higher permeability due to their lower clay content and higher sand content (Ružičić et al., 2019b). Given that monitoring well Vg-6 is primarily recharged by precipitation, the interaction between that specific source of recharge, urban runoff and the groundwater system could be the mechanism responsible for causing the elevated Cl^{-}, Na^{+} and K^{+} concentrations in this well. Moreover, the remaining monitoring wells can be grouped as follows: Čp-23, P-3, Vg-4 and Vg-5/2 with the Sava River fraction ranged from 47.1 to 54.2% and precipitation fraction ranged from 45.7 to 52.9%; and Lg-4, P-7 and Vg-1 group where the Sava River fraction ranged from 66.7% to 83.3%, while the precipitation fraction ranged from 16.6 to 33.3% (Buškulić et al., 2025). The Sava River contribution to aquifer recharge is lower compared to previous research (Parlov et al., 2019), likely due to hydrometeorological conditions that prevailed during the sampling period. During the sampling period, from March 2021 to February 2022, the average monthly precipitation was 55.7 mm (Buškulić et al., 2023a), which is 30% lower than the monthly average precipitation of 80 mm characteristic for the study area (Buškulić et al., 2023b). In 2021, precipitation was 26% below average, and in 2022, it was 30% lower than the long-term average (Bačani, 2024). Additionally, the average air temperature also contributed to this, given that warm thermal conditions prevailed during 2021 and very warm during 2022.

Following the initial findings on the origin of nitrate, the origin of nitrate was further determined using nitrate stable isotopes, isotopic values of local nitrogen sources and the Bayesian isotope mixing model (Buškulić et al., 2025). The $\delta^{18}O_{NO3}$ values observed in the groundwater and the Sava River samples were lower than the values characteristic for NO_3^- based fertilizers and atmospheric deposition of NO_3^- (see Figure 6 in Buškulić et al., 2025), confirming no contribution from these two sources. The lack of atmospheric NO_3^- deposition supports the statement that NO_3^-concentrations in the Zagreb alluvial aquifer system cannot be solely attributed to natural sources. The $\delta^{15}N_{NO3}$ values in the groundwater and the Sava River ranged from 2.6 to 18.3‰ and from 4.4 to 13.1‰, respectively. $\delta^{18}O_{NO3}$ values ranged between -6.7 and 9.7‰ for groundwater and from -0.8 to 3.9‰ for the Sava River. Based on these results, the nitrate isotopic composition of groundwater and Sava River samples indicated that NO_3^- contamination during the sampling period originated from following nitrate sources: soil NH_4^+, NH_4^+ in mineral fertilizers and precipitation, and manure/septic waste (see Figure 6 in Buškulić et al., 2025). Among the total number of groundwater samples, 66% of samples exhibited $\delta^{15}N_{NO3}$ isotope signatures higher than 9‰, indicating contamination predominantly originating from manure and/or septic waste. The remaining samples fell within the overlapping range reported for soil NH_4^+, NH_4^+ in fertilizers and precipitation. The $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ isotope signatures for monitoring well Vg-6 confirmed that NO_3^- contamination mainly originated from organic wastes (manure and/or sewage), as 11 out of 12 analyses exhibited $\delta^{15}N_{NO3}$ values higher than 9‰. These findings are consistent with the molar ratios and correlations analyses. However, the isotopic composition of nitrate did not support the influence of mineral fertilizer for monitoring wells Vg-1 and Vg-10/2. In contrast, this method suggested the influence of mineral fertilizer most likely for the P-3, P-7 and Vg-5/2 monitoring wells. Furthermore, the $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values from Lg-4 suggested contamination solely from manure and septic waste, as indicated by $\delta^{15}N_{NO3}$ values higher than 9%. However, such elevated $\delta^{15}N_{NO3}$ values could be the result of denitrification process rather than the source of contamination. The statistical analyses confirmed significant variations in $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ across groundwater samples from different locations ($p < 0.05$).

A Bayesian isotope mixing model was used to quantify spatial and temporal proportional contributions from three local nitrogen sources in the study area (see Table 1 in Buškulić et al., 2025). Regarding spatial variations in the proportional contributions, the model outputs showed a similar pattern across all sampling sites including surface water (see Figure 7 in Buškulić et al., 2025), while there were noticeable seasonal variations in the proportions for groundwater (see Figure 8 in Buškulić et al., 2025). Groundwater and the Sava River showed similar proportional contributions of different local N sources likely due to their continuous mixing. The proportional contributions were ranked as follows: organic wastes > mineral fertilizers > soil (Buškulić et al., 2025). The model was not able to discern which source of contamination, sewage, septic waste or manure, was dominant, as all three organic inputs used in the mixing model had similar isotopic composition. Organic wastes showed the highest proportional contributions, ranging from 56 to 68%, in agreement with results obtained using $\delta^{15}N_{NO3}$ isotope signatures, which illustrated that more than half of groundwater samples indicating contamination due to the application of manure, leakage from the sewage system in urban areas and/or septic tanks leakage in rural areas. The proportional contribution of mineral fertilizers contributed moderately, with a range of 21 to 27%. This partly aligned with the results obtained using other approaches that detected the influence of mineral fertilizers only on individual groundwater sampling sites (e.g., Lg-1 and Vg-10/2). This is not a surprise given that nitrate concentrations represent the result of mixing of individual nitrate contamination sources, which makes it difficult to disentangle by using methods such as molar ratios, correlation analyses and isotopic $\delta^{15}N_{NO3}$ vs. $\delta^{18}O_{NO3}$ bivariate plots. Soil contributions showed the lowest contributions, ranging from 10 to 17% and making it the least significant source which was consistent with the results obtained using other approaches. Regarding seasonal changes, the proportional contribution of organic wastes in groundwater was lowest during summer (75%) and highest during spring (84%) (Buškulić et al., 2025). Conversely, the highest proportional contributions of soil and mineral fertilizers were observed in the summer period (9% and 16%, respectively), while the lowest were in the spring (5% and 11%, respectively). Additionally, for surface water, the model outputs showed a significant difference compared to groundwater, attributed to the fact that only three values per season were available for the Sava River water. Therefore, the limited data available for the Sava River increased the uncertainty of the analysis, making the results unreliable for further consideration.

The results agree with findings from a previous study (Kovač, 2017; Kovač et al., 2018a) which stated that nitrate origin in the Zagreb aquifer system area is predominantly organic. However, findings of this research for the first time cannot exclude mineral fertilizers as the

NO_3^- source and possible cause of elevated NO_3^- concentrations. Coupled with organic wastes (sewage, manure and septic waste), mineral fertilizers are the significant source of nitrate contamination. Thus, the 3rd hypothesis is not confirmed. Given that mineral fertilizers could not be identified as a potential nitrate source at each sampling site using the methods employed prior to the Bayesian isotope mixing model, the mixing model results indicate the importance of employing multi-method approaches and using the isotopic composition of local N sources for more accurate identification of nitrate origin. Additionally, results suggest that biogeochemical process, most likely the nitrification, masked the nitrogen isotopic signature typically associated with mineral fertilizers.

The conceptual models of nitrogen species dynamics through the unsaturated and saturated zone of the Zagreb aquifer system, as the final step of the research, were developed based on the knowledge obtained and interpreted during this study. The models refer only to the area of Eutric Cambisols on the Holocene deposits, illustrating the dominant sources of N and the dominant processes that influence the transformations and dynamics of N. The conceptual model of N species dynamics influenced by only natural factors is shown in Figure 5. Nitrogen enters the system through precipitation deposition as NH_4^+ and NO_3^-. Nitrates being consumed through the assimilation process, while ammonium ions infiltrate downwards. The dominant sources of NO_3^- within the shallow soil are NH_4^+ from precipitation and NH_4^+ originating from mineralization of soil organic N. The rate of nitrification is relatively slow, influenced by factors such as elevated water content, lower pH, the presence of organic carbon and fine-grained sediments. Due to plant root penetration through the shallow soil, NO_3^- and NH_4^+ are taken up by plants through assimilation process. Infiltration of precipitation from the shallow to deeper soil occurs only during periods of intense and prolonged rainfall. NH_4^+ is dominant form of N within soil layer with notable presence of silt and clay, characterized by low permeability and reduced aeration. Due to the higher clay content, NH_4^+ tends to adsorb to the soil particles. The adsorption process helps retain NH_4^+ and prevents its infiltration into deeper soil. In the deeper soil, NO_3^- sources are predominantly linked to soil organic N and its mineralization into NH_4^+. Under specific controlling factors which contribute to aerobic conditions, such as lower water content, elevated pH, the partial presence of organic carbon and significant presence of coarse-grained sediments, NH_4^+ undergoes rapid nitrification and converts into NO_2^- and NO_3^- (dominant forms of N). Nitrogen primarily migrates from the soil zone to deeper parts of the unsaturated zone in the form of NO_2^- and NO_3^- at concentrations lower than 2.7 mg/L and 6.8 mg/L, respectively. It can be assumed that the process of oxidation of NO_2^- to NO_3^- will most likely occur in the unsaturated zone, under aerobic conditions. Thus, nitrogen mostly enters the groundwater of the Zagreb alluvial aquifer system in the form of NO_3^-.

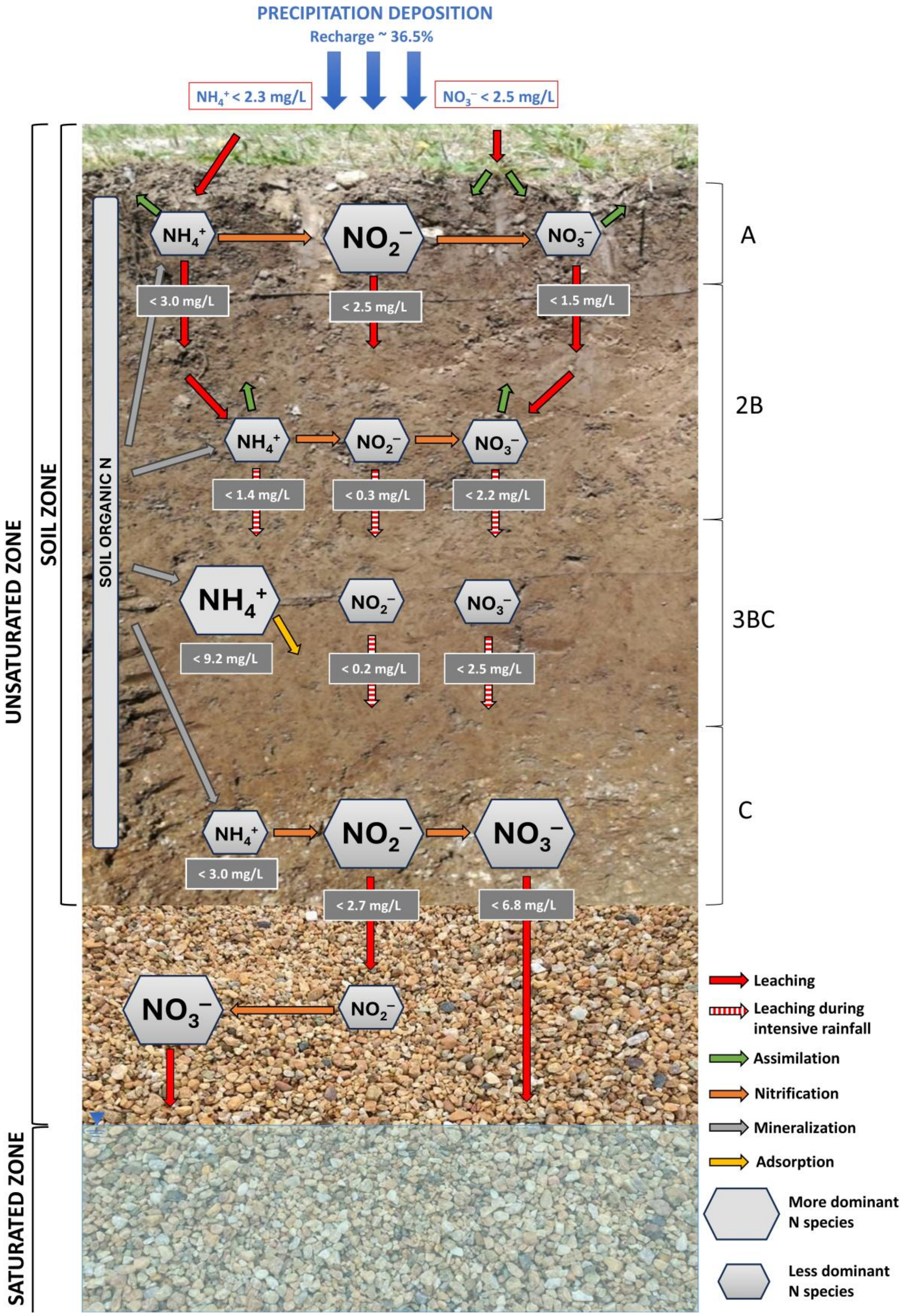

Figure 5. Conceptual model of N species dynamics through the unsaturated and saturated zone of the Zagreb aquifer system within the area of Eutric Cambisols on the Holocene deposits influenced by natural factors.

The conceptual model of N species dynamics influenced by both natural and anthropogenic factors is shown in Figure 6. Given that model refers to the area of Eutric Cambisols on the Holocene deposits, groundwater recharge was estimated without monitoring wells Vg-6 and Vg-5/2 since they located in the area of Fluvisols, as well as without Lg-1 and Vg-10/2, which recharge comes from a different source. Accordingly, groundwater is predominantly recharged by the Sava River with an average contribution of 63.5%. The highest recorded NO_3^- concentration in the Sava River was 6.5 mg/L, which enables the transport of NO_3^- from surface water to the groundwater at concentrations only lower than this value. Average recharge from precipitation amounts to 36.5%. Since Eutric Cambisols exhibit lower permeability, higher capacity for water retention and only high intensity precipitation events enable infiltration to deeper soil layers, precipitation does not represent a significant source of atmospheric NO_3^-. Instead, precipitation is a key mechanism of N species leaching from shallow to deeper parts of soil, especially N forms from anthropogenic sources. The basis for the development of this model was dominant sources and processes of N under natural conditions, to which anthropogenic sources were added and behavior of nitrogen from such sources was assessed. The dominant anthropogenic NO_3^- sources are NH_4^+ mineral fertilizers, septic waste, sewage and manure, while dominant natural NO_3^- sources are soil organic N, precipitation NH_4^+ and Sava River. The dominant processes include infiltration of precipitation (leaching), assimilation, mineralization and nitrification within the shallow soil, as well as leaching during intense rainfall, adsorption, mineralization and nitrification within the deeper soil. Organic N from septic waste, sewage or manure, upon entering the system, undergoes process of mineralization into NH_4^+. The NH_4^+ from organic wastes (such as septic waste, sewage or manure), mineral fertilizers or precipitation deposition infiltrates into deeper soil or undergoes process of nitrification. The highest recorded NO_3^- content in the deepest soil was 6.8 mg/L and the maximum NO_3^- content in the Sava River was 6.5 mg/L, which represents the maximum NO_3^- concentration that could infiltrate from natural environmental source towards the aquifer. Other contributions to groundwater nitrate concentrations, which are below 31.9 mg/L, must be linked to the anthropogenic sources. Given that no significant concentrations of nitrites were measured in the groundwater, this further confirms the process of oxidation of NO_2^- to NO_3^- in the unsaturated zone.

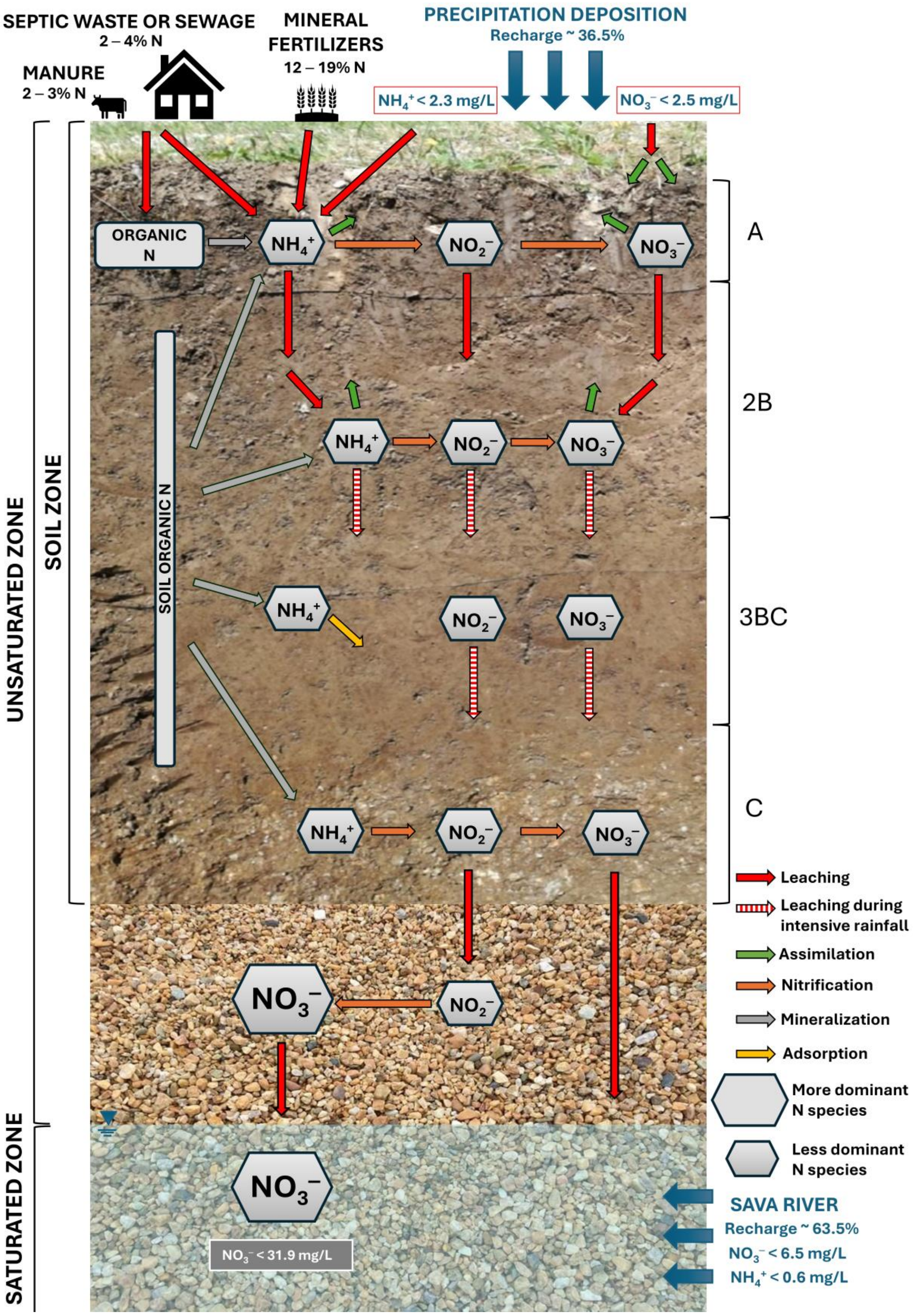

Figure 6. Conceptual model of N species dynamics through the unsaturated and saturated zone of the Zagreb aquifer system within the area of Eutric Cambisols on the Holocene deposits influenced by natural and anthropogenic factors.

A multi-method approach has resulted in a more detailed determination of NO_3^- origin and proved to be crucial in identifying nitrate sources. It is not surprising that in dynamic systems such as unconfined aquifers, the separate application of the individual methods cannot provide a clear understanding. In such systems, there are various sources of contamination, and it is necessary to use and combine numerous types of data and methods if more reliable conclusions about nitrate origin and processes that affect them want to be achieved. The findings highlighted the importance of employing a diverse range of analytical methods to obtain reliable and comprehensive understanding of NO_3^- contamination. By integrating multi-method approaches, stakeholders can better understand the complexity of groundwater contamination and take more targeted actions to safeguard the water supplies for future generations.

4. CONCLUSION

The Zagreb aquifer system is a crucial groundwater source for potable water and contains strategic groundwater reserves of the Republic of Croatia. However, the rapid growth of the City of Zagreb and industrial development have a significant impact on groundwater quality, leading to high vulnerability to contamination through recent years. The conducted research provided a comprehensive understanding of nitrate dynamics in the unsaturated and saturated zone within the area of Eutric Cambisols on the Holocene deposits by utilizing historical and recent soil water, precipitation, groundwater, surface water, soil and contaminant source data. Samples were collected in the field, analysed and interpreted using various methods. All research objectives were achieved, resulting in conceptual model of nitrogen species dynamics in the context of nitrogen species content, biogeochemical and physical processes, nitrate origin and recharge sources for the entire system (soil, unsaturated and saturated zone). Through the investigations presented in three scientific papers, two research hypotheses were confirmed, whereas one hypothesis was not. The key findings of this research are:

- ambient background values, obtained using the model-based objective methods, must be statistically significant and determined using as wide as possible dataset. Reducing the size of the dataset, i.e., data selection, is acceptable only in case of statistically non-significant value obtained using original data without selection. If some of the model-based objective method yields a statistically non-significant result, further data selection and re-estimation of the ABV should be made until result are proven to be statistically significant. Moreover, ABV of nitrate concentrations determined at the level of the Zagreb aquifer system, differs from the background value of nitrate determined for the catchment area of the Velika Gorica well field (from 7.1 to 7.5 mg/L).
- the Sava River is the main source of recharge of the Zagreb aquifer system, but the results also indicate that in the southern part of the aquifer groundwater flows from the south-western part of the aquifer and is recharged from the surrounding hills.
- the combination of a nitrous oxide isotopic analyzer and the titanium(III) reduction method provides reliable measurements of $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$, with optimal stability achieved under specific target N_2O gas concentration inside the analyser, reagent-to-sample ratio during sample preparation and reaction time.
- the shallowest soil horizon (A) showed higher NO_2^- concentrations indicating the dominance of the nitritation process. Dominant NH_4^+ in the soil horizon 3BC was a

result of notable presence of silt and clay, characterized by low permeability and reduced aeration. In the deepest soil horizon (C), from 0.9 to 1.17 meters of soil profile, N was mainly present as NO_2^- and NO_3^- which confirmed the dominance of soil NH_4^+ nitrification. This outcome is attributed to lower soil moisture content, elevated pH, the partial presence of TOC and significant presence of gravel, which contributes to aerobic conditions suitable for the process of nitrification. Hence, soil moisture content, pH, organic carbon availability and soil texture are important and key controlling factors influencing the vertically distribution of N species in the soil zone. Furthermore, the results suggested that N primarily migrates, from 1.17 meters to deeper parts of the unsaturated zone, in the form of NO_2^- and NO_3^-. Considering that NO_3^- are very soluble and have leaching potential, the aerobic conditions of the deepest soil horizon promote nitrate stability and the potential for their transport. Findings of this research indicate that it is extremely important to monitor parameters within the soil horizons, especially in the deepest one, in order to get information about dominant N species which can enter the unsaturated zone and aquifer. Long-term goals should involve modeling transport of N compounds in soil, unsaturated zone and saturated zone in order to enhance comprehension of the entire aquifer system and facilitate sustainable management of the Zagreb aquifer system.

- the origin of nitrate in the catchment area of the Velika Gorica well field was mainly related to the organic sources and the process of the nitrification prevailed in the most observed locations. The denitrification process occurred at only one specific site and its influence on the nitrate dynamics in the groundwater is likely negligible. For the first time, isotopic composition of local nitrogen sources had been determined, which showed that contribution from soil and mineral fertilizer exists and varies from both spatial and temporal point of view. Furthermore, determination of isotopic composition of NO_3^- sources present the crucial part from the interpretation point of view. From that perspective, more detail inspection of NO_3^- sources in future research must be done in the study area with focus to the evaluation of isotopic composition of mineral fertilizers, especially those which contain both ammonium and nitrate nitrogen in their formula. Further research should monitor and investigate the soil zone located in an area influenced by anthropogenic sources of nitrate, as this would obtain more details about nitrate behavior in the study area.
- multitude of analyses is necessary when determination of NO_3^- origin and related processes presents the main goal of the research. Although hydrogeochemical, molar

ratios and correlation analyses were found to be useful in previous research, in the areas where NO_3^- concentrations are the consequence of different sources of contamination, it must be considered with great caution. Such methods clearly suggested that most part of nitrate concentrations were associated with the multiple contamination inputs, which turned out to be correct, but at the same time did not recognize the influence of mineral fertilizer. Even though they can be indicative and helpful within the interpretation procedure, their usage without nitrate isotopic composition should be avoided. Therefore, the importance of employing multi-method approaches for more accurate NO_3^- source identification in regions facing similar environmental challenges is clear.

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