NITRATE MIGRATION IN PLANT-SOIL-GROUNDWATER SYSTEM

Martina Šturm

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Evaluation Board:

Assoc. Prof. Dr. Marina Pintar, Member, Biotechnical Faculty, Jamnikarjeva ulica 101, Ljubljana

Assist. Prof. Dr. Nina Kacjan Maršić, Member, Biotechnical Faculty, Jamnikarjeva ulica 101, Ljubljana
Assist. Prof. Dr. Nives Ogrinc, Member, Jožef Stefan Institute, Jamova cesta 39, Ljubljana

MEDNARODNA PODIPLOMSKA ŠOLA JOŽEFA STEFANA
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GIBANJE NITRATA V SISTEMU RASTLINA-TLA-PODZEMNA VODA

Doktorska disertacija

Supervisor: Assist. Prof. Dr. Sonja Lojen

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Abstract

Nitrogen (N) is an essential nutrient for plant growth and one which has the greatest effect on the yield. Since effective indigenous sources are insufficient by far to support current levels of global agricultural outputs, N is intensively applied by farmers in the form of N-based fertilisers. However, excessive rates of fertiliser N to obtain maximum levels of crop production are associated with potential environmental, health and economic risks. The pollution of groundwater by nitrate is reported to be one of the most serious issues of environmental concern in Slovenia, considered in the context of agricultural pollution. Though it is not possible to prevent nitrate leaching, it has been demonstrated that improved management practices can reduce the potential for nitrate contamination of groundwater as a result of increased fertiliser N use efficiency.

The aim of this doctoral work was to follow the migration of nitrate in the plant – soil – groundwater system on an experimental field cultivated with white cabbage (*Brassica oleracea* var. *capitata* L.) in order to find the best N management practice for growing brassicas which would give optimal yields in terms of quantity and quality and at the same time pose the least negative environmental risk (N losses). For the first time in Slovenia, the effect of different fertilizer types and the effect of the so far poorly studied split and combined usage of synthetic (inorganic) and organic fertilizers on the isotopic composition of total N ($\delta^{15}N$) were investigated, and thus the suitability of the $\delta^{15}N$ signature as a tool to distinguish between organic and conventionally grown vegetables was evaluated.

On an experimental field, located above a shallow groundwater body and cultivated with white cabbage, four different fertilization and irrigation practices were compared, namely: pre-plant broadcast fertilization and drip irrigation covering 50% of the crop's water requirements, fertigation with drip irrigation covering 100% of the crop's water requirements, farmer's practice consisting of pre-plant broadcast fertilization and irrigation on the day before and after transplanting using a tank sprinkler and an unfertilized control with farmer's practice of irrigation. Nitrate fertilizer, labelled with ^{15}N isotope, was used as a tracer. Nitrate migration and distribution was followed by analysing nitrate and total N in plants, soil, soil solution and groundwater for content and isotopic composition of. In order to do so, different methods for isolation of nitrate N from groundwater (anion exchange method) and soil solution (Teflon trap diffusion method) were introduced, which in Slovenia have not been used previously. The studied management practices were evaluated based on N mass balance calculations (inputs – outputs) and yield quality in terms of nitrate content.

The effect of different fertilizers on $\delta^{15}N$ levels of vegetables was studied in a greenhouse pot experiment with lettuce (*Lactuca sativa* L.), grown under seven different treatments comprising of single and split application of synthetic (inorganic) or organic fertilizer, as well combined usage of both fertilizers. Furthermore, in this work $\delta^{15}N$ signatures of 14 different organic and conventionally grown vegetable varieties commercially available on the Slovenian market, as well as $\delta^{15}N$ signatures of some synthetic (inorganic) and organic fertilizers commonly used in Slovenia were determined, which should serve as a baseline for future studies.

The results of the field experiment did not confirm our hypothesis that fertigation with drip irrigation covering 100 % of the crop's water requirements is the best practice for growing cabbage. Under the given environmental conditions, farmer's practice with pre-plant broadcast fertilization and irrigation on the day before and after transplanting was found to be the most appropriate practice from the economic (highest yield) as well as the environmental (lowest N losses) points of view. The results obtained in the greenhouse pot experiment provided an important contribution to our knowledge in regard to the effect on plant $\delta^{15}N$ of split N fertilization, which could cover up the use of prohibited synthetic (inorganic) N fertilisers in organic production. The experiment revealed that based on $\delta^{15}N$ it is possible to differentiate between organic and conventionally grown produce when fertiliser is applied in a single application, whereas in a split application it is not possible to detect low or moderate rates of synthetic fertiliser illegally applied to a lettuce crop. The analysis of different vegetable varieties of organically and conventionally grown vegetables from the Slovenian market confirmed that due to several limitations of the method applied, the $\delta^{15}N$ signature of the produce could only be used as an additional supportive and not as a single definitive tool to control organic vegetable production.

Povzetek

Dušik (N) je esencialno hranilo za rast rastlin, ki v največji meri vpliva na količino pridelka. V želji po pridelavi zadostnih količin hrane za potrebe stalno naraščajoče populacije ga kmetje intenzivno in pogosto v prekomernih količinah dodajajo v obliki gnojil, kar lahko vodi v potencialne okoljske in zdravstvene probleme ter ekonomsko škodo. V Sloveniji predstavlja onesnaženje podzemne vode z nitrati enega izmed najbolj perečih problemov s stališča kmetijskega onesnaževanja. Spiranja nitrata ni mogoče popolnoma preprečiti, vendar pa z uvajanjem dobrih kmetijskih praks, ki omogočajo večji odvzem N z rastlinami in boljši izkoristek gnojila, lahko to spiranje omejimo.

Namen doktorskega dela je bil s pomočjo sledila v obliki nitratnega N gnojila, obogatenega s težjim N izotopom ^{15}N, slediti migraciji nitrata v sistemu rastlina – tla – podzemna voda in na ta način najti najprimernejšo kmetijsko prakso za gojenje kapusnic, ki ob minimalnem negativnem vplivu na okolje (izgube N) daje optimalen pridelek v smislu količine in kakovosti. Prvič v Sloveniji smo proučevali tudi vpliv uporabe različnih gnojil ter vpliv do sedaj zelo slabo raziskanega dognojevanja in kombinirane uporabe organskih in sintetičnih gnojil na izotopsko sestavo celotnega dušika ($\delta^{15}N$) v zelenjavi in s tem ovrednotili primernost uporabe $\delta^{15}N$ v zelenjavi kot orodja za ločevanje med ekološko in konvencionalno pridelano zelenjavo.

Na poskusnem polju smo na primeru mladega zelja (*Brassica oleracea* var. *capitata* L.) med seboj primerjali štiri različne prakse gnojenja in namakanja: založno gnojenje s kapljičnim namakanjem, ki pokriva 50 % potreb rastlin po vodi, fertigacijo s kapljičnim namakanjem, ki pokriva 100 % potreb rastlin po vodi, kmetovo prakso, to je založno gnojenje in zalivanje z razpršilcem ter negnojeno kontrolo s kmetovo prakso zalivanja. Gibanju in porazdelitvi nitrata smo sledili s pomočjo analize koncentracij in izotopske sestave celotnega dušika in nitrata v rastlini, tleh, talni raztopini in podzemni vodi. V ta namen smo uvedli metodi za izolacijo nitratnega N iz vzorcev podzemnih vod (metoda z ionskimi izmenjevalci) ter talne raztopine (difuzijska metoda), ki se v Sloveniji pred tem nista uporabljali. Proučevane kmetijske prakse smo ovrednotili na podlagi izračuna masne bilance N (vnosi–iznosi) ter kakovosti pridelka glede na vsebnost nitrata.

Vpliv različnih gnojil na $\delta^{15}N$ v pridelku smo proučevali na primeru lončnega poskusa s solato (*Lactuca sativa* L.), pri katerem smo izvedli sedem različnih obravnavanj, ki so zajemala tako enkratno aplikacijo organskih in sintetičnih N gnojil kot tudi dognojevanje z različno kombinacijo obeh gnojil ter negnojeno kontrolo. Prvič so bile v okviru doktorskega dela določene tudi $\delta^{15}N$ vrednosti v 14 vrstah komercialno dostopne konvencionalno in ekološko pridelane zelenjave s slovenskega trga ter $\delta^{15}N$ vrednosti v nekaterih sintetičnih in organskih gnojilih, pogosto uporabljenih v Sloveniji, ki naj bi služile kot temeljno izhodišče za nadaljne raziskave.

Rezultati poljskega poskusa niso potrdili postavljene hipoteze, da je fertigacija v kombinaciji s 100 % pokrivanjem potreb rastlin po vodi, najprimernejša praksa za gojenje kapusnic. Pri danih okoljskih pogojih je namreč ustaljena kmetova praksa z založnim gnojenjem ter zalivanjem dan pred in po presaditvi sadik dosegla največji pridelek ob najmanjših izgubah N v okolje in je tako najprimernejša praksa za pridelovanje mladega zelja na obravnavanem tipu tal. Poskus v rastlinjaku je pokazal, da v primeru enkratnega gnojenja z organskimi in sintetičnimi gnojili na podlagi izotopskega zapisa N v rastlini

lahko razlikujemo med solato, gnojeno samo s sintetičnim oz. organskim gnojilom, da pa se z dognojevanjem z različno kombinacijo obeh gnojil te razlike zmanjšajo ali celo zabrišejo, tako da na podlagi $\delta^{15}N$ v rastlini ni več mogoče zanesljivo ločiti med ekološko in konvencionalno pridelavo. Slednja ugotovitev predstavlja pomemben doprinos k razumevanju obravnavane problematike. Tudi analiza ekološko in konvencionalno pridelane zelenjave s slovenskega trga kaže na to, da se zaradi določenih pomanjkljivosti metode $\delta^{15}N$ zapis v pridelku lahko uporablja le kot dodatno, podporno in ne kot edino orodje za kontrolo ekološke pridelave zelenjave.

Abbreviations

ANOVA	=	Analysis of Variance
ANCA–SL	=	Automated nitrogen and carbon analyser with a preparation module for solid and liquid samples
atom % ^{15}N	=	Absolute abundance of ^{15}N isotope in per cent
CRM	=	Certified reference material
C/N	=	Organic carbon to total nitrogen ratio
DM basis	=	Dry matter basis
DAT	=	Days after transplanting
δ	=	Delta, relative isotopic composition, value given in parts per thousand or per mil (‰)
$\delta^{15}N$	=	Isotopic composition of nitrogen
FIA system	=	Flow Injection Analysis system
FW	=	Fresh weight
IRM	=	In–house reference material
IRMS	=	Isotope Ratio Mass Spectrometry
LSD	=	Least significant difference
LQD	=	Limit of quantification
KAN	=	Potassium ammonium nitrate fertiliser
MAFF	=	Ministry of Agriculture, Forestry and Food
$N\text{-}NO_3^-$	=	Nitrate nitrogen
n.d.	=	Not determined
Ndff	=	Nitrogen derived from the fertiliser
Ndfs	=	Nitrogen derived from soil
N:P:K	=	Fertiliser containing three main nutrients, i.e. nitrogen (N), phosphorus (P) and potassium (K)
OJ RS	=	Official Journal of the Republic of Slovenia
PTFE	=	Teflon – Polyfluoroethylene
SD	=	Standard deviation
SDW	=	Subsample dry weight
SFW	=	Subsample fresh weight
T	=	Temperature
UV–VIS Spectrometry	=	Ultraviolet–Visible Spectroscopy

1 Introduction

Nitrogen (N) is an essential nutrient for plant growth and in the desire to produce more food, farmers apply it intensively, and often in excessive quantities, in the form of nitrogen–based fertilisers. Since only a fraction of the applied fertiliser N (on average less than 50%) is taken up by the crop, the remainder is subject to loss, representing both economic and environmental risk. As huge quantities of fertiliser N are involved in agricultural production systems, the economic losses are enormous. In addition, excessive rates of fertiliser N to obtain maximum levels of crop production are associated with potential environmental, ecological and health risk. If fertiliser application exceeds plant demands and the denitrification capacity of the soil, nitrate not taken up by the crop may potentially contribute to ground and surface water pollution through nitrate leaching and soil erosion (Gastal and Lemaire, 2002, Wang et al., 2002, Chen et al., 2004, Almasri and Kaluarachchi, 2007 and references therein), possibly raising the nitrate concentrations in groundwater above the maximum allowed level of 50 mg L^{-1}, set by the European Commission Nitrate Directive (91/676/EEC). In some areas, problems related to the presence of abundant N from anthropogenic sources (including, but not limited to, fertilisers) have been reported. Estimates of the global anthropogenic inputs of about 80 million tons per year and a global agricultural output of 23 million tons N per year imply low overall efficiency of N utilization in agriculture for food production. The gaseous losses to the atmosphere are estimated at 26–60 million tons N per year, whereas between 32 and 45 million tons N per year are received by ground– and surface waters through leaching and runoff (van Cleemput et al., 2008 and references therein) (Figure 1).

In Slovenia, average nitrogen input from mineral fertilisers is low (47 kg ha^{-1}), however the average N input from organic manure is higher (90 kg ha^{-1}, in regions with higher stocking rate, e.g. Maribor, even up to 138 kg ha^{-1}). The average nitrogen deposition from the atmosphere is 17 kg N ha^{-1} (Leskošek, 1993 – Kmečki glas, in Matičič, 1999). In all regions, net–balance surplus is below 100 kg ha^{-1}, with an average net–balance surplus about 50 kg ha^{-1} (Matičič, 1999).

Nitrate is often the major source of nitrogen available to higher plants (Marschner, 1995). Its uptake and distribution in plants is of major importance with respect to both environmental concerns and the quality of plant products (Gastal and Lemaire, 2002). The higher the N uptake, the less N is remaining in the soil available for potential leaching or gaseous losses. However, high uptake can on the other hand cause excessive nitrate accumulation in plants, especially in most leafy vegetables (Chen et al., 2004), and as reported in Commission Regulation (EC) No.1881/2006, vegetables are the major source of nitrate in the human intake. Nitrate is relatively non–toxic but its metabolites (nitrite) may produce a number of deleterious health effects (e.g. methaemoglobinaemia, carcinogenesis) (Santamaria, 2006), so care should be taken, especially for pregnant women and babies, not to exceed the acceptable daily intake of 3.65 mg nitrate per kg body weight.

Slovenia is a rich country as far as abundance of water is concerned. Groundwater is a source used for water supply in our country. The pollution of surface and ground waters is mostly caused by the chemicals used in industry, urban wastes and agriculture. Agricultural pollution sources in Slovenia are intensive farming practices, particularly fertilisation, livestock rising and plant protection. The pollution of groundwater by nitrate is one of the most serious issues of environmental concern in Slovenia, considered in context of agriculture

pollution. A little less than one third of our territory is agricultural land (600 000 ha) and only 38% is arable land. An important feature of the Slovenian territory is the predominance of hilly areas. Over a total area of agricultural land, plains, where most of intensive agriculture takes place, account only for 28% (Matičič, 1999). From this reason, intensive vegetable production is frequently performed in fields located above shallow groundwater bodies which are very susceptible to nitrogen leaching and prone to pollution of groundwater. Hence, in order to reduce the burden to the natural environment caused by excessive N fertilisation, optimal crop cultivation methods are sought to diminish the negative effect of N compounds on the environment, and ensure high and quality yields at the same time (Rahn, 2002). While it is not possible to prevent nitrate leaching, improved management practices leading to increased fertiliser N use efficiency can reduce the potential for nitrate contamination of groundwater (Bijay-Sighn et al., 1995; Cassman et al., 2002; Di and Cameron, 2002).

In spite of the development of new technologies for improving the efficiency of use/recovery of applied fertiliser N by crops over recent decades, mainly in the developed world, fertiliser N use efficiency has remained low in many regions of the world due to land degradation, changes in land use (and cropping systems) and the use of inappropriate land management practices in response to socioeconomic and other pressures on agricultural production systems. Fertiliser N not taken up by the crop to which it is applied is very likely to be lost to the environment. This is an important issue for developing sustainable agricultural systems, in view of the need to support further intensification of agriculture to produce enough food for the growing population while preserving the natural resource base. It is now widely accepted that efficiency of fertiliser use should be evaluated not only agronomically (recovery and yield increases, and product quality), but also environmentally and socioeconomically. The role of fertiliser N should be evaluated within the context of all potential inputs of N to the system as a whole (van Cleemput et al., 2008).

Compared with more intensive conventional farming systems, organic farming is considered an effective mean of reducing nitrogen losses (Berntsen et al., 2006). Furthermore, organically produced foods are reported to be produced in a more environmentally compatible manner as well as safer for health of the consumer. Due to increased concern about food quality and safety, demand as well as supply of organic produce is constantly increasing. With increasing demand for organic produce in our diet, there is a need to find suitable authentication tools to rapidly distinguish if organically certified produce is indeed organically grown, and not simply labelled as such (Woese et al., 1997; Siderer et al., 2005) and produced with synthetic (inorganic) fertilisers (Rogers, 2008). Nitrogen isotopic signature of the produce is reported to be a promising indicator.

1.1 Nitrogen cycle in agricultural system

Nitrogen is one of the most important nutrients for crop growth, second only to water. All vital biological processes are related to the existence of functional plasma, of which N is a basic constituent (protein, nucleic acids). N is also a basic constituent of many other compounds of primary physiological importance to plant metabolism, such as chlorophyll, nucleotides, proteins, alkaloids, enzymes, hormones and vitamins (Marchner, H. in Zapata, Chapter 1, p 1). In agricultural systems, N is obtained from the soil through mineralization of soil organic matter and from external sources, both organic and inorganic (Zapata, 2008). However, N inputs from indigenous sources are insufficient by far to support current levels of global agricultural output. Therefore, they must be supplemented by applications of N fertilisers (IFA, France, 2007). Nitrogen is the major nutrient which can be controlled by the producer.

On Earth, there are two pools of N, with relatively little exchange between them: the

gaseous dinitrogen (N_2, relatively inert) of the atmosphere, and of N that is chemically bound to other elements such as carbon, hydrogen or oxygen and has been described as "reactive nitrogen" for its tendency to react with other elements (Galloway et al., 2004). It exists in many different chemical forms (different oxidation states) and passes around natural and agricultural ecosystems in a cycle (Figure 1). Put simply, N moves from the soil to the plant, and back from the plant to the soil, often with animals or humans as intermediates. The real situation is however more complex as N compounds undergo a number of transformations in the soil (mineralization, immobilization, nitrification and denitrification) and are exchanged between soil and the atmosphere (through volatilization, denitrification, biological N fixation, atmospheric deposition) and between soil and the hydrosphere (through leaching, erosion/runoff, irrigation). These transformations and fluxes constitute the soil N cycle. In natural ecosystems, the cycle is more or less closed, with N inputs balancing N losses. In agricultural systems, the cycle is disturbed by the export of substantial amounts of N in harvested products. Consequently, application of fertilisers containing N and other crop nutrients is essential to balance inputs and outputs and so maintain or improve soil fertility, increase agricultural productivity and, in turn, preserve natural ecosystems and wild habitats from conversion to farming (IFA, France, 2007).

The various forms of nitrogen determine its availability to plants or whether nitrogen escapes and is no longer available to them. The presence of useable nitrogen and its losses affects the sustainability of production. As mentioned before, mismanaged nitrogen can result in economic loss to the producer, environmental repercussions, or both. For an optimal yield, the N supply must be available according to the needs of the plant, matching its pattern and total amount (Zapata, 2008).

Chemical forms of nitrogen:

- Inorganic forms:
  - **Nitrogen gas (N_2)** → makes up 78 per cent of atmosphere. It is not directly available for use by plants but is directly used in nitrogen fixation (e.g. legumes has developed symbiotic systems with N_2–fixing bacteria) and industrial fertiliser manufacture.
  - **Ammonia (NH_3)** → in this form the nitrogen is unavailable to plants. It is used as a building block of N fertilisers.
  - **Nitrate (NO_3^-)** → is the most common form available to plants. In this form, nitrogen is mobile, leachable and usually the end product of mineralisation.
  - **Ammonium (NH_4^+)** → is a less common plant–available form of nitrogen when compared to nitrate but is a preferred nitrogen source. Plants use less energy for uptake in this form. There are many beneficial side effects and nitrogen in this form is less likely to be lost than in other forms.
  - **Nitrite (NO_2^-)** → is an intermediate in the conversion of ammonium to nitrate. It is a less common plant–available form but more toxic to plants and more prone to gaseous losses than nitrate.
  - **Nitrous Oxide (N_2O)** → is a greenhouse gas and is lost through denitrification and may be harmful to the ozone layer.
  - **Nitric Oxide (NO)** → this form is lost through denitrification and may be harmful to the ozone layer.
- Organic nitrogen compounds (e.g. amines, proteins and nucleic acids) are complex and unavailable to plants. They are the end products of immobilization.

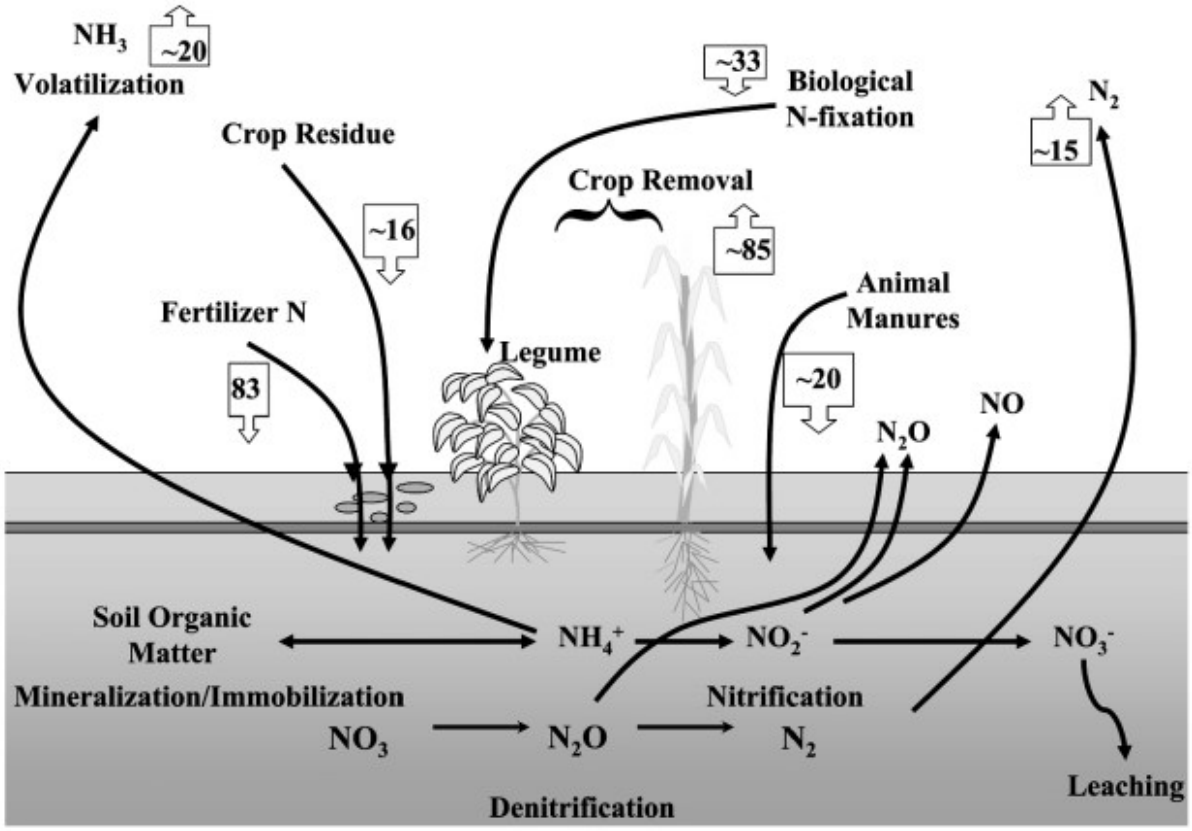


Figure 1: *A simplified view of the nitrogen cycle in crop production with estimated global N flows* (inputs and losses, million tonns N year^{-1}) (From Mosier et al., 2004).

Plants use nitrogen by absorbing either nitrate or ammonium through the roots. Nitrogen is readily transported through the plant from older tissue to younger tissue. Therefore, a plant deficient in nitrogen will show yellowing in the older leaves first due to the underdevelopment or destruction of chloroplasts and an absence of the green pigmented chlorophyll (http://www.hsc.csu.edu.au/agriculture/production/3363/nitrogen.htm). Lack of chlorophyll inhibits the capacity of the plant to assimilate CO_2 and synthesise carbohydrates, leading to poor and premature flowering and fructification, with shortening of the growth cycle. N deficient plants respond quickly to the addition of N fertilisers if applied in a timely manner and properly. Adverse effects on annual plants caused by early–stage lack of N cannot usually be corrected by late application of N (Zapata, 2008 and references therein).

N cycling in soil is closely related to organic matter turnover. Since there are no minerals containing bioavailable N in soil, reserves of N depend on the soil organic matter content. Thus, micro–organisms are responsible for soil–N transformations, which play a key role in determining the availability of N for plant growth and crop production. Mineral N is highly mobile in the soil and, if not taken up by plant roots and microbes, it can be lost through leaching and gaseous emissions, mainly by denitrification and volatilization, creating environmental hazard. Changes in the N cycle associated with excessive soil–N loading can have detrimental effects on terrestrial and aquatic ecosystems, such as eutrofication, algal blooms and “dead zones” (oxygen depleted coastal waters) as well as surface waters and groundwater contamination, increased greenhouse gas emissions, etc. Similarly, the use of reactive (fixed) N affects human health both in positive and in negative ways (see Chapter 1.1.1), depending on the rates of reactive N used in the ecosystem. Negative health effects of highly reactive N are both direct (pollution of air and water) and indirect (ecological feedback to disease) (Zapata, 2008 and references therein).

1.1.1 Impact of nitrate on human health

As mentioned previously, nitrate is a naturally occurring form of nitrogen and is an integral part of the nitrogen cycle in the environment. It is found in the air, soil, water and food (particularly in vegetables) and is produced naturally within the human body. Nitrate is also used as a food additive (e.g. meat), mainly as a preservative anti microbial agent (Santamaria, 2006 and references therein).

Until recently nitrate was perceived as a purely harmful dietary component which causes infantile methemoglobinaemia (known also as the blue baby syndrome), carcinogenesis (the formation of N–nitroso compounds), and possibly even teratogenesis (birth defects). In addition, Parslow et al. (1997) found a positive relationship between the incidence of childhood–onset insulin–dependent diabetes mellitus and levels of nitrate in drinking water. Nitrate alone is non–toxic, but approximately 5% of all ingested nitrate is converted in saliva and the gastrointestinal tract to the more toxic nitrite. The only chronic toxic effects of nitrate are those resulting from the nitrite formed by its reduction by bacterial enzymes (Santamaria et al., 2006, and references therein). Vegetables, an important component of the human diet and a major source of nitrate (Table 1), constitute nearly 72 to 94% of the average daily human dietary intake (Dich et al, 1996; in Anjana et al., 2007).

Some recent studies (Santamaria, 2006 and Anjana et al., 2007) on the other hand, suggest that nitrate is harmless and rather beneficial, which is based on the hypothesis that nitric oxide (NO) formed in the stomach from dietary nitrate has a antimicrobial effects on gut pathogens and a role in host defence. Some of these studies have revealed a negative correlation between nitrate intake and gastric cancer because vegetables are an excellent source of vitamins, minerals and biologically active compounds, while others report the effect of nitrate on the reduction of hypertension and cardiovascular diseases.

However, the studies reporting potential beneficial effects of nitrate are still scarce, hence an intake of vegetables and consumption of drinking water (max allowed level for groundwater is 50 mg NO_3^-–L^{-1}, set by the European Commission Nitrate Directive (91/676/EEC)) with such a high nitrate content that acceptable daily intake (3.65 NO_3^- per kg of body weight) is exceeded for a prolonged period, should be avoided (Boink and Speijers, 2001). Thus, in order to gain as much as possible from the indisputable benefits of vegetables, a reduction in nitrate levels is highly desirable for consumers and probably profitable for farmers (Auserwald et al., 1999; Santamaria, 2006).

Table 1: Classification of some frequently grown vegetables according to NO_3^- content (mg kg^{-1} FW; Santamaria, 2006).

Very low (<200)	Low (200–500)	Middle (500–1000)	High (1000–2500)	Very high (>2500)
Asparagus	Broccoli	Cabbage	Celeriac	Celery
Garlic	Carrot	Radicchio	Endive	Lamb's lettuce
Onion	Cauliflower	Turnip	Kohlrabi	Lettuce
Green bean	Cucumber		Leek	Radish
Pea	Pumpkin		Parsley	Red beetroot
Pepper				Spinach
Potato				Swish chard
Tomato				

1.1.2 Slovenian legislation dealing with nitrates in drinking water and foodstuffs

There are several regulations in force in Slovenia that are supposed to control water and food quality in connection with nitrates (Table 2). Slovenian legislation is quite strict as far as standards on drinking water, food quality or quality of agricultural products is concerned regarding nitrate (Matičič, 1999). Slovenia developed Operative programme for environmental protection with nitrates from agriculture use for years 2004–2008 which is focused on nitrate pollution input into the water from agriculture sources (Kanduč and Šturm, 2010).

Table 2: Legislation dealing with nitrates in drinking water, foodstuffs and soil.

Matrix	Legislation
Groundwater	Decree concerning the protection of waters against pollution caused by nitrates from agricultural sources Official Journal of the Republic of Slovenia (OJ RS) No. 113/09.
	Operational programme for the protection of water against pollution caused by nitrates from agricultural production for 2004-2008.
	Decree on the quality of underground water, OJ RS, No. 11/02, 41/2004 ZVO-1, 100/2005.
	Decree on the water protection zone for the aquifer of Ljubljansko polje, OJ RS, No. 120/04, 7/2006.
Soil	Decree on the limit input concentration values of dangerous substances and fertilisers in soil, OJ RS, No. 84/05, 62/2008, 113/2009.
	Decree on the input of hazardous substances and plant nutrients into the soil, OJ RS, No. 68/96, 35/2001.
	Guidelines (Guidelines) concerning good agricultural practice in manuring, OJ RS, No. 34/2000, 130/2004.
	Mineral Fertilisers Act, OJ RS, No. 29/2006.
	Regulation on statutory management requirements and good agricultural and Environmental conditions for farming, OJ RS, No. 21/2005, 114/2005, 76/2006, 34/2007.
	Regulation about cross compliance, OJ RS, No. 11/2009, 7/2010.
Foodstuffs	Rules on contaminants in foodstuffs, OJ RS, No. 69/2003, 20/2004, 17/2005, 29/2007.

1.2 Principles and applications of nitrogen isotopes in fertiliser experiments

1.2.1 Nitrogen isotopes

Element N has six isotopes, i.e. atoms with the same atomic number (7 protons) but differing in mass number (number of protons and neutrons in the nucleus) varying from 12 to 17. Of the six isotopes, ^{15}N and ^{14}N are stable isotopes while others are radioactive (undergoing disintegration or decay, emitting radiation) with relatively short half–lives, therefore making it impossible to conduct experiments with plants during growth stage (van Cleemput et al., 2008).

The naturally occurring isotope ratios of $^{15}N/^{14}N$ are measured against atmospheric N_2 as a reference and are termed $\delta^{15}N$ and expressed as delta (δ) values, as follows:

$$\delta^{15}N_{sample}\ (‰) = \left(\frac{^{15}N/^{14}N_{sample}}{^{15}N/^{14}N_{standard}} - 1\right) \times 1000 \qquad (1)$$

where standard denotes atmospheric nitrogen with a $^{15}N/^{14}N$ ratio of 0.00368 (i.e. $^{15}N/^{14}N$ = 1/272) and a $\delta^{15}N$ value of 0 ‰.

There is a persisting and perhaps widespread view that the behaviour of ^{15}N in soils and plants is too complex to permit variations in its natural abundance to be used as a tracer or even as a probe to explore plant–soil relationships in natural ecosystem (Hauck et al., 1972; Stewart, 2001). Hence, ^{15}N enriched (or depleted) fertilisers are being produced commercially by different processes of separation (e.g. distillation process, laser separation, gaseous diffusion process, centrifuge process) (Figure 2), which can be used as tracers in various types of experiments.

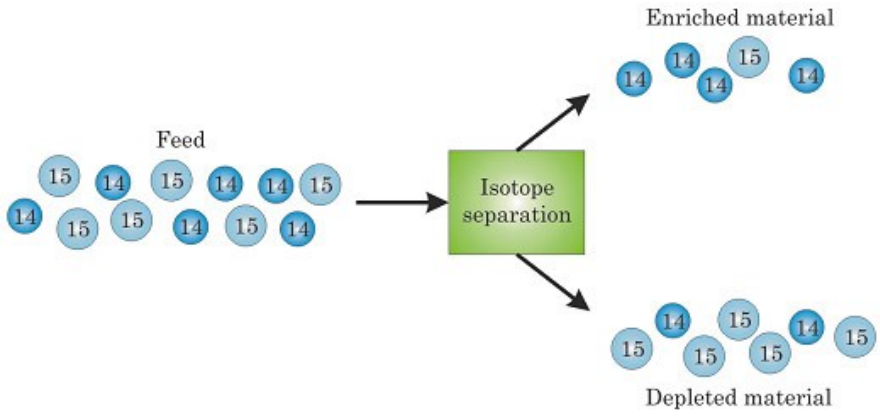


Figure 2: *Hypothetical N isotope separation process, with ^{14}N as the preferred isotope* (From: http://www.klydon.co.za/images/Isotope.jpg).

$\delta^{15}N$ (natural abundance) and ^{15}N–enrichment are quite different approaches to studying plant N relations. Enriched ^{15}N labelling has been used extensively to trace N sources and/or the amounts of N which move from a source to a sink, hence the term ”trace”. N which is ^{15}N–enriched can be used in this way to create a strong ^{15}N signal, which is different enough from natural abundance ratios that natural variations in the N cycle are insignificant in

comparison, and can be ignored (Kendall, 1998). $\delta^{15}N$ values are very small compared to those of ^{15}N–enriched tracers. The enrichment of the isotope is usually expressed as atom% excess, which is the % of atoms as the minor isotope in excess of their background abundance. In air, ^{15}N comprises only 0.3663% of total N_2 and a compound containing 1 atom% ^{15}N has 0.6337 atom% ^{15}N excess (Unkowich et al., 2001), whereas ^{15}N–enriched tracers may contain >99% ^{15}N. A difference in ^{15}N–enrichment of 1 atom percent (e.g. between 5 atom% and 6 atom%) is equivalent to a change of 3046‰. Atom% ^{15}N values are expressed as follows:

$$\text{atom}\,\%\ ^{15}\text{N} = \left[{}^{15}\text{N}/{}^{14}\text{N}_{\text{sample}}/({}^{15}\text{N}/{}^{14}\text{N}_{\text{sample}} + 1)\right] \times 100 \qquad (2)$$

For example, the $\delta^{15}N$ value of ammonium sulphate that is 20 atom % ^{15}N is calculated as follows (Kendall, 1998):

$$\delta^{15}\text{N}_{\text{air}} = \left\{\left[(20/80)/(1/272)\right] - 1\right\} \times 1000 = +67000\,‰ \qquad (3).$$

In our work, both the natural abundance and the ^{15}N enriched approach were used for food authenticity and nitrate leaching studies, respectively.

1.2.2 Nitrogen isotopic composition in plants

N–autotrophs can utilize a variety of materials from purely inorganic compounds (NH_4^+, NO_3^- N_2, and NO_2^-) to amino acids, and can have a wide range in $\delta^{15}N$ values depending on environmental conditions. In general, microorganisms and plants preferentially uptake ammonium and the soil nitrate is preferentially assimilated by tree roots relative to soil ammonium (Kendall, 1998; and references therein). According to Larcher (2003) most plants prefer NO_3^- as long as pH in the rooting zone remains favourable. In nutrient-poor, acid and wet soils plants take up mostly NH_4^+. Plant uptake depends on temperature and is smaller at low temperatures (Larcher, 2003).

Most plants have $\delta^{15}N$ values in the range of -5 to +2‰ (Fry, 1991; Kendall, 1998). N fixing plants have $\delta^{15}N$ values close to zero as they incorporate N_2 from the atmosphere. Other plants cannot fix N_2 and incorporate N by assimilating NH_4^+ or NO_3^- from soil instead. The $\delta^{15}N$ values of plants are strongly dependent on those of the soil, which are in part controlled by plants (Sharp, 2007). It has been demonstrated that different plant parts (e.g. seed, herbage, crows, and roots) of crops grown on ^{15}N enriched soil may contain N of different isotopic composition from each other. Roots are known to have higher ^{15}N enrichments than foliage or seeds (Danso, 1985 and references therein).

Recent investigations have concluded that there is negligible fractionation during plant uptake in most natural N–limited systems (Nadelhofer and Fry, 1994); nevertheless, tree tissues and litter have slightly lower $\delta^{15}N$ values than soil (Kendall, 1998). In non–limited systems, preferential uptake of lighter ^{14}N isotope by plant results in a few permil fractionations between plants and dissolved inorganic nitrogen. Several studies show that the $\delta^{15}N$ of NO_3^- in soil solution and consequently in plants generally is isotopically lighter that the $\delta^{15}N$ of NH_4^+, due to fractionation during nitrification. Processes which lead to strong losses of N, such as clear felling followed by nitrification and NO_3^- leaching losses, may lead to increases in plant $\delta^{15}N$, even when the plants take up NO_3^- (Högbom et al., 2002, in Falkengren-Grerup et al., 2004).

1.2.3 Nitrogen isotopic composition in soil

The $\delta^{15}N$ in soils is affected by many factors including soil depth, vegetation, climate, cultural history, soil age, etc. The $\delta^{15}N$ of total soil N ranges from about -10 to +15‰, with most soils having $\delta^{15}N$ values in the range of 2 to 5‰ (Kendall, 1998). Cultivated soils have slightly lower $\delta^{15}N$ values (0.6 ± 2.6‰) than uncultivated soils (2.7 ± 3.4‰). In general, the soil nitrate produced from fertiliser (average $\delta^{15}N$ value = 4.7 ± 5.4‰) and animal waste (average $\delta^{15}N$ value = 14.0 ± 8.8‰) are isotopically distinguishable but they both overlap with the composition of nitrate in precipitation and natural soils (Kendall, 1998). The loss of the bioavailable, ^{15}N depleted ammonium to plant uptake, nitrification, and leaching coupled by recycling of the ^{15}N enriched biomass, will inevitably lead to increases in $\delta^{15}N$ of the total soil nitrogen (Kendall, 1998).

Because nitrate is more mobile in soils than ammonium (adsorption of NH_4^+ to soil particles, e.g. clay), it is less likely to accumulate and, therefore, readily leaches from soils. Although it has often been assumed that nitrate is the most abundant N solute in catchment waters, several recent studies have found that dissolved organic nitrogen is actually the dominant N solute (Kendall, 1998, and references therein). Soluble dissolved inorganic nitrogen (DIN; mainly NO_3^-) constitutes about 1% of the N in the soils, and hence is a very small pool whose $\delta^{15}N$ is much more sensitive to change then the larger organic pool. Turnover times of DIN in various soils are in the order of days (Davidson et al, 1990; Högberg, 1997; Kendall, 1998).

Most of the nitrogen in soils is bound in forms not readily available to plants; hence, the $\delta^{15}N$ of total soil N is generally not a good approximation of the $\delta^{15}N$ of nitrogen available for plant growth (Kendall, 1998). Nitrate $\delta^{15}N$ values usually increase with depth in surface soils, though values can decrease below the rooting zone (50-500 cm) where concentrations are low and N pools are mainly derived from leaching from above.

Mineralization followed by nitrification and leaching is probably a major cause of enrichment in total soils. Other processes can also produce increase in $\delta^{15}N$ of nitrate with depth. Also seasonal changes in soil temperatures can affect the $\delta^{15}N$ of nitrate, resulting in higher values in the summer in unfertilized fields (Ostrom et al., 1998), due to higher temperatures which cause higher volatilization and denitrification. In well-oxigenated vadose zones, there may be little or no change in the $\delta^{15}N$ of nitrate past the root zone, indicating little denitrification or other nitrogen cycling reactions during transport (Gormly and Spalding, 1979). Delwiche and Steyn (1970) noted that where there is a significant change in texture in the profile (e.g. a point where sand content in unusually high), there is a significant enrichment in ^{15}N. But they could not demonstrate a consistent relationship between ^{15}N content of N and soil particle size or total N content to the soil (Kendall, 1998). Plants are integrators of the $\delta^{15}N$ of available N sources, and although there are complexities caused by storage effects, perhaps plant, especially fine roots, would provide the simplest and best estimate of the $\delta^{15}N$ value of available N in the soil (Högberg, 1997; Kendall, 1998).

1.2.4 ^{15}N methodology

The large size of the native N pool in soils, compared to fertiliser N inputs, and the inherent variability of native soil, makes assessment of the fate of fertiliser N virtually impossible to asses without the use of a tracer to differentiate the fertiliser input from background soil N. Consequently as mentioned previously, ^{15}N–enriched substrates have been widely used in studies following the fate of N in soil–plant systems (Fillery and Recous, 2001). Following the tracer yields data with which one can quantify the fate of the added fertiliser N as it passes into various partitions – the portion taken up by the plants, the portion remaining in the soil N

pool, the portion lost by denitrification into the atmosphere, and the portion leached into runoff waters. Such data leads to recommendations for fertilisation that yield the greatest benefit to food crops and the least possible pollution of drinking water by nitrate runoff.

The increasing concern for maximizing the efficiency of fertiliser nitrogen use in crop production is reflected in the experimental increase in the use of ^{15}N to study the uptake of applied nitrogen (Hauck, 1976). With the ^{15}N labelling method, the isotopic signature of the enriched tracer can be pre–determined to ensure significant differences in atom% ^{15}N between the source and background level, even when fractionation occurs. This technique has been used extensively for a number of years (Broadbent and Nakashima, 1974, Fried et al., 1975, Hardarson et al., 1983, Mulvaney and Boast, 1986, Bronson et al., 2000, Bedard-Haughn et al., 2003, Zamora et al., 2009, and many others), and has been accepted by the scientific community at large as the most reliable way to follow the flow and fate of N in systems (Bedard-Haughn et al., 2003). When ^{15}N enriched material is applied it becomes part of the overall N–cycle and the path of ^{15}N through the various soil and plant N pools can be followed (Bedard-Haughn et al., 2003). The isotopic method is the only direct means of measuring N uptake from applied fertiliser. The recovery data are known to be the ″real coefficient of utilization″ (van Cleemput et al., 2008).

Examples of ^{15}N methodology application (Fillery and Recous, 2001):

1. Measurements of ^{15}N enrichment in individual soil N pools, and in plant material, can be employed to follow the fate of applied ^{15}N fertilisers. Analysis of added ^{15}N in the NH_4^+, NO_3^- and organic N pools and in plant material enables the recovery of added ^{15}N to be determined.
2. Experiments measuring the difference between added ^{15}N and recovered ^{15}N after a particular time interval can be used as a measure of the loss of fertiliser–derived N from soil–plant systems.
3. ^{15}N–enriched techniques have been used to determine the efficiency of uptake of N sources by crop plants.

In order to follow and understand the pathway of nitrogen through different reservoirs (plant–soil–groundwater), various ^{15}N–enriched fertilisers can be used. From literature it seems that $(NH_4)_2SO_4$ is most commonly used, although no explanation is given for this preference. However, Vallis et al. (1967, in Danso et al., 1985) observed a greater uptake of N from $K^{15}NO_3$ than from $(^{15}NH_4)_2SO_4$ and suggested that ammonium would probably be better than the nitrate form of fertiliser when the aim is to have much of tracer incorporated in the internal soil nitrogen cycle. On the other hand, nitrate is better when the objective is to minimise microbial transformation before uptake. According to Hystead and Lowe (1977), in order not to disturb the soil N cycle, labelled N should be added in a form already present in the soil (Danso et al., 1985).

Just as with the different forms, many methods of ^{15}N application can be used, including broadcast at planting, broadcast and incorporated one week before planting, sprayed or injected into soil as solution and banding. The greatest problems with the different methods of application may, however, be related to uniformity of fertiliser distribution in the soil and how each method affects the rate of release of ^{15}N (or tie–up in the soil colloid or organic matter). Certainly, surface application, unless followed by irrigation or rainfall, may lead to vertical gradients in the ^{15}N composition of soil. Solution application has the advantage that small amounts of ^{15}N–labelled fertiliser (which would be difficult to distribute evenly on the soil surface) can be dissolved in a large volume of water and applied uniformly to cut down on spatial variability (Danso et al., 1985 and references therein).

1.3 The use of N isotopic signature for controlling organic vegetable production

Compared to conventional farming, organic farming is reported to reduce negative environmental impacts and to produce healthier foods and hence became one of the preferred farming practices. It has experienced a rapid growth over recent years. In 2000, 115 organic (eco-) farms were registered in Slovenia, whereas in 2009 number of farms amounted to 1853 (SURS). In Slovenia, bio–agriculture has been practiced for many years, but European comparable inspection and certification system of organic farming has started to develop only a few years ago. The first step in the "legalization" of organic production is to establish standards on the example of those used in organic associations in the neighbouring countries. In Slovenia, such standards were prepared in May 1996, a year later a brochure, entitled "Recommendations for organic farming in Slovenia", was printed by the Ministry of Agriculture, Forestry and Food (MAFF). Rules on organic production and processing of agricultural products and/or foods came in force in 2001 (OJ RS, No. 32/2001, 52/2003, 128/2006). The Union of Slovenian Organic Farmers Associations created and promotes a single collective mark for organic food, which, after fulfilling the required criteria, any producer who is a member in one of these associations can use to label his products (Bratuša, 2010).

Since organic products attain higher prices on the market, mainly due to higher production costs and costs connected with the certification process, there is concern among users over mislabelling conventionally grown crops as “organic”. Growers must undergo testing and follow rigorous guidelines to obtain a certificate to produce and sell authentic organic produce, however, current chemical analytical controls performed on organic vegetables consist of searching for pesticide residues and contaminants (Woese et al., 1997) rather than confirmation of growing regime (Rogers, 2008). Nevertheless, in the last decade or so, studies have begun to look for additional ways to test organic products for authenticity (Nelson et al., 2004 and references therein). Some of these tests are oriented towards using stable nitrogen isotopes as indicators of fertilisation with synthetic (inorganic) N fertilisers (Evans, 2001; Choi et al., 2002; Choi et al., 2003; Bateman et al., 2005; Rapisarda et al., 2005; Yun et al., 2006; Bateman et al., 2007; del Amor et al., 2008), while other researchers are investigating whether concentrations of trace elements could be used to differentiate between organic or conventional produce (Gundersen et al., 2000; Kelly and Bateman, 2010).

The possible use of nitrogen isotopes to differentiate between crops grown with or without inputs of synthetic (inorganic) nitrogen (N), which are prohibited in organic agriculture, is based on the hypothesis that the application of synthetic (inorganic) nitrogen fertilisers with $\delta^{15}N$ values close to 0 ‰ will result in the $\delta^{15}N$ values of plants grown in conventional regimes being lower than those in organic regimes due to the different fertiliser production processes (Bateman et al., 2005). Synthetic (inorganic) nitrogen fertilisers tend to have $\delta^{15}N$ values within a few per mil of zero since their nitrogen is derived from atmospheric nitrogen (with $\delta^{15}N = 0$ ‰) and there tends to be little fractionation during the production process (Bateman et al., 2005, del Amor, 2008). Animal manures with $\delta^{15}N$ values around 5 ‰, on the other hand, have been reported to produce nitrate with $\delta^{15}N$ values in the range of 10 to 22 ‰ (Kreiler, 1979; Bateman et al., 2005) due to the preferential volatilization of ^{15}N depleted ammonia from the manure.

Table 3 presents $\delta^{15}N$ values for different synthetic (inorganic) and organic fertilisers, obtained from literature. The $\delta^{15}N$ in the compiled literature dataset are in the range close to 0 ‰, whereas fertilisers that may be permitted in organic agriculture have greater $\delta^{15}N$ values and a much broader range in $\delta^{15}N$ values, due to very different origin (manure, seaweed, fish meal, hoof and horn, bonemeal, dried blood) (Bateman and Kelly, 2007).

Table 3: Review of the $\delta^{15}N$ values (in ‰) in different synthetic (inorganic) and organic fertilisers.

Fertiliser type	Mean	Std	Min	Max	n	Reference
Synthetic (inorganic)	+0.4	1.8	-5.9	+2.8	44	IEH Lab., 2010
	–1.6	0.2	–1.2	–1.7	12	Rogers, 2008
	+0,2	1.3	–1.7	+3.9	22	Vitoria et al., 2004
	–0.2	2.1	–5.9	+6.6	29	Bateman and Kelly, 2007
	/	/	–4.0	+4.0	/	Kendall, 1998
Manure/compost	+8.1	3.9	+3.5	+16.2	11	Bateman and Kelly, 2007
	+8.3	5.4	+4.4	+26.7	9	IEH Lab., 2010
	+6.3	3.7	+2.7	+11.3	12	Rogers, 2008
	/	/	+2.0	+30.0	/	Kendall, 1998
Seaweed based	+2.5	1.5	+0.6	+5.4	9	Bateman and Kelly, 2007
Mammalian, non manure*	+5.9	1.0	+4.1	+6.8	9	Bateman and Kelly, 2007
	+5.6	2.4	+4.4	+7.7	12	IEH Lab., 2010
Fish based	+7.1	3.6	+2.1	+10.6	4	Bateman and Kelly, 2007
	+8.6	4.6	+7.2	+17.2	14	IEH Lab., 2010

*Mammalian, non manure source comprises of dried blood, hoof and horn, and bonemeal.

In recent years, many studies have been conducted to test this hypothesis on different pot/greenhouse grown (Bateman et al., 2005; Yun et al., 2006) and commercially available (Bateman et al., 2007; Kelly et al., 2010) crops, e.g. maize, cabbage, lettuce, tomato, and carrot. As summarized by Kelly and Bateman (2010), previous studies have demonstrated that the nitrogen isotope composition of a crop may be used to distinguish between crops grown using conventional synthetic (inorganic) fertilisers and crops grown under organic conditions (Choi et al., 2003), as long as the crops are not nitrogen–fixing plants that remove the nitrogen from the air ($\delta^{15}N_{air}$ = 0‰) rather than using nitrogen reserves from the soil (Rogers, 2008) and that the timing of application (Choi et al., 2002), irrigation water (Bateman et al., 2005) and the chemical form of synthetic (inorganic) fertiliser (Evans, 2001) are, along with some other factors (i.e. soil type, variations in local agricultural practices, etc.), also important in determining how fertiliser $\delta^{15}N$ impacts crop $\delta^{15}N$. With the exception of Yun et al. (2006) who studied the interactive effects of N fertiliser source and timing of fertilisation on $\delta^{15}N$ signatures in Brassica campestris L. cv. Maeryok (Chinese cabbage), most popular vegetable in Asia, most studies (e.g. Bateman et al., 2005; Bateman et al., 2007; Choi et al., 2003; Choi et al., 2002) have dealt only with $\delta^{15}N$ variations in plants after a single application of organic or synthetic (inorganic) nitrogen fertiliser. Therefore, the effect on plant $\delta^{15}N$ of split N fertilisation, which might enable growers to cover up the use of synthetic (inorganic) fertilisers, is not well studied and needs to be extended to other plant species that are popular in Europe, as we know that almost a quarter of the world's organic land is in Europe (i.e. 8.2 Mha, which is 1.7 % of the European agricultural area), and the sales of organic products achieved in 2008 approximately 18 000 M€, where the vegetable crops market constitutes an important part (Willer and Kilcher, 2009).

2 Aims and Hypothesis

The goals of the thesis were as follows:

- To study the effect of different fertilisation and irrigation practices on yield, fertiliser use efficiency, yield quality (nitrate content) and the potential of N losses in order to obtain data which could lead to recommendations for growers growing vegetables (white cabbage) on a sandy-loam soils inside groundwater protection areas
- To check the suitability of the nitrogen isotopic fingerprint ($\delta^{15}N$) in organically and conventionally grown vegetables as a potential marker to identify the use of synthetic (inorganic) N fertilisers in organic production:
  - on a pot grown lettuce (single, split and combined application of organic and synthetic (inorganic) N fertilisers)
  - in organically and conventionally grown vegetables available on the Slovenian market
- To introduce an ion exchange method for the preparation of freshwater (ground and surface water) samples for determination of the isotopic composition of nitrate nitrogen at natural ^{15}N abundance into a routine laboratory practice
- To introduce and compare different methods for the preparation of soil solution samples for determination of the isotopic composition of nitrate nitrogen at ^{15}N enriched and natural ^{15}N abundance

The hypothesis considering nitrate leaching under different fertilisation and irrigation practices were as follows:

- Irrigation, temporally and spatially adjusted to plant requirements, decreases nitrate leaching bellow the root zone to the groundwater
- Fertigation (effectively applied fertilisation through precision irrigation) is the most appropriate practice for growing white cabbage from environmental and economical point of view

The hypothesis considering the applicability of $\delta^{15}N$ signature as a marker of organic produce, were as follows:

- The application of synthetic (inorganic) N fertilisers will result in the $\delta^{15}N$ values of plants grown in conventional regimes being lower than those in organic regimes
- It is not possible to reveal little or moderate rates of synthetic (inorganic) fertiliser illegally applied to a lettuce crop in organic production
- The best assessment of whether the N isotope approach is an appropriate tool to discriminate between organically and conventionally grown produce will be obtained by analyzing commercially available organic and conventional vegetables from Slovenian market

3 Materials and Methods

3.1 Field experiment

White cabbage (*Brassica oleracea* var. *capitata* L.) is an important vegetable in Slovenia. It is frequently grown in fields located above shallow groundwater bodies which are very susceptible to nitrogen leaching and prone to pollution of groundwater, which in Slovenia represents the most important source of drinking water. Therefore, a field experiment was conducted with white cabbage to study the effect of different fertilisation and irrigation practices on yield, N–fertiliser use efficiency and consequently yield quality (i.e. nitrate content) and the potential for N losses (i.e. N surplus after harvest) in order to obtain data which could lead to recommendations for farmers growing white cabbage on sandy–loam soils inside groundwater protection areas. The study was preformed between April and June, 2007, with ^{15}N enriched potassium nitrate fertiliser as a tracer.

3.1.1 Experimental site

The experimental field (40°4`42`` N, 14°35`46`` E, 30 m wide and 70 m long) was located above the sandy-gravel aquifer of Ljubljansko polje, east of Ljubljana (Figure 3). The agricultural soil was classified as gleyic fluvisol and endogleyic fluvisol (World Reference Base for Soil Resources, 2006). The chemical and physical properties of the soil are presented in Table 4 and Appendices 1-2.

Table 4: Chemical and physical properties of soil on the Ljubljansko polje experimental area.

Parameters	Value
pH ($CaCl_2$; 1:5)	7.4
Total organic C	
Content (mg kg^{-1})	14600
Total N	
Content (mg kg^{-1})	1400
atom % excess	0.003
2 M $CaCl_2$ extractable NO_3^--N	
Content (mg kg^{-1})	1.6
2 M $CaCl_2$ extractable NH_4^+-N	
Content (mg kg^{-1})	6.8
Organic N[a]	
Content (mg kg^{-1})	1391.6
C/N weight ratio	12.2
Texture	Loam and sandy loam

[a]The content of organic N was determined as the difference in N between total N and inorganic N.

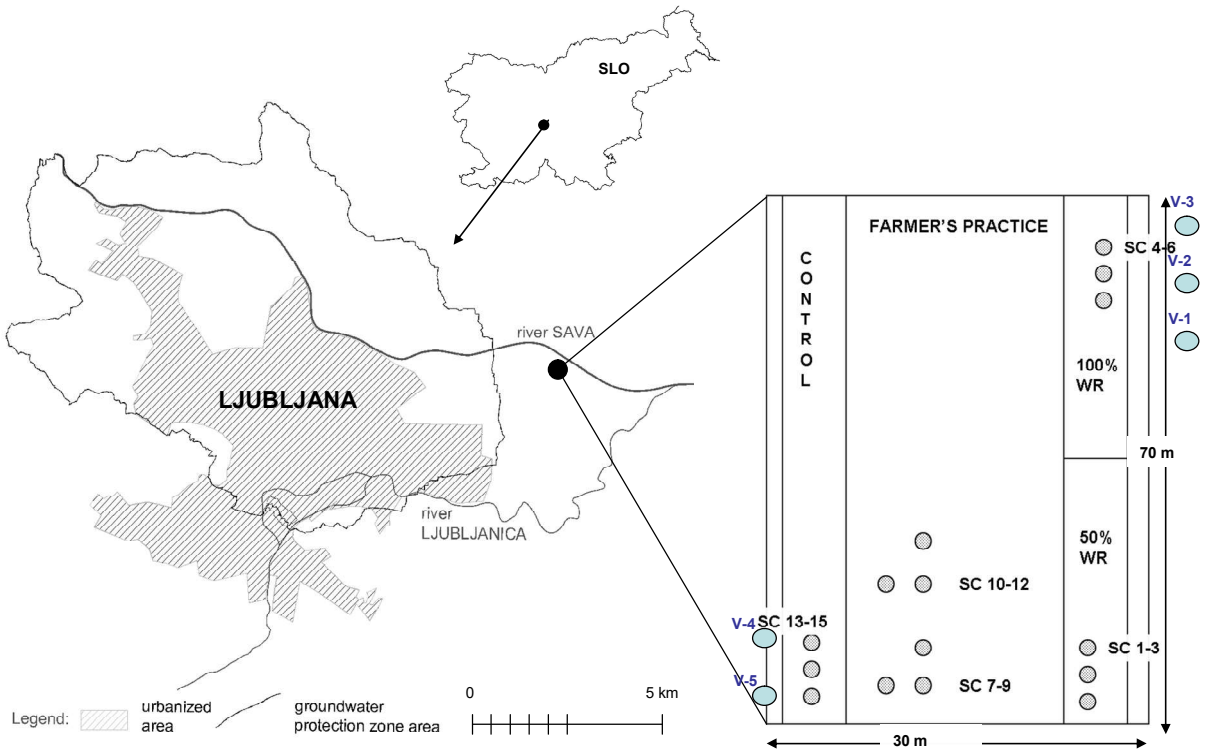


Figure 3: *City of Ljubljana with water protection zone and experimental site in Sneberje, Slovenia, marked with black dot.* Field layout (white cabbage growing period; right hand side) shows gray dots as suction cups (SC) where soil water was sampled (SC 1-3 on 50 % irrigation, SC 4-6 on fertigation, SC 7-12 on farmer's practice and SC 13-15 on control plot) and blue dots as wells (V-1 to V-5) where groundwater was sampled, where V-4 and V-5 are located before the field and V-1 to V-3 are located after the field, considering the groundwater stream (Zupanc et al., submitted).

3.1.2 Weather conditions

The mean annual precipitation in the study area for the 1971–2000 reference period was 1368 mm, and the average annual air temperature was 10.2 °C, measured at the meteorological station Ljubljana–Bežigrad (299 m.a.s.l., 46°3`57`` N, 14°31`2`` E). Data for precipitation, air temperature, wet deposition of nitrate and ammonium for the growing season were obtained from the Environmental Agency of the Republic of Slovenia and are presented in Table 5.

During the growing period of white cabbage (April–June, 2007), the mean temperature was above the 30 year average by 4.6 °C in April and by about 2 °C in May and June. Precipitation was below the 30 year average in all by 199 mm, with the greatest shortfall in April (97 mm below average) and June (74 mm below average).

Table 5: Weather data for the growing period of white cabbage (April – June, 2007) and for the 1971 – 2000 reference period. Data were obtained from Environmental Agency of the Republic of Slovenia.

Month	Mean T (°C)		Precipitation (mm)		Wet deposition (g m^{-2})	
					$N\text{-}NH_4^+$	$N\text{-}NO_3^-$
	2007	**1971–2000**	**2007**	**1971–2000**	**2007**	**2007**
April	14.6	10.0	6	103	0.007	0.008
May	17.2	15.0	113	113	0.040	0.032
June	20.9	18.1	80	154	0.053	0.038
Sum			**199**	**370**	**0.100**	**0.078**

3.1.3 Experimental set–up

In the experiment, four different fertilisation and irrigation treatments were applied, namely: (1) unfertilised control plots with the farmer's practice of irrigation; (2) treatment with the farmer's practice of fertilisation and drip irrigation covering 50% of the crop's water requirements; (3) treatment by fertigation with drip irrigation covering 100% of crop's water requirements, and (4) treatment with farmer's practice of fertilisation and irrigation. Farmer's practice consisted of broadcast fertiliser application the day before transplanting plus irrigation the day before and the day after transplanting (DAT), using a tank sprinkler. Tap water was used for irrigation (1.9 mg N L^{-1}, 0.369 at% ^{15}N; most of the N was present in nitrate form). The two treatments with drip irrigation covered 100% and 50% of crop water requirements, determined by the Penman-Monteith method (Allen et al., 1998). Each treatment was replicated three times. Fertilised plots (6.5 m^2) were divided into 3 subplots (2.6 m^2), and ^{15}N labelled fertiliser was applied on the middle subplot.

The following fertiliser norm was followed: 200 kg N ha^{-1}, 80 kg P ha^{-1}, 300 kg K ha^{-1}, 280 kg Ca ha^{-1} and 30 kg Mg ha^{-1}. The amount of fertiliser applied was determined according to the Regulations on Integrated Production of Vegetables (Official Journal of the Republic of Slovenia (RS) 63/2002) and Technological Instructions for Vegetable Production (Ministry of Agriculture, Food and Forestry (MAFF), 2003). ^{15}N labelled KNO_3 fertiliser (Shanghai Research Institute, Shanghai, China) was used as a tracer. In fertigation treatment, the total amount of P and K and 30 % of the total N rate were applied as unlabelled granular fertiliser as a pre-plant broadcast application and the remaining N as ^{15}N labelled solution via fertigation**.** During the growing season, fertigation was performed three times, i.e. at 57, 66 and 75 DAT. The labelled KNO_3 + unlabelled water soluble $Ca(NO_3)_2$ were dissolved in tap water and applied as solution with 3.52 ± 0.04 at% ^{15}N. On plots with farmer's practice of fertilisation, unlabelled $Ca(NO_3)_2$ (0.365 at% ^{15}N) was applied as a broadcast application, followed by the application of the labelled fertiliser, which was applied as a solution. We assumed that $Ca(NO_3)_2$, which was applied as a broadcast application the day before transplanting, was dissolved after the irrigation within a few hours and mixed with the labelled fertiliser in the soil. In all treatments, for unlabelled subplots only unlabelled $Ca(NO_3)_2$ was applied.

Cabbage (*B. oleracea* var. *capitata* L.) was sown in plug trays, containing Klasmann tray substrate TS 3 (i.e. a mixture of poorly to moderately decomposed white peat and highly decomposed black peat, fertilised with 1–1.5 mg m^{-3} NPK (14:16:18), pH between 5.5–6.5, electrical conductivity between 30 and 40 mS m^{-1} (± 25 %)), and transplanted to the field 48 days later. It was grown in the field for 76 days, from April 11 to June 27, 2007.

3.1.4 Sampling

Plant samples. Samples were taken at 59, 68 and 78 DAT. At each sampling event, three plants were destructively sampled from each subplot of each replicate. For determination of isotopic composition, only the samples from ^{15}N labelled subplots were used. Cabbage heads were divided into 3 equal parts, inner, middle and outer parts. Fresh weight was determined for all aboveground plant parts.

Soil samples. Soil samples were taken before planting (10.4.2007) from the upper 25 cm layer.

Soil water samples. Soil water was sampled at three different occasions during cabbage growing period and at eight occasions after harvest (untill December, 2007) after individual rain events using porous ceramic suction cups (SDEC, France). Three suction cups were installed per plot/treatment at 50 cm depth.

Groundwater samples. Groundwater was sampled bi-weekly. At each sampling event, two litres of groundwater sample was taken at 6 m depth from each well.

3.2 N isotopes as a screening tool to control organic vegetable production

3.2.1 $\delta^{15}N$ signature of synthetic and organic fertilizers

Isotopic composition of four different types of organic fertilisers, namely farmyard manure, compost and two commercially available organic fertilisers (Biogrena and Valentin naravno organsko gnojilo), and seven different types of synthetic (inorganic) fertilisers, namely N:P.K (7:20:20; 15:15:15), KAN, KNO_3, $Ca(NO_3)_2$ (2 different manufacturers) and $MgNO_3$, frequently used in Slovenia, were determined.

3.2.2 Pot experiment

The effect on plant $\delta^{15}N$ of split N fertilization, which might enable producers to cover up the use of prohibited synthetic (inorganic) N fetilisers was studied on a lettuce (*Lactuca sativa* L.) since lettuce is a popular organically produced crop in Slovenia as well as in Europe. In addition, lettuce was chosen as it is a vegetable with short growing period (e.g. as compared to cabbage) and can easily be grown in a greenhouse pot experiment.

3.2.2.1 Experimental set–up

A pot experiment with lettuce (*Lactuca sativa* L.) was performed in a greenhouse (15–25 °C, 80% transparency to visible irradiation; solar radiation was 173, 266 and 210 hours in April, May and June, respectively) at the Biotechnical Faculty of Ljubljana, Slovenia (Figure 4). Lettuce was sown in plug trays containing Klasmann tray substrate and individually transplanted into pots 35 days later at the five to seven true leaf stage. Plants were grown in pots for 50 days, from April 24 to June 10, 2009.

Seven treatments were applied in a completely randomized design with three replications, as follows: an unfertilized control (C), a single basal organic fertilisation of 40 mg N kg^{-1} soil (O), a single basal synthetic (inorganic) fertilisation of 40 mg N kg^{-1} soil (S), a basal synthetic (inorganic) fertilisation of 20 mg N kg^{-1} soil followed by an additional organic fertilisation of 20 mg N kg^{-1} soil (S+O), a basal organic fertilisation of 20 mg N kg^{-1} soil followed by an additional synthetic (inorganic) fertilisation of 20 mg N kg^{-1} soil (O+S), a basal synthetic (inorganic) fertilisation of 20 mg N kg^{-1} soil followed by an additional synthetic (inorganic) fertilisation of 20 mg N kg^{-1} soil (S+S), a basal organic fertilisation of 20 mg N kg^{-1} soil followed by an additional organic fertilisation of 20 mg N kg^{-1} soil (O+O). The levels of applied N are typical of those used in lettuce cultivation by local farmers. As the basal application, N–based fertilisers (calcium nitrate and organic fertiliser) were mixed thoroughly with 7.5 kg of soil per pot. Organic fertilisation with pulverized (to pass a 2 mm sieve) fertiliser was performed two days before transplanting, whereas synthetic (inorganic) fertiliser was applied on the day of transplanting. Additional application of N fertilisers was performed immediately after sampling at 30 days after transplanting (DAT).

During the experiment, plants were irrigated manually with tap water (3.5 mg $N–NO_3^-$ L^{-1}, $NH_4^+ + NO_2^-$ <0.02 mg N L^{-1}; $\delta^{15}N$ = 4.6‰) every two days at the beginning of the experiment, but as the plants grew bigger, watering was performed daily. All treatments received the same amount of irrigation. Watering was adjusted by weighing and kept near to the field capacity.

Figure 4: *Greenhouse pot experiment with lettuce.*

3.2.2.2 Sampling and sample preparation

During the experiment, aboveground lettuce samples were taken at 0, 20, 30 and 50 DAT. At each sampling event three plants were destructively sampled for each treatment. Each plant was divided into three parts, i.e. inner, middle and outer leaves and fresh weight was measured for each part. Dry weight was determined after drying in a drying chamber at 60 °C till constant weight.

3.2.3 $\delta^{15}N$ of some organically and conventionally grown vegetables available on the Slovenian market

Fourteen different types of organically grown vegetables were selected from organic markets from certified producers (labelled as “ekološki”) and their conventionally grown counterparts from different supermarkets in Slovenia. In the selection of organic produce, labelled and certified foods were preferentially chosen, however some additional samples grown in accordance with organic production but not certified, were also analysed. Conventionally grown (nonorganic) vegetables were sampled from bulk produce display areas in the supermarkets.

3.3 Analytical methods

3.3.1 3.3.1 Total nitrogen and ^{15}N in soils, fertilisers and plants

3.3.1.1 Total N in plants and soils

Total N in finely ground samples of soil and white cabbage was determined at the Biotechnical Faculty, Centre for Pedology and Environmental Protection, after incineration at 900°C in a VarioMAX CN analyser and determined by a Thermal Conductivity Detector (TCD) (ISO 13878). Measurement uncertainty was 9 %.

N content in lettuce samples (pot experiment) was determined simultaneously with $\delta^{15}N$ determinations on ANCA–IRMS. Estimated measurement uncertainty, based on a long term measurements of an in–house reference materials Sunflower with 7.79 g N kg^{-1}, was 7 %.

3.3.1.2 Isotopic composition of N

Soil samples. Soil samples were air-dried; roots and gravel/sand were removed. Samples were homogenized during grinding to fine powder using an agate mortar and pestle. For the determination $\delta^{15}N$ abundance, about 50–70 mg of dried and homogenized sample was weighed into tin cups.

Fertiliser samples. Synthetic fertilisers were homogenized during grinding to fine powder using an agate mortar and pestle and transferred into tin cups. Organic fertilisers were air dried and homogenized during grinding to fine powder using an agate mortar and pestle and transferred into tin cups for isotopic analysis.

Plant samples. Vegetables were washed thoroughly with distilled water (Mili–Q) and chopped into small pieces. Plant subsamples were dried at 60 °C and homogenized during grinding to fine powder using the agate mortar and pestle. DM was determined after drying at 60 °C until constant weight. For the determination of $\delta^{15}N$ abundance (and N content), about 8–11 mg of dried and homogenized sample was weighed into tin cups for determination on ANCA–IRMS.

3.3.2 N-NO_3^- in plants

$N–NO_3^-$ content in plants (white cabbage) was determined on the Centre of Soil and Environmental Sciences, Biotechnical Faculty, University of Ljubljana. It was determined in water extracts, using UV/VIS spectrometer, Pekin–Elmer, Lambda 2 with FIA–system (VDULFA, 1976), with measuring uncertainty of 19 %.

3.3.3 $N–NO_3^-$ and $^{15}N–NO_3^-$ in water

In order to measure $^{15}N–NO_3^-$, NO_3^- must be removed from the solution and concentrated in a form that can be introduced to a mass spectrometer. During this process, sufficient nitrogen must be obtained to make a measurement on a mass spectrometer while avoiding contamination and fractionation (Holmes et al., 1998). Accurate and reliable methods that are also simple and inexpensive are highly desirable for routine laboratory use because a large number of analyses can be performed with the same input of resources (Mulvaney et al.,

1997).

3.3.3.1 N-NO_3^- in water

Nitrate content in groundwater and soil water samples was determined at the Drinking Water and Sewerage System Public Utility, Ljubljana, by ion chromatography (ISO 10304-01:2007). Their reported (personal communication) expanded measuring uncertainty was determined, as follows:

$$U\,(k=2) = 2 * \left(0.08 * LQD + 0.02 * Cx\right) \text{mg L}^{-1} \quad (4)$$

where LQD is a limit of quantification (0.5 mg N L^{-1} for nitate) and Cx is nitrate conecntration of the sample.

When necessary, to get a quick result, nitrate content was determined in our laboratory to assure that the amount of N is in the correct range for mass spectrometry. Groundwater and soil water samples were filtered through 0.45µm filter (MILLIPORE, Durapore® membrane filters, Cat. No. HVLP04700) using peristaltic pump. Afterwards, N–NO_3^- concentrations were determined spectrophotometrically using a Hach Lange DR 2800 spectrophotometer (Düsseldorf, Germany). In our case, for one IRMS measurement between 200 and 300 µg of N is necessary, which corresponds to 0.88–1.33 mg NO_3^-.

3.3.3.2 ^{15}N–NO_3^- in soil solution

The speed and labour efficiency of ^{15}N determinations from aqueous solutions such as soil solutions or soil extracts are often limited by sample preparation (Goerges and Dittert, 1998). In the past, several diffusion methods based on the conversion of NO_3^- to ammonium (NH_4^+) and subsequent diffusion of gaseous ammonia (NH_3) into acid buffer traps for NO_3^--N isotope analyses were elaborated (e.g. Adamsen and Reeder, 1983; Brooks et al., 1989; Sørensen and Jensen, 1991; Sigman et al., 1997; Mulvaney et al., 1997; Goerges and Dittert, 1998; Downs et al., 1999).

In this work, soil water samples were prepared for ^{15}N–NO_3^- determination by diffusion after Sigman et al. (1989), Goerges and Dittert (1998) and Downs et al. (1999) with some modifications described in IAEA TCS No. 14 (2001). Firstly, two different diffusion methods, namely the Hook method and the Teflon trap method were compared at natural abundance and ^{15}N enriched levels for their suitability for routine laboratory use and compared with the anion exchange method (described in section 3.3.3.3).

Since the hook method is not suitable for larger volumes (due to the problems with moist vapour which may appear and cause the ammonia to drop out of the filter trap back into the solution), for the comparison of both diffusion methods soil water samples were pre–concentrated by evaporation in a drying oven at 60 °C to 3 mL. However, the Teflon trap method was tested also for larger volumes, namely 200 mL, to verify the suitability of the method for larger volumes.

For the diffusion method first ammonium was removed from soil water samples by raising the pH above 10 by the addition of 1M NaOH (Acros Organics, Belgium, extra pure). Samples were incubated in dark place in opened vials over night (3 mL) or 14 days (200 mL). After incubation, Devarda's alloy (Merck KGaA, Germany) was added to the samples to reduce nitrate to ammonium, which was again liberated from the sample due to high pH and concentrated on small filter discs containing 10 µL of 2.5 M $KHSO_4$ (IAEA, Vienna, Austria). After the addition of 0.2 mg of Devarda's alloy, the vials were closed immediately and incubated at room temperature overnight (or 14 days in the case of 200 mL samples). After incubation (room T, i.e. 20-23 °C, shaken manually twice a day), the filter discs were either

placed above the sample on a stainless steal wire hook, attached to the vial lid (the hook method, Figure 5, right hand side) or placed into the sample so that the acidified filter disc (Schleicher & Schuell QF cut into circles using a paper punch), sealed between two layers of a Teflon tape, was floating on the soil water sample surface (the Teflon trap method) (Figure 5, left hand side). For the Teflon trap method, aliquots of KCl (Sigma–Aldrich, Germany, p.a.) were added to achieve 2M KCl solution. This decreased the solubility of the NH_3 and increased the osmotic pressure of the solution, which maintained the integrity of the diffusion sandwiches (USGS, RSIL Lab Code 2898). After incubation, the filter discs were removed from the sample vials, dried in a desiccator over a concentrated H_2SO_4, sealed in tin cups and analysed for ^{15}N as $(NH_4)_2SO_4$.

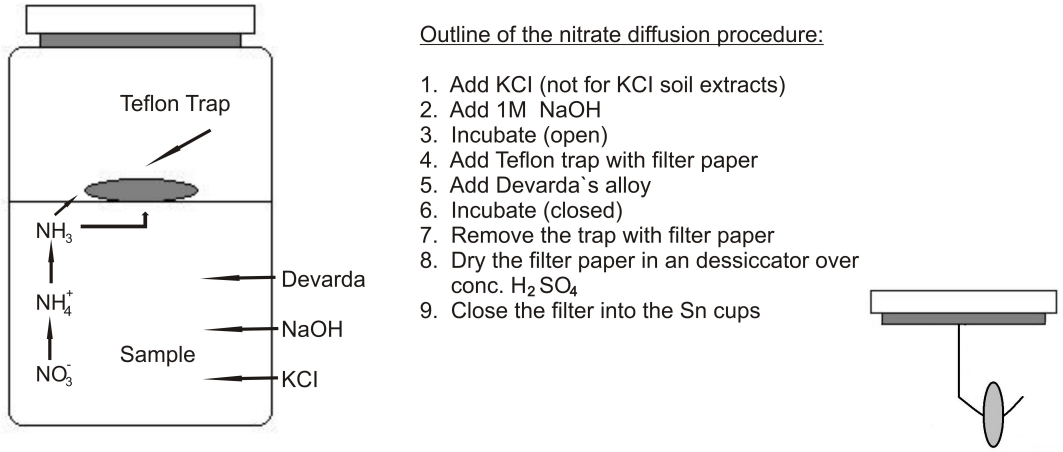


Figure 5: *Outline of the nitrate diffusion procedure (modified after Holmes et al., 1998) with scheme of the "Teflon trap method" (left hand side) and the "Hook method" (right hand side).*

3.3.3.3 $^{15}N_{tot}$ and $^{15}N–NO_3^-$ in groundwater

$\delta^{15}N$ in groundwater was monitored in order to see whether or not the applied labelled fertiliser is causing pollution of groundwater aquifer. Since the $\delta^{15}N$ of the applied fertiliser was highly above the $\delta^{15}N$ natural level, the measurement of the isotopic composition of total N alone gave satisfactory enough information on whether the ^{15}N labelled fertilizer has reached the groundwater, hence $\delta^{15}N$ of total N was determined for all samples, whereas the determination of $\delta^{15}N$ of nitrate was only determined occasionally.

For $\delta^{15}N$ of total N as well as for $\delta^{15}N$ of nitrate N, water samples were filtered through 0.45 µm filter (Silva et al., 2000; Hannon and Böhlke, 2008). For total N, samples were evaporated to dryness on a hot plate at 60 °C. Afterwards, dry precipitate was scrapped from the beaker, transferred into Sn cups and closed with tweezers for IRMS analysis.

3.3.3.3.1 Anion exchange method

Different versions of a method for concentrating nitrate on anion exchange resin are described by Kendall et al. (1995), Chang et al. (1999) and Silva et al. (2000). In this work, however, samples were prepared following the method by Silva et al. (2000) and modified by Fukada et al. (2003). The procedure of the method (Figure 6) which was introduced into our laboratory as a part of presented doctoral work is described below. Briefly, nitrate from the sample is concentrated on the anion exchange resin and afterwards eluted with 3 M HCl. Acid eluate is neutralised with the addition of Ag_2O which reactes with HNO_3 to form $AgNO_3$. The solution is filtered and dried at 60 °C. The dry $AgNO_3$ is scrapped from the beaker and transferred to silver capsules containing brown sugar (1:2.5 ratio) for the analysis on IRMS.

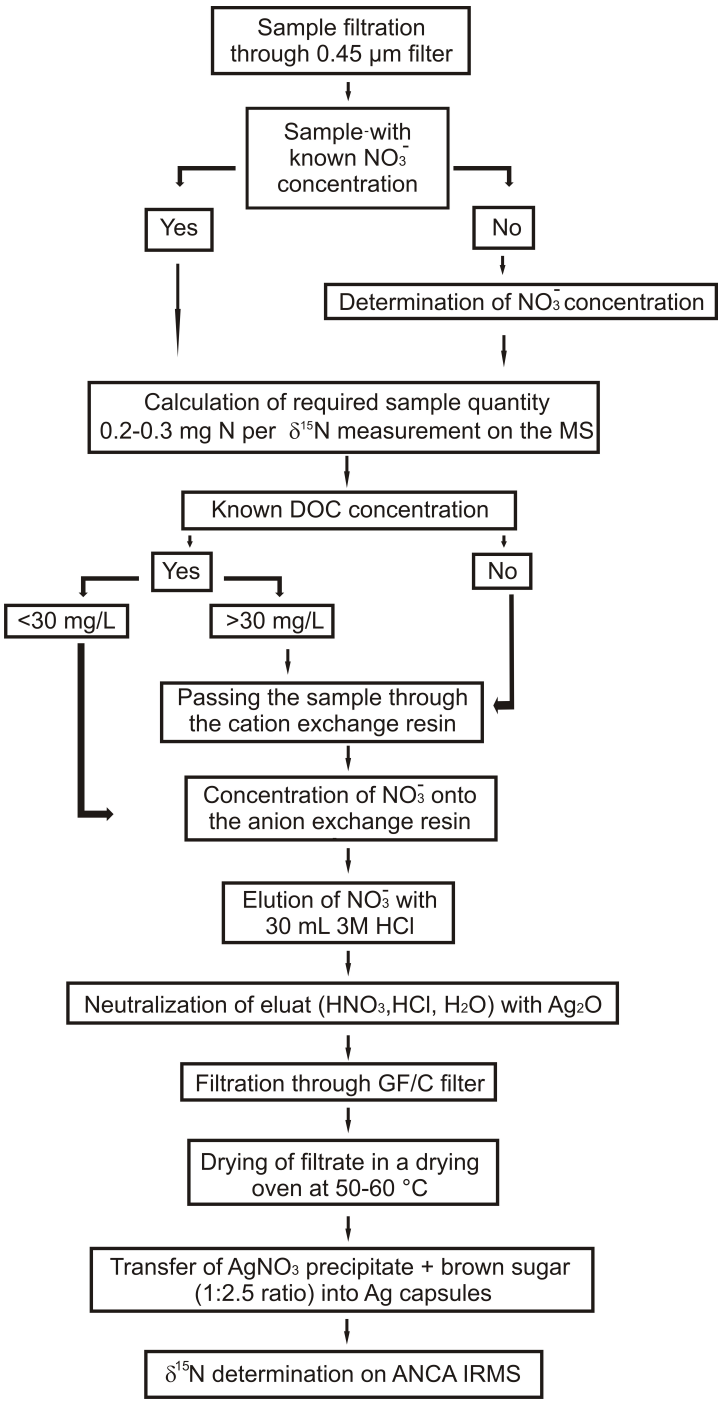


Figure 6: *Scheme of the procedure of the anion exchange method.*

Preparation of cation and anion exchange resins

Before use, cation (AG®50W-W8 100-200 Mesh, Hydrogen form, BIO-RAD, cat.#142–1441) and anion (AG®1-X8 200-300 Mesh, Chloride form, BIO RAD, cat.#140–1441) exchange resins has to be properly cleaned which was performed as follows: anion/cation exchange resin was put into 250 mL beaker and poured over with 10 times its volume of distilled (mili-Q) water. The solution was stirred manually with glass rod and when the resin settled water was decanted. The procedure was repeated 5 times. Afterwards, about 5 mL of the resin was transferred to the plastic ion exchange column, covered with mili-Q water and closed with a

cover on the bottom and top of the column. Thus prepared columns were stored in the refrigerator till use.

Passing the sample through the cation exchange resin

Since the concentration of dissolved organic carbon was unknown, before passing the sample through the anion exchange resin, water samples were preferentially passed through the cation exchange resin to remove the dissolved organic carbon (DOC) from the sample, since it might clog the anion exchange resin (Figure 7). Flow on a peristaltic pump was set at 16.7 mL min^{-1}.

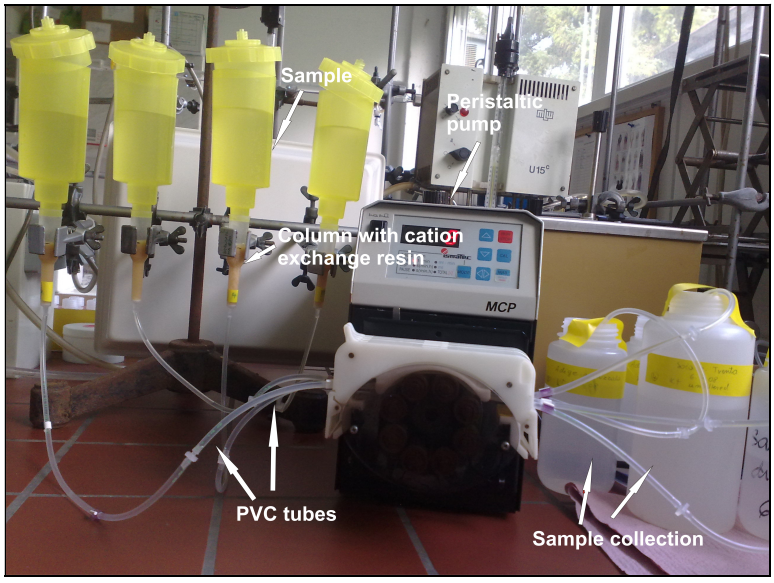


Figure 7: *Passing the sample through the cation exchange resin.*

Passing the sample through the anion exchange resin

Afterwards, the sample was passed through the anion exchange resin. The flow on the peristaltic pump was set at 8.2 mL min^{-1}. During the procedure, nitrate from the sample was exchanged with Cl^- ions from the anion exchange resin and was thus concentrated on an anion exchange resin. Anion resins containing nitrate were kept in refrigerator till further analysis.

Cleaning of Ag_2O

Commercial Ag_2O (Carlo Erba, Italy; G250), needed for the neutralization step (see paragraph below), can contain substantial amount of NO_3^- which can disfigure the isotopic value of the sample and should therefore be thoroughly cleaned before use. The cleaning procedure was preformed as follows: 250 g of Ag_2O and about 1500 mL of distilled water (mili-Q) was put into 2000 mL beaker and boiled and stirred for 1 hour on a heating magnetic stirrer. Afterwards, water was decanted and the procedure was repeated 9 more times. After the last repetition, the solution containing Ag_2O was filtered through Whatman GF/C filter and dried at 105 °C.

Nitrate elution and neutralization of the acid eluate

The nitrate was eluted from the anion resin with 30 mL of 3M HCl in ten 3 mL increments. During elution the flow on the peristaltic pump was set at 8.2 mL min^{-1}. After each elution of 3 mL increments, before adding the next 3 mL of HCl, the flow on the pump was set at 110 mL min^{-1} to remove the last HCl drops and thus dry the resin.

The collected eluate was immediately placed in a cold water bath (about 2 °C) and neutralized with about 13 g of Ag_2O, added in 1 g increments to allow the heat of reaction to dissipate without producing a vapour. The sample was mixed between each incremental

addition of Ag_2O with a glass stirring rod to break the crust, which tends to encapsulate the unreacted reagent. A final pH of about 5.5–6.0 is checked by pH paper. During the neutralization, Ag_2O reacted with eluted HNO_3 to produce $AgNO_3$, AgCl and water. The resulting silver chloride precipitate was removed by vacuum filtration (Whatman GF/C) and the solution, containing $AgNO_3$, was evaporated in a drying oven at 60 °C.

Dry $AgNO_3$ was scrapped from the beaker, sealed in silver cups together with brown sugar (1:2.5 ratio; added for batter combustion efficiency) and analysed for ^{15}N by IRMS.

3.3.3.4 Verification of the diffusion methods

Our aim was to select the best method for NO_3^- isolation from soil solution samples for ^{15}N–NO_3^- determination. In order to do so, two diffusion methods, i.e. the Hook method and the Teflon trap method, and the anion exchange method were compared, analyzing 3 and 200 mL of prepared KNO_3 standard solution (containing 200–300 μg of N) at a natural abundance level (3.5‰ ^{15}N). In addition, results of the ^{15}N determinations of soil solution samples at ^{15}N enriched levels (0.569-2.285 at% ^{15}N) after nitrate isolation by the both diffusion methods were also compared.

3.3.3.4.1 ^{15}N natural abundance standard solutions

No significant differences ($p<0.01$) in $\delta^{15}N$ have been observed between the solid KNO_3 and the NO_3^- that was isolated from the prepared KNO_3 standard solution (3 mL), following different methods of isolation (Table 6, Figure 8).

Table 6: $\delta^{15}N$ (‰) of solid KNO_3 and of nitrate, isolated from the prepared KNO_3 standard solution, following different methods of isolation (3 mL samples).

Method	N	Mean	Minimum	Maximum
KNO_3 (solid)	12	+3.4 ± 0.2	+3.2	+3.8
Diffusion (Hook method)	6	+3.5 ± 0.2	+3.3	+3.8
Diffusion (Teflon trap method)	6	+3.4 ± 0.1	+3.2	+3.5
Anion exchange method	12	+3.5 ± 0.2	+3.2	+3.7

We found that the addition of reagents, especially KCl and Devarda's alloy, which might have traces of N (max. 0.001 % in the KCl and Devarda's alloy used in our study), did not affect $\delta^{15}N$ value in 3 mL samples. For larger additions where higher amount of KCl are necessary to prepare 1M KCl solution, this effect could be substantial for natural abundance samples (however, the effect is negligible at enriched levels). In our study the effect of reagents was not quantified. However, according to Stephan and Kavanagh (2009) who stressed that the reagent effect (the reagent N/target N ratio) is minimized when diffusion is performed on samples containing ≥30 μg target N. Hence, the reagent effect in our case is expected to be minimal since 200–300 μg of N was needed for the measurement on IRMS.

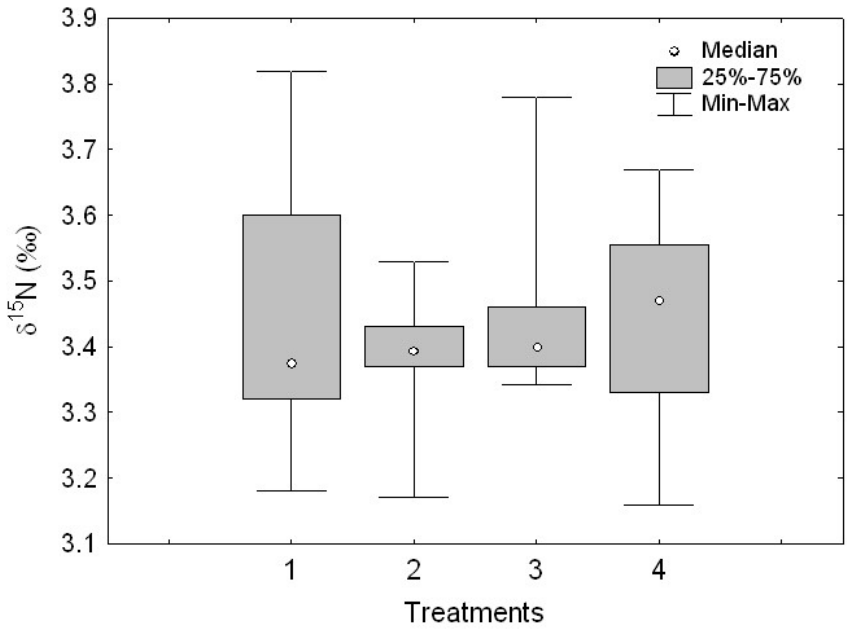


Figure 8: *$\delta^{15}N$ (‰) of solid KNO_3 (1), of NO_3^-, isolated from the working standard solution by the Teflon trap microdiffuion method (2), by the Hook microdiffuison method (3) and by the anion exchange method (4) (3 mL samples).*

With larger volumes, a problem of incomplete recovery was observed. While the lighter isotope ^{14}N is more reactive compared to the heavier isotope ^{15}N, ^{14}N preferentially diffuses from the solution and consequently preferentially bounds onto the filter trap, which leads to the isotopic fractionation, giving erroneously light $\delta^{15}N$ values. However, as reported by Holmes et al. (1998), we also observed that fractionation is consistent across samples incubated under identical conditions (Table 7). Our results demonstrate and confirm that the use of a correction factor which eliminates the fractionation effect associated with incomplete recovery and that of reagents is appropriate and necessary. Hence, for larger volumes (200 mL) with low nitrate concentrations and natural ^{15}N abundance level, a correction factor was implemented after Holmes et al. (1998) (Equation 5), i.e. the fractionation of standards was determined (observed $\delta^{15}N$ – actual $\delta^{15}N$) and this value was added to the observed sample $\delta^{15}N$ values to correct for fractionation. This allows reliable correction of $\delta^{15}N$ values when ammonium recovery is less than 100 % and isotope fractionation occurs, causing erroneously light measured ^{15}N:^{14}N ratio in the sample (depleted in ^{15}N).

$$\delta^{15}N_{corrected} \pm SD_{corrected} = (I + X) \pm \sqrt{(SD^2_{standard} + SD^2_{sample})} \quad (5)$$

Table 7: Calculation of the mean fractionation of KNO_3 standard (200 mL samples).

Replication	$\delta^{15}N$ (‰)			
	True value (T)	Measured value (M)	Fractionation (T – M)	Mean ± SD (X ± SD)
1	+3.5	–5.5	9.0	9.2 ± 0.1
2	+3.5	–5.6	9.1	
3	+3.5	–5.7	9.2	
4	+3.5	–5.8	9.3	
5	+3.5	–5.8	9.3	

An example of the implementation of the correction factor to a sample with the measured $\delta^{15}N$ value of –3.3 ± 0.4‰ is presented in the Equation 6:

$$\delta^{15}\mathrm{N}_{\mathrm{corrected}} \pm \mathrm{SD}_{\mathrm{corrected}} = (-3.3 + 9.2) \pm \sqrt{(0.1^2 + 0.4^2\mathrm{sample})} = 5.9 \pm 0.4\ ‰ \quad (6).$$

According to literature (Goerges and Dittert, 1998; IAEA TCS 14, 2001; Stephan and Kavanagh, 2009), time of the diffusion can be shortened by increasing the T of the diffusion above room T and by performing the diffusion on the shaker. This might also result in higher recovery. However, this will be the case of future studies.

3.3.3.4.2 ^{15}N enriched soil solution samples

Diffusion methods were compared also on the "real" ^{15}N enriched soil solution samples, obtained from the field experiment in Sneberje (Table 8, Figure 9).

Table 8: Nitrate nitrogen at% ^{15}N in soil solution samples, isolated with different diffusion methods.

Sample	Teflon Trap Method (at% ^{15}N)	Hook Method (at% ^{15}N)	Difference (%)
1	0.337	0.345	2.4
2	0.351	0.354	8.5
3	0.398	0.414	4.0
4	0.417	0.428	2.6
5	0.447	0.458	2.5
6	1.344	1.361	1.3
7	1.360	1.415	4.0
8	1.409	1.424	1.1
9	1.429	1.469	2.8
10	1.496	1.573	5.1
11	1.497	1.521	1.6
12	1.500	1.488	0.8
13	1.610	1.582	1.7
14	1.919	1.919	0.0
15	2.366	2.513	6.2
16	2.370	2.435	2.7

Volumes of soil solution samples were chosen depending on the NO_3^- concentrations, so as to contain 200–300 µg of N. For method comparison, samples were pre–concentrated to 3 mL by evaporation (60 °C) on a sand bath, when necessary. The results of compared diffusion methods were in good agreement ($R^2 = 0.9974$).

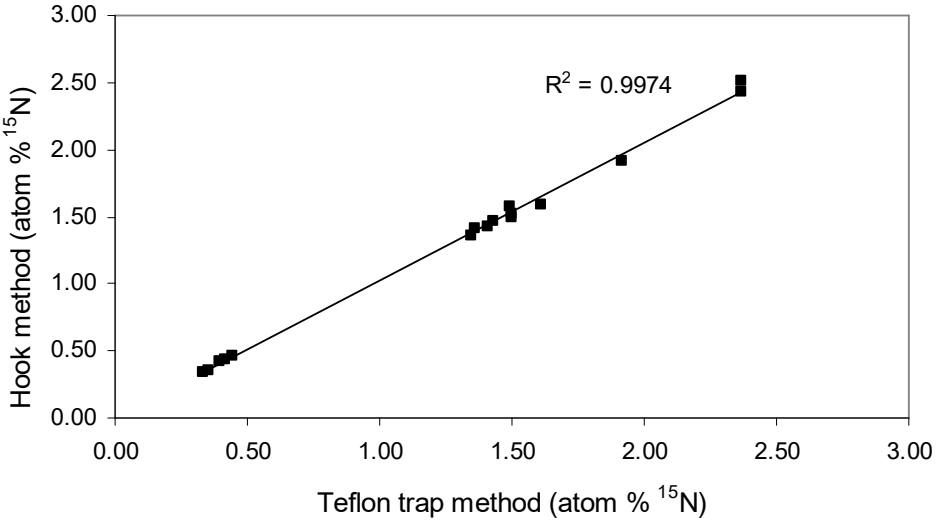


Figure 9: *Comparison of nitrate ^{15}N determinations in soil solution after diffusion with the Hook method and the Teflon trap method.*

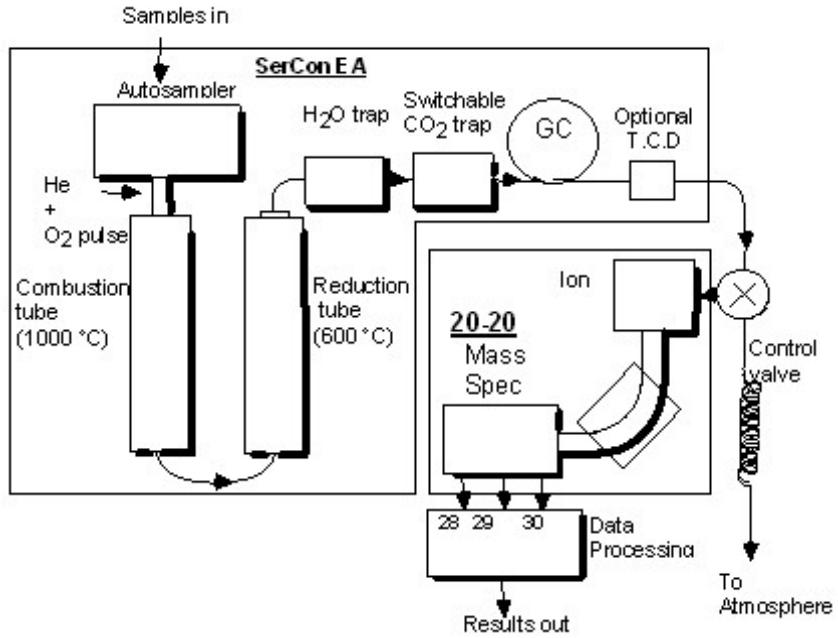
In the case of soil solution samples with high NO_3^- concentrations, the anion exchange method was found to be less appropriate in comparison to the diffusion methods due to high costs of Ag_2O and due to a time consuming and labour intensive procedure. Since the Teflon trap method is suitable also for larger volumes (200 mL), pre-concentration of soil solution samples is usually not needed, which means shorter time of sample preparation and consequently less chance for contamination during the preparation. Hence, the Teflon trap method was found to be most suitable among the tested methods for the routine laboratory use. However, under experimental conditions (room T, manual shaking twice a day) the use of standard solution with known isotopic value and the correction of the measured values for fractionation when NO_3^- recovery is bellow 100 % is necessary when analysing samples of larger volume and natural abundance of ^{15}N.

3.3.4 Determination of ^{15}N by Isotope Ratio Mass Spectrometry (IRMS)

Nitrogen contents and stable nitrogen isotope measurements were performed by isotope ratio mass spectrometry (IRMS) using an Europa 20–20 mass spectrometer connected to an ANCA–SL preparation module for solid and liquid samples (Europa Scientific, Crewe, UK) (Figure 10).

An appropriate amount of plant, soil, and $AgNO_3$ samples or filter traps were put into tin capsules ($AgNO_3$ into silver capsules to avoid corrosion), sealed with tweezers and put into auto sampler of the automatic C/N elemental analyser (ANCA), connected to a mass spectrometer (ANCA–MS) (Figure 10). During analysis, a capsule drops from the auto sampler into a combustion chamber (a quartz tube heated to 1020 °C through which a He carrier stream flows) containing a catalyst (Cr_2O_3 granules), finely divided CuO wire (to oxidize hydrocarbons), and Ag wool (to remove S and halogens). Concurrently, a pulse of O_2 is admitted to promote flash combustion of the Sn, which increases the temperature to around 1700 °C, ensuring complete oxidation of the sample. The combustion products (CO_2, N_2, NO_x, and H_2O) are swept into a tube containing Cu wire at 600 °C, where NO_x species are reduced to N_2, followed by $Mg(ClO_4)_2$ trap for removal of H_2O and switchable Carbosorb® trap for removal of CO_2. Gas is purified by gas chromatography and a fraction of the effluent is admitted to the mass spectrometer for determination of $\delta^{15}N$ (Mulvaney, 1986). The

measurements of $\delta^{15}N$ and atom % ^{15}N, namely natural abundance samples and enriched samples, were run separately.



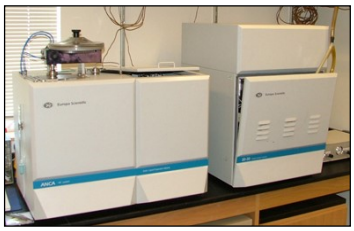


Figure 10: *Left hand side: Diagram of the automated N/C elemental analyzer with continuous flow interface online (ANCA IRMS).* (From: Callisto CF_IRMS). Right hand side:Europa Scientific ANCA-IRMS (From: http://ecosystems.mbl.edu/silab/equipment.html).

Stable isotope results of natural abundance samples are reported as relative values, as deviations versus international standards, i.e. atmospheric air for nitrogen, and are expressed in δ − notation in per mil (‰) by Equation 1 (see page 7).

The results of the ^{15}N labelled samples are reported in atom % (Equation 2), which is the absolute abundance of an isotope, it is the percentage of atoms which occur as the various isotopes, i.e. Nitrogen ^{14}N = 99.63 %, ^{15}N = 0.37 %) (Seely & Lajtha, 1997).

All samples were measured in duplicates. $\delta^{15}N$ and atom % ^{15}N values were accepted when sample standard deviation was ≤0.2 ‰ and ≤0.006 %, respectively.

3.3.4.1 QA/QC of the IRMS measurements

For QA/QC purposes for IRMS, international certified reference materials (CRM) and in–house reference materials (IRM) (calibrated against CRM), were used (Tables 9–11). In each measuring batch at least three different reference materials were included with N content and $\delta^{15}N$ embracing the N content and $\delta^{15}N$ of the samples.

Table 9: Reference materials and in–house reference materials used for IRMS determinations of nitrogen content (% N) in plant samples.

Reference material	Material	% N	Type of RM
IAEA–PLANT RM	Plant	1.90 ± 0.03	RM
WEPAL 1	String bean	2.54 ± 0.11	IRM
WEPAL 2	Melon	2.87 ± 0.17	IRM
WEPAL 3	Lucerne	2.79 ± 0.12	IRM
WEPAL 4	Sunflower	7.97 ± 0.95	IRM

Table 10: Certified reference materials and in–house reference materials for IRMS determinations of the isotopic composition of N in samples at ^{15}N natural abundance values.

Reference material	Material	$\delta^{15}N$ (‰)	Type of RM
IAEA–N–1	Ammonium sulphate	+0.4 ± 0.2	CRM
IAEA–N–2	Ammonium sulphate	+20.3 ± 0.2	CRM
USGS–32	Potassium nitrate	+180 ± 0.2	CRM
USGS–34	Potassium nitrate	–1.8 ± 0.2	CRM
Europa N	Ammonium sulphate	+2.50 ± 0.2	IRM
KNO_3, Merck	Potassium nitrate	+3.5 ± 0.2	IRM
WEPAL 2	Melon	+12.6 ± 0.6	IRM
WEPAL 3	Lucerne	–0.6 ± 0.6	IRM
WEPAL 4	Sunflower	+7.6 ± 0.6	IRM

Table 11: Certified reference materials, reference materials and in–house reference materials used for IRMS determinations of the isotopic composition of N in samples at ^{15}N enriched values.

Reference material	Material	atom % ^{15}N	Type of RM
USGS 32	Potassium nitrate	+0.432 ± 0.367	CRM
IAEA–305 B	Ammonium sulphate	+0.502 – 0.504	RM
IAEA–311	Ammonium sulphate	+2.03 – 2.06	RM
IAEA–PLANT RM	Plant	+1.187	RM
WEPAL 1	String bean	+1.009 ± 0.054	IRM
Europa N	Ammonium sulphate	+0.367	IRM

Uncertainty of IRMS measurement with a coverage factor k=1 was calculated using equation (7):

$$\sqrt{(\text{St.dev.})^2 + (\text{unc\_method})^2} \qquad (7)$$

where *St. dev.* is standard deviation of 3 measurements and *unc_method* is the estimated uncertainty of the method used.

Uncertainty of isotopic analysis was determined based on standard deviations of long–term measurements (1–2 years) of IAEA-N-2, IAEA Plant RM for nitrogen at natural abundance and enriched level, respectively. The certified data for ^{15}N can be found on website: http://curem.iaea.org/catalogue/SI/index.html. The obtained estimated uncertainties for IRMS for ^{15}N were, as follows (Šturm et al., 2009; IJS-DP-10311):

^{15}N natural abundance: 0.12 ‰
^{15}N enriched abundance: 0.003 at%

In order to accurately measure the isotopic composition of plant samples, firstly the minimal amount of N needed for measurement on IRMS was determined using an in-house reference material Melon with $\delta^{15}N$ of +12.6±0.6‰. It was found that below a peak area of $4.3*10^{-8}$, the $\delta^{15}N$ determinations were too high, whereas between peak areas of $7.6*10^{-8}$ and $1.6*10^{-6}$, $\delta^{15}N$ determinations obtained were in good agreement with $\delta^{15}N$ values defined for the reference material used (Figure 11).

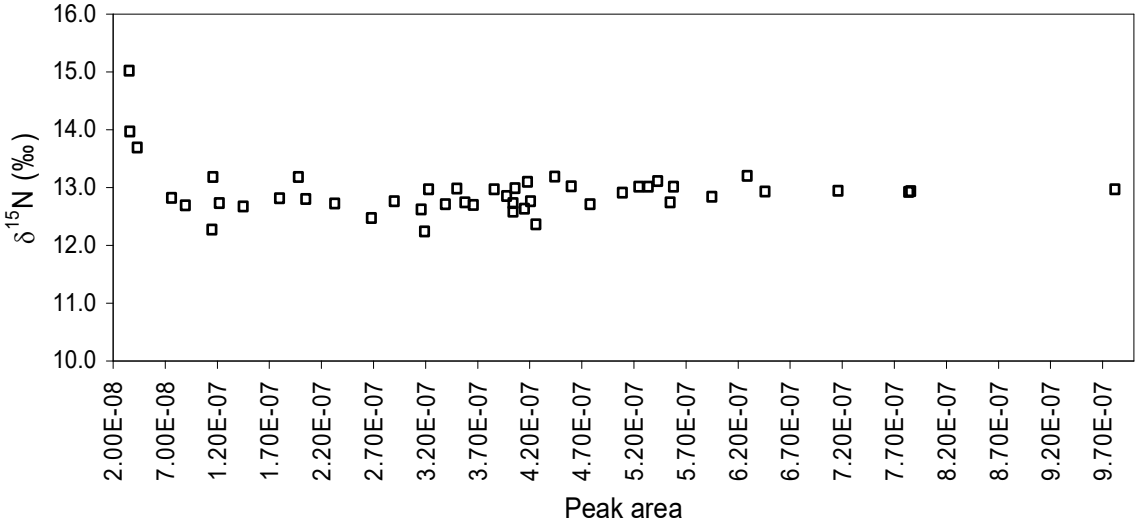


Figure 11: *Determination of an optimal peak area for $\delta^{15}N$ determiantions in plant samples*.

The accuracy of ^{15}N measurements in plant samples at natural and enriched levels was verified also with successful participations in the international interlaboratory studies: WEPAL International Plant-analytical Exchange Program, IPE 2009.2 and IPE 2010.2 (Šturm et al., 2009, IJS-DP-10311; Šturm and Lojen, 2010, IJS-DP-10505).

3.3.5 Calculations used in experiments with ^{15}N

For all field and greenhouse experiments with ^{15}N labelled materials, the following primary data need to be recorded for each plot and treatment (van Cleemput et al., 2008):

- ✓ Dry matter yield for the whole plant or subdivided into parts. This parameter is utilized to estimate the amounts of total N uptake, and is determined from the isotope plot. Dry matter yields can be calculated for whole plant or separately by plant part (vegetative and reproductive, such as shoots and pods, straw and spikes, etc.) and than summed up to obtain the total biomass or total dry matter produced by the crop at harvesting time.

- ✓ Total N concentration (% total N in dry matter) of the whole plant or plant parts (e.g. by dry combustion, Dumas).

- ✓ Plant % ^{15}N abundance (mass spectrometry).

- ✓ Fertiliser % ^{15}N abundance (mass spectrometry).

- ✓ ^{15}N labelled fertilisers used and N rate of application.

The first parameter to be determined when studying fertiliser N uptake by the isotopic method is the portion of N in the plant derived from the ^{15}N labelled fertiliser (Ndff). The information to calculate this parameter is obtained from the plant % ^{15}N abundance and fertiliser % ^{15}N abundance data. ^{15}N abundance data must be converted into atom% ^{15}N excess by subtracting the natural abundance (0.3663 atom% ^{15}N) from the % ^{15}N abundance of the sample (van Cleemput et al., 2008).

Afterwards, following calculations can be made (Equations 8–14) (IAEA, Training Course Series No.14, 2001, van Cleemput et al., 2008):

- Biomass produced/dry matter yield per hectare (kg ha^{-1})

$$\text{Dry matter yield (kg ha}^{-1}\text{)} = \text{FW (kg)} \times \frac{10000\ (\text{m}^2\ \text{ha}^{-1})}{\text{area harvested (m}^2\text{)}} \times \frac{\text{SDW (kg)}}{\text{SFW (kg)}} \quad (8)$$

- Fertiliser N uptake or fertiliser N yield (kg ha^{-1})

$$\text{N yield (kg ha}^{-1}\text{)} = \text{dry matter yield (kg ha}^{-1}\text{)} \times \frac{\%\ \text{N}}{100} \quad (9)$$

- Nitrogen in plant tissue derived from the fertiliser (Ndff; i.e. the portion of total N in the plant, that was derived from the applied fertiliser) and from soil (Ndfs, i.e. the portion of total N in the plant, that was derived from soil N):

$$\%\ \text{Ndff} = \frac{\text{atom \% } ^{15}\text{N excess}_{\text{plant}}}{\text{atom \% } ^{15}\text{N excess}_{\text{fertilizer}}} \times 100 \quad (10)$$

$$\%\ \text{Ndfs} = 100 - \%\ \text{Ndfs} \quad (11)$$

- Fertiliser N uptake/yield (kg ha^{-1}):

$$\text{Fertilizer N yield (kg ha}^{-1}\text{)} = \text{N yield (kg ha}^{-1}\text{)} \times \frac{\%\ \text{Ndff}}{100} \quad (12)$$

- Fertiliser N use efficiency/fertiliser N recovery/real coefficient of utilization:

$$\%\,\text{Fertilizer N use efficiency} = \frac{\text{Fertilizer N yield}}{\text{Rate of N application}} \times 100 \quad (13)$$

where FW is sample fresh weight per area harvested and SDW and SFW are subsample dry and fresh weight, respectively. Total N accumulation (kg ha^{-1}) was calculated by multiplying the dry matter yield of plant parts and the mean N concentration in the plant parts.

- The fraction of NO_3^-–N in soil water derived from the fertiliser (Ndff):

$$\text{Ndff} = \frac{\text{atom \% } ^{15}\text{N excess}_{\text{water sample}}}{\text{atom \% } ^{15}\text{N excess}_{\text{fertilizer}}} \quad (14)$$

Statistical analyses

Data were verified statistically with the Analysis of variance (ANOVA) module using Statistica 6.0 (StatSoft, Tulsa, OK, USA) package. Means were separated by post hoc least significant difference test (Gaussian normal distribution) or by the nonparametric Mann-Whitney U test (non Gaussian distribution). In the field and pot experiment, differences were considered to be significant at $p<0.05$, whereas in the method comparison study, differences were considered to be significant at $p<0.01$.

4 Results and Discussion

4.1 Field experiment with white cabbage

In order to get data which could lead to recommendations for growers growing white cabbage on sandy-loam soils inside groundwater protection areas, the effect of different fertilisation and irrigation practices on yield, N–fertiliser use efficiency and consequently yield quality (i.e. nitrate content in plant) of white cabbage (*Brassica oleracea* var. capitata L.) as well as the potential for N losses (i.e. N surplus after harvest) were investigated. Four different fertilisation and irrigation practices were applied, i.e. unfertilized control, irrigated in accordance with the farmer's practice (1), pre–plant broadcast fertiliser application with drip irrigation covering 50% of crop's water requirements (2), fertigation with drip irrigation covering 100% of crop's water requirements (3) and farmer's practice, which consisted of pre–plant broadcast fertiliser application with irrigation on the day before and after transplanting. The potential for N losses was determined in accordance with the mass balance calculations.

4.1.1 Yield and dry matter

Yield. The study revealed that the treatment used significantly affected cabbage yield (Table 12, Figure 12). Fertigation did not increase the yield compared to broadcast fertiliser application, as was also found by Hagin (1999) and Kacjan-Maršić (2004). The highest yield was found for treatment with farmer's practice, lower for fertigation and the lowest for treatments with broadcast fertilisation and irrigation covering 50% of the crop's water requirements and for the control.

Table 12: Yield and dry matter (DM) in white cabbage.[*]

Treatment	Yield (t ha^{-1})	DM (%)
1[#]	47 a	11.03 a
2	58 ab	8.79 b
3	72 b	9.32 b
4	93 c	9.02 b

[*] Different letters denote significant difference between treatments at the $p<0.05$.

[#] Treatments: 1–control, 2–broadcast fertilisation with 50% irrigation, 3–fertigation with 100% irrigation, 4–broadcast fertilisation with farmer's practice of irrigation.

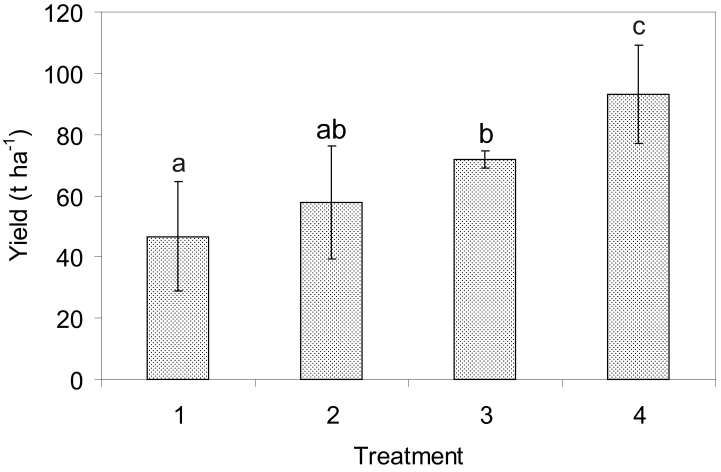


Figure 12: *Cabbage yield under different treatments: 1–control, 2–broadcast fertilisation with 50% irrigation, 3–fertigation with 100% irrigation, 4–broadcast fertilisation with farmer's practice of irrigation.* Data are means ± standard deviation (n=3). Bars designated by different letters are significantly different at the $p<0.05$.

Dry matter. We found a significant effect of nitrogen fertilisation on dry matter content in cabbage (Table 13). The average dry matter content was 9.04% in fertilized plants and 11.03% in unfertilized plants. In comparison to the control, fertilisation caused a considerable decline in dry matter content. A similar observation was made by Smolen and Sady (2009), who studied the effects of various nitrogen fertilisation and foliar nutrition regimes on the dry matter content in carrot roots (*Daucas carota* L.). Sørensen (1999), who studied the effects of nitrogen on vegetable crop production and chemical composition, also reported that increased N supply decreased the dry matter content.

4.1.2 Total N and nitrate content in plants

Results of total N and NO_3^- determination in aboveground plant parts are presented in Tables 13–14 and Figures 13–14.

Total N content. Fertilisation caused a significant increase in total nitrogen in cabbage heads, compared to the control. Under fertilised conditions, a significantly higher N content was determined in crops under farmer's practice of irrigation (Treatment 4), whereas the difference between the two treatments with drip irrigation (covering 50% and 100% of the crop's water requirements; Treatments 2 and 3, respectively), was not significant. Under all treatments, a decline in N content was observed towards the final harvest in inner, middle and outer leaves of cabbage due to the dilution effect during plant growth.

At final harvest, a statistically significant difference in N content was found between different leaves under each treatment. On fertilized plots as well as under control, most of the N was accumulated in the outer leaves and least in the middle leaves of the plants, indicating the highest uptake of N at early growth stage (Gastal and Lemaire, 2002).

Nitrate content. It was found that fertilisation significantly increased the nitrate content in crops compared to the control, as also found by various authors, who studied the NO_3^- contents in cabbage (Turan, 2005), carrot (Smolen and Sady, 2009 and references therein), and in rape, Chinese cabbage and spinach (Chen et al., 2004). The highest mean nitrate content at final harvest was found for farmer's practice (1326 mg kg^{-1} fresh weight; Treatment 4) and for treatment with fertigation (1132 mg kg^{-1}; Treatment 3), lower for treatment with broadcast fertilisation and irrigation covering 50% of the plant's water requirements

(Treatment 2), and the lowest for the control (345 mg kg^{-1}; Treatment 1).

Table 13: Nitrate content in different leaves of cabbage at final harvest.*

Treatment	NO_3^- (mg kg^{-1} fresh weight)			N content∞
	I§	M	O	(% DM)
1#	344.0 a	228.2 a	324.4 a	1.62 x
2	544.5 a	845.1 a	1222.4 b	2.70 y
3	819.9 a	753.3 b	1478.7 c	2.52 y
4	775.2 a	1305.6 b	1685.7 b	2.86 z

* Different letters denote significant difference between different leaves (a, b, c) and between treatments (x, y, z) at the $p<0.05$. Data are means for n=9 plants per treatment.

Treatments: 1–control, 2–broadcast fertilisation with 50% irrigation, 3–fertigation with 100% irrigation, 4–broadcast fertilisation with farmer's practice of irrigation.

§ Different letters denote different plant leaves: inner (I), middle (M) and outer (O) leaves.

∞ N content represents weighted average content in aboveground cabbage at final harvest.

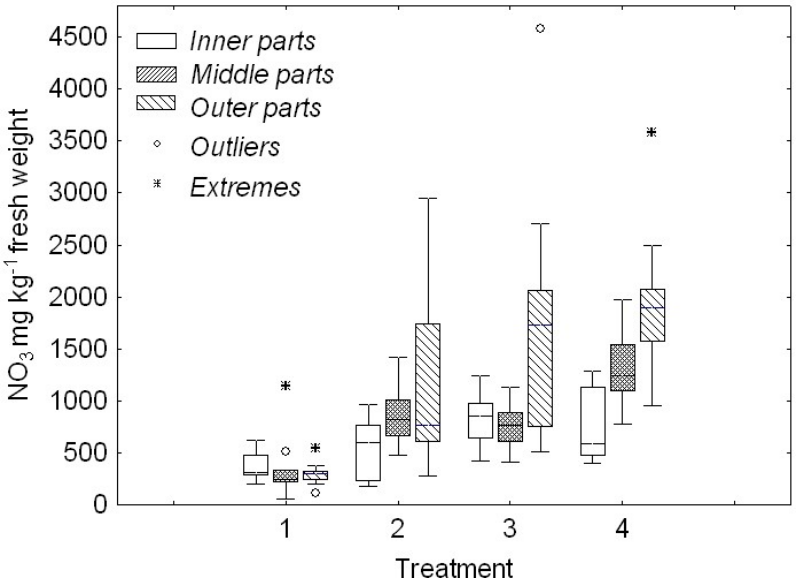


Figure 13: *Nitrate content in inner, middle and outer fresh cabbage leaves at final harvest.* Treatments: 1–control, 2–broadcast fertilisation with 50% irrigation, 3–fertigation with 100% irrigation, 4–broadcast fertilisation with farmer`s practice of irrigation. Box gives lower and upper qartiles and median; whiskers show 25th and 75th percentiles; bars represent the nonoutlier range.

No significant intra–plant variations were found in the control, whereas for fertilized treatments with broadcast application, a significantly lower content was found in the inner, younger parts and the highest in the outer, older leaves of the plants. For treatment with fertigation (Treatment 3), the lowest nitrate content was found in the middle parts and the highest in the outer, older leaves of cabbage.

Table 14: N content of different leaves of cabbage at 59, 68 and 78 days after transplanting.*

Treatment	% N (DM basis)								
	59 DAT			68 DAT			78 DAT		
	I§	M	O	I	M	O	I	M	O
1#	2.59 a	1.85 b	2.40 a	2.02 ab	1.67 a	2.23 b	1.62 b	1.30 a	1.93 b
2	3.28 a	2.94 a	4.29 b	2.62 a	2.58 a	3.49 b	2.65 ab	2.37 a	3.08 b
3	3.14 a	2.42 b	3.64 c	2.67 a	2.45 a	3.31 b	2.38 a	2.11 a	3.06 b
4	3.25 a	3.11 a	4.89 b	3.17 a	3.03 a	4.34 b	2.71 a	2.60 a	3.26 b

* Different letters denote significant difference between different leaves at the $p<0.05$. Data are means for n=9 plants per treatment.

Treatments: 1–control, 2–broadcast fertilisation with 50% irrigation, 3–fertigation with 100% irrigation, 4–broadcast fertilisation with farmer's practice of irrigation.

§ Different letters denote different plant leaves: inner (I), middle (M) and outer (O) leaves.

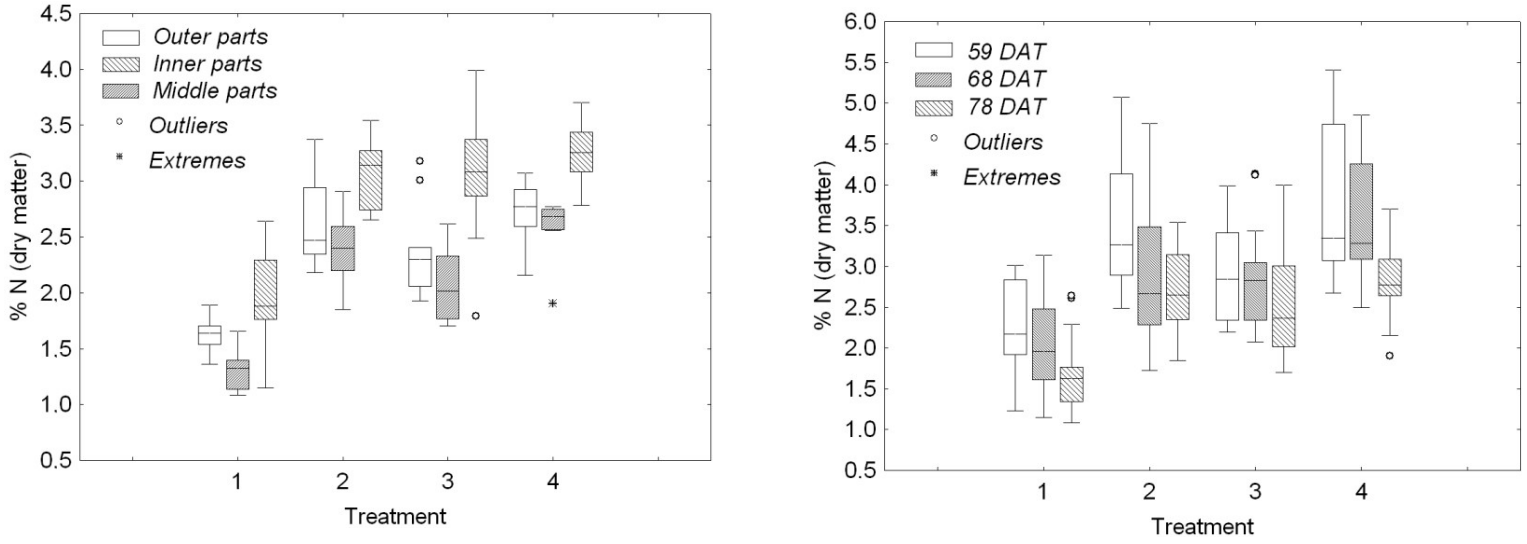


Figure 14: *Intra–plant variation of N content in different leaves at final harvest (left hand side) and N content in cabbage at 59, 68 and 78 DAT (right hand side). Treatments: 1–control, 2–broadcast fertilisation with 50% irrigation, 3–fertigation with 100% irrigation, 4–broadcast fertilisation with farmer`s practice of irrigation.* Box gives lower and upper quartiles and median; whiskers show 25th and 75th percentiles; bars represent the nonoutlier range.

4.1.3 ^{15}N in cabbage

Plants from control plots were significantly depleted with ^{15}N compared to crops from fertilized plots, since the control plants reflected the isotopic composition of soil N (with low ^{15}N content) (Figure 15, Appendix 3). On fertilized plots, the lowest ^{15}N values were observed in plants from treatment by fertigation (Treatment 3). At final harvest, the difference in at% ^{15}N excess between treatments by fertigation and farmer's practice (Treatments 3 and 4, respectively) became insignificant (1.17, 1.19 at% ^{15}N excess, respectively), whereas cabbage under treatment by broadcast fertilisation with irrigation covering 50% of the crop's water requirements was still significantly more enriched in ^{15}N (1.55 at% ^{15}N). No significant differences in at% ^{15}N were observed between outer, middle and inner leaves of cabbage (Figure 16).

During the growing season, two different trends and a significant difference in the at% ^{15}N excess were observed between fertilised treatments. With time, enrichment with ^{15}N in cabbage increased in treatment by fertigation (Treatment 3) and decreased in both treatments with broadcast application (Treatments 2 and 4). The variation in at% ^{15}N excess in cabbage could be attributed to the different availability of soil- and fertiliser-N with time of growth (Choi et al., 2002). Decreasing enrichment with ^{15}N with time in cabbage under the two treatments with broadcast application indicates an increased accumulation of N from soil (Choi et al., 2002) and the increasing enrichment under treatment with fertigation, on the other hand, indicates increased accumulation of the enriched fertiliser N with growth time. When available fertiliser N was low in the soil, the relative contribution of soil– to plant N increased with time (Yun and Ro, 2009).

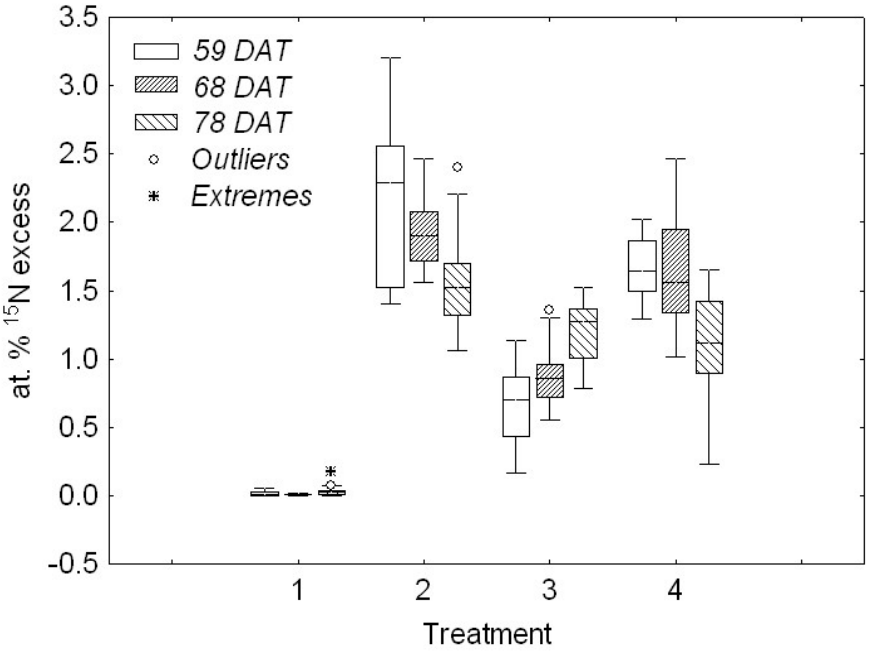


Figure 15: *atom % ^{15}N excess in aboveground cabbage leaves at 59, 68 and 78 DAT*. Treatments: 1–control, 2–broadcast fertilisation with 50% irrigation, 3–fertigation with 100% irrigation, 4–broadcast fertilisation with farmer's practice of irrigation. Box gives lower and upper quartiles and median; whiskers show 25th and 75th percentiles; bars represent the nonoutlier range.

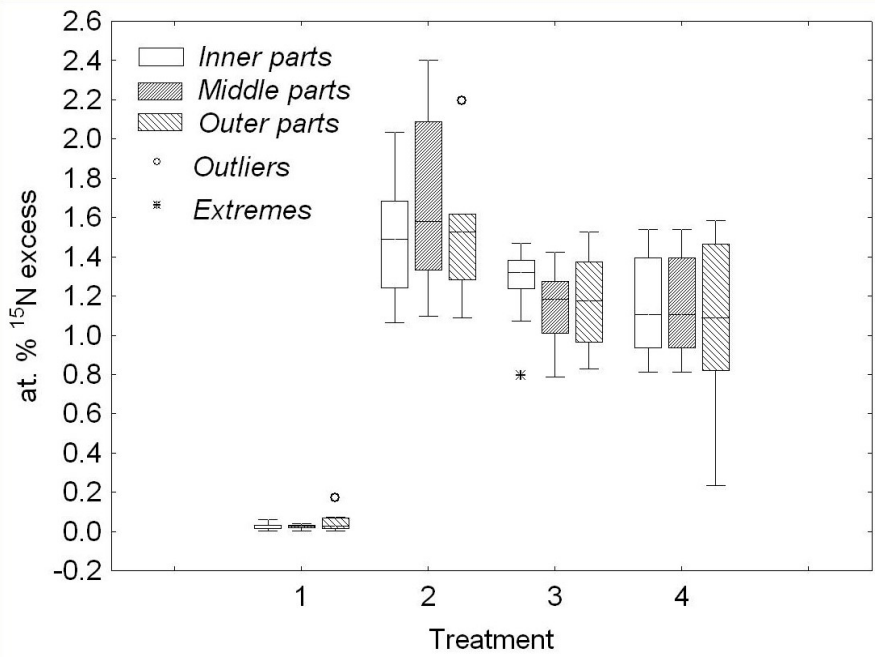


Figure 16: *atom % ^{15}N excess in different cabbage leaves at final harvest*. Treatments: 1–control, 2–broadcast fertilisation with 50% irrigation, 3–fertigation with 100% irrigation, 4–broadcast fertilisation with farmer's practice of irrigation. Box gives lower and upper quartiles and median; whiskers show 25th and 75th percentiles; bars represent the nonoutlier range.

4.1.4 Nitrogen uptake and fertiliser use efficiency

Fertiliser use efficiency deserves careful attention if agriculturists are to produce maximum crop yields and prevent pollution of natural waters with plant nutrients (Bijay-Singh et al., 1995). This depends largely on the synchrony between plant N demand and the quantities of N supplied by the fertiliser and by the soil. Consequently, it is strongly affected by N management methods, as well as by crop management practices (van Cleemput et al., 2008). Since only a fraction of the applied fertiliser N (on average less than 50 %) is taken up by the crop, the remainder is subject to loss, representing both an economic cost and an environmental risk (van Cleemput et al., 2008, references therein). Even though low fertiliser use efficiency does not always imply that unused N will leach into groundwater and does not necessarily pose a hazard to the environment (as long as excess water does not leach it beneath the root zone (Bijay-Singh et al., 1995)), maximizing the efficiency of fertiliser nitrogen can reduce the risk of nitrate pollution from leaching (Bijay-Singh and Sekhon, 1979).

As reported also by van Cleemput et al. (2008), the use of ^{15}N-labelled fertiliser revealed differences in the uptake of soil N between plants fertilized with N and those unfertilized. The portion of N derived from the soil exceeded the portion of N derived from the fertiliser (Table 15). In experiments with ^{15}N-labelled fertiliser, it is often observed that the uptake of unlabelled N is greater by plants receiving labelled fertiliser than in the unfertilized control (Powlson and Barraclough, 1993). This can occur entirely as a result of ″pool substitution″, i.e. labelled inorganic N from the added fertiliser taking the place of unlabelled inorganic N that would otherwise have been immobilized (taken up by microorganisms, where N is incorporated into proteins, nucleic acids, and other organic N constituents of microbial cells and cell walls and as such becomes a part of the biomass). The greater the amount of labelled N that is added, the smaller will be the proportion of unlabelled N that is immobilized and the

more unlabelled N will remain in the inorganic pool available for uptake by plants (Powlson and Barraclough, 1993).

Under the field experimental conditions, N uptake was 84 kg N ha^{-1} for the control and 138–246 kg ha^{-1} for fertilized treatments (Table 15). The proportion of N taken up from soil or fertiliser varied between treatments. The highest uptake of soil N was calculated for farmer's practice (162.4 kg ha^{-1}), lower for the control (84.2 kg N ha^{-1}) and the lowest for treatment with broadcast fertilisation with irrigation covering 50% of the crop's water requirements (77.1 kg N ha^{-1}), whereas higher fertiliser N utilization was determined under farmer's practice (41.8%), compared to treatment with broadcast fertilisation with irrigation, covering 50% of crop's water requirements (30.3%) (Table 16).

Fertigation is expected to increase the nutrient uptake efficiency, thereby minimizing leaching losses compared to the application of fertiliser in dry granular form broadcast over a large soil area at less frequent intervals (Alva, 2009). However, among the practices tested in this study on a sandy loam agricultural soil and under the environmental conditions with precipitation somewhat below and temperature above the 30 year average (Table 5), fertigation with three-split applications of the fertiliser (Treatment 3) did not result in the lowest N leaching potential. The highest yield, N uptake and lowest leaching potential, but also highest NO_3^- content at final harvest, were determined for treatment with broadcast fertilisation and farmer's practice of irrigation (Treatment 4).

Table 15: N uptake, fertiliser N yield and soil N yield of cabbage at final harvest (kg ha^{-1}).*

Treatment	N uptake by crops	Fertiliser N yield	Soil N yield
1#	84.2 a	–	84.2
2	137.7 b	60.6	77.1
3§	168.8 b	–	–
4	246.0 c	83.6	162.4

* Different letters denote significant difference between treatments at the $p<0.05$.

#Treatments: 1–control, 2–broadcast fertilisation with 50% irrigation, 3–fertigation with 100% irrigation, 4–broadcast fertilisation with farmer`s practice of irrigation.

§In treatment by fertigation the pre–plant broadcast application was made with unlabelled fertiliser, and ^{15}N was applied by fertigation only, therefore the % Ndff, % Ndfs, fertiliser N yield and % fertiliser N utilization were not calculated for this treatment.

Table 16 presents the balance of N inputs and outputs for the experiment. The inputs involve N in fertilisers, N from wet deposition and irrigation N, and the outputs comprise only N uptake by the aboveground biomass of plants (van Eerdt and Fong, 1998, Oenema et al., 2003, Ju et al. 2006). The N surplus, i.e. the difference between inputs and outputs, represents the N that was lost by volatilization of ammonia, denitrification or leaching, or stored in various soil fractions (Ju et al., 2006). The control (Treament 1) and farmer's practice (Treament 4) had a negative balance (–79.5 and –41.3 kg N ha^{-1}, respectively), whereas treatment via broadcast application and irrigation covering 50% of the crop's water requirements and treatment with fertigation (Treatment 3) with irrigation covering 100% of the crop's water requirements (Treatment 2) had positive balances of N inputs and outputs (+68.0 and +37.8 kg N ha^{-1}, respectively).

Calculation of the N budget (Table 16) indicates a higher potential for N losses in treatments with broadcast fertiliser application with 50% irrigation (highest N surplus; Treatment 2) and fertigation with 100% irrigation (Treatment 3), compared to farmer's practice of fertilisation and irrigation (Treatment 4), where crop N uptake exceeded N inputs by over 40 kg N ha^{-1}, thus resulting in soil N depletion to that extent.

Table 16: N balance of N inputs and outputs and fertiliser use efficiency.

Treatment	Inputs[#]	Outputs	N surplus	Fertiliser use efficiency
	kg N ha^{-1}			%
1[*]	4.7	84.2	–79.5	–
2	205.7	137.7	+68.0	30.3
3	206.6	168.8	+37.8	–
4	204.7	246.0	–41.3	41.8

[*] Treatments: 1–control, 2–broadcast fertilisation with 50% irrigation, 3–fertigation with 100% irrigation, 4–broadcast fertilisation with farmer's practice of irrigation.

[#]Inputs comprise fertiliser N, irrigation N and N from wet deposition. Outputs comprise only the uptake by the aboveground biomass of crops. N surplus represents N that was lost by ammonia volatilization, denitrification or leaching, or stored in various soil fractions.

Regarding the results obtained, under these environmental conditions and soil type the application of N fertiliser should be increased by about 40 kg ha^{-1} under farmer's practice of fertilisation and irrigation, decreased by about 68 kg ha^{-1} under treatment with broadcast fertiliser application with 50% irrigation, and decreased by 38 kg ha^{-1} or apply N in more frequent fertigation events under treatment with fertigation with 100% irrigation, in order to gain the same yield without depletion of the soil N and without environmental risk.

4.1.5 Nitrate content and ^{15}N in soil water

Due to small sample volumes or even lack of samples in some treatments, especially in treatments with farmer's practice of irrigation, it was not possible to determine the nitrate content and ^{15}N concentration for all sampling dates. Results of determination of nitrate and ^{15}N concentrations in soil water sampled during and after the growing period of white cabbage are presented in Tables 17–18 and Figure 17.

Nitrate content. According to Alva (2009), as mentioned above, fertigation is expected to increase the nutrient uptake efficiency, thereby minimizing leaching losses compared to the application of fertiliser in dry granular form broadcast over a large soil area at less frequent intervals. However, among the tested practices and under the environmental conditions in our study, fertigation (Treatment 3) with three split applications of the fertiliser did not result in the lowest N leaching and is hence not the most appropriate method for growing cabbage. As shown on Figure 18, higher nitrate content in soil water samples was determined under fertigation treatment (26.0 and 30.2 mg L^{-1} on 23.5. and 6.6.2007, respectively) as well as under treatment 2, with broadcast fertiliser application and drip irrigation covering 50% of crop's water requirements (30.2 mg L^{-1} on 6.6.2007), compared to the treatment with farmer's practice (19.3 and 8.0 mg L^{-1} on 23.5. and 6.6.2007, respectively). Seventy days after harvest, nitrate content in soil water was comparable under all treatments.

^{15}N in nitrate. In natural (unlabelled) system, NO_3^- in soil leachate is subject to considerable isotopic variation and $\delta^{15}N$ may provide more information on the predominance of microbial processes in soils than on origin. A major difficulty associated with the use of $\delta^{15}N$ to assess origins is the ability to distinguish between isotope behaviour that is conservative and that which is a reflection of processes (e.g. denitrification, nitrification, ion exchange) that alter isotope abundances (Ostrom et al., 1998). Additional complexity may arise because source materials, such as soils, may contain several isotopically heterogeneous forms of N (Tiessen et al., 1984; Ledhard et al., 1984) and therefore, may not behave

functionally as a single pool of N with distinct and characterizable isotope ratio (Ostrom et al., 1998). However, in our study this problem was overcame with the use ^{15}N labelled fertiliser.

The portion of N in soil water derived from the fertiliser is presented in Table 18. On 23.5.2007 isotopic composition could only be determined for the sample from the fertigation treatment (Treatment 3). It was found that 5.4% of nitrate N in the analysed sample originated from the applied fertiliser and 94.6% (100% - Ndff) originated from soil N. On the second sampling event (4.6.2007), the highest nitrate concentration under treatment 2 coincides with the calculated highest portion of N derived from the fertiliser (46.7%; only 8.5% for the fertigation treatment), which confirms that this treatment results in the highest fertiliser leaching and is hence the least appropriate for growing white cabbage on this type of soil among tested practices (at least under current environmental conditions). Twenty one days after harvest (18.7.2007), the portion of N derived from the fertiliser was still higher under treatment 2 (33.8%) compared to fertigation treatment (11%). Seventy days after harvest, highest portion of N derived from the fertiliser was determined under farmer's practice (16.5%), lower under treatment 2 (decreased for 22% between 18.7. to 5.9.2007) and lowest under fertigation treatment (decreased for 6% between 18.7. to 5.9.2007); however, as mentioned above, nitrate concentrations were relatively uniform under all treatments.

Table 17: Nitrate content (mg L^{-1}) in soil water during and after growth of white cabbage.

Treatment	23.5.2007	4.6.2007	18.7.2007	5.9.2007
1*	10.0	10.5	n.d.#	18.4
2	n.d.	30.8	9.9	16.4
3	28.4	30.2	13.8	16.2
4	19.3	8.0	2.1	18.7

*Treatments: 1–control, 2–broadcast fertilisation with 50% irrigation, 3–fertigation with 100% irrigation, 4–broadcast fertilisation with farmer`s practice of irrigation.
#n.d. – not determined.

Table 18: Isotopic composition (atom % ^{15}N excess) of soil water nitrate with the portion of total N derived from the fertiliser (Ndff).

Treatment	23.5.2007		4.6.2007		18.7.2007		5.9.2007	
	at%^{15}N excess	% Ndff	at%^{15}N excess	% Ndff	at%^{15}N excess	% Ndff	at%^{15}N excess	% Ndff
1*	n.d.#	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2	n.d.	n.d.	1.645	46.7	1.191 ± 0.072	33.8 ± 2.0	0.404	11.5
3	0.191	5.4	0.299 ± 0.086[d]	8.5 ± 2.4	0.389	11.0	0.161 ± 0.046	4.6 ± 1.3
4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.582 ± 0.028	16.5 ± 0.8

*Treatments: 1–control, 2–broadcast fertilisation with 50% irrigation, 3–fertigation with 100% irrigation, 4–broadcast fertilisation with farmer`s practice of irrigation.
#n.d. – not determined.

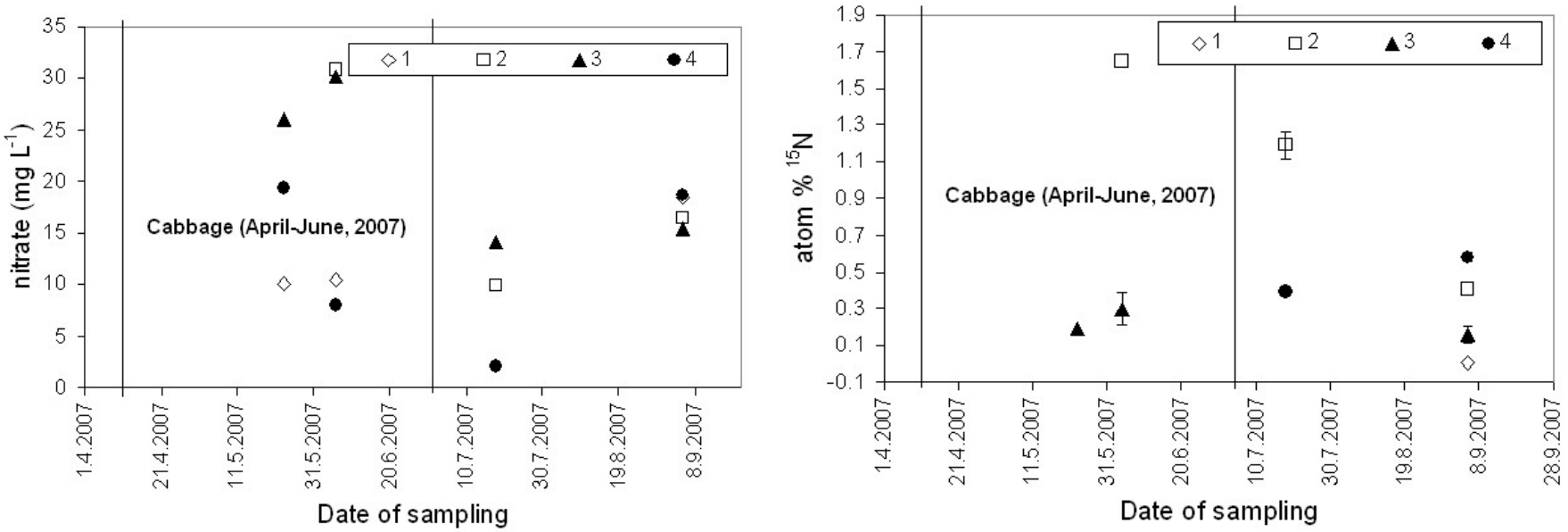


Figure 17: *Nitrate content (left hand side) and atom % ^{15}N excess (right hand side) in soil water sampled using porous ceramic suction cups during and after the growth of white cabbage.* Treatments: 1–control, 2–broadcast fertilisation with 50% irrigation, 3–fertigation with 100% irrigation, 4–broadcast fertilisation with farmer`s practice of irrigation. Vertical lines denote the cabbage growing period.

4.1.6 Nitrate content and ^{15}N in groundwater

Nitrate content and $\delta^{15}N$ of nitrate in groundwater was followed in five piezometers (namely, V-1 to V-5), placed around the experimental field (Figures 3), in the period between 1. 10. 2006 and 25. 7. 2008, in order to detect the potential pollution, caused by the applied labelled fertiliser on the field (before white cabbage, labelled fertiliser was already applied on the field during the endive vegetation period between 10.8. and 26.10.2006).

Nitrate content. Figure 18 and Appendix 4 present the fluctuation of nitrate content in groundwater sampled below the experimental field between May, 2006 and July, 2008. Nitrate content determined in our study, was constantly bellow maximum allowed level of 50 mg L^{-1} (ranging between 20.5 and 30.8 mg L^{-1}), set by the World Health Organization and was in good agreement with previous study of Urbanc et al. (2003) who studied nitrates in groundwater of Ljubljansko polje (4.9-33.2 mg L^{-1}). No extraordinary deviations in NO_3^- content have been observed during the white cabbage vegetation period. Groundwater seasonal pattern of nitrate concentration was in accordance with the highest Sava river discharge peak, recorded during spring time (March 2007, 2008) (Drolc and Zagorc Končan, 1996; 2007; Drolc et al., 2007; Zupanc et al., submitted) and not with increased NO_3^- concentrations measured in soil solution.

^{15}N in total N and nitrate N. Considering the isotopic composition of the labelled fertiliser used (3.5 atom % ^{15}N, that is about 8950 ‰), which is high above the natural abundance values, natural $\delta^{15}N$ values of total N and nitrate of the groundwater samples analysed (ranging from 4.7 to 13.8 and from 6.0 to 8.3, respectively) indicate that during the observed time, the N from the applied labelled fertiliser did not reach the groundwater aquifer (Figure 19, Appendix 4).

Groundwater of Ljubljansko polje is rapidly charged and discharged, which means that pollutants are relatively quickly washed away from the aquifer; however there is a constant risk of sudden influx of pollutants from surface as well as the Sava river into the aquifer (Auersperger et al., 2005). The aquifer of Ljubljansko polje is fed by two components, by the Sava River and by rainfall. This two components are exposed to several sources of pollution, therefore a correlation exist between their mixing ratio and groundwater chemical composition (Urbanc and Jamnik, 2007). Kanduč (2006) found $\delta^{15}N$ values of suspended organic matter of the Sava river amounting of 7.0 ± 1.0‰ and 5.5 ± 0.7‰, measured in September, 2004 and May, 2005, respectively. Similar values were obtained by Urbanc and Jamnik (2007), who studied distribution and origin of nitrate in the groundwater of Ljubljansko polje between autumn 2002 and summer 2004, and report mean $\delta^{15}N$ values ranging between 4.2 and 9.4‰. The values obtained by Kanduč (2006) and Urganc and Jamnik (2007) are in good agreement with $\delta^{15}N$ values of total N and nitrate obtained in our study which again confirms that the values obtained are in ″normal″ range and were not affected by the ^{15}N labeled fertiliser.

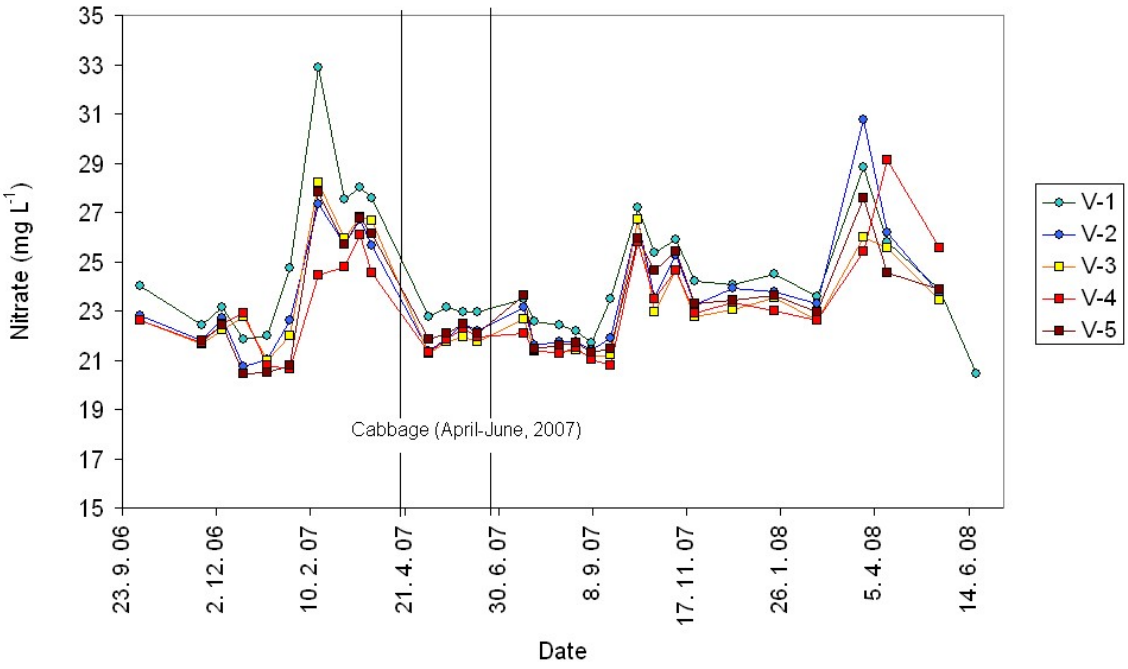


Figure 18: *Nitrate fluctuation in groundwater sampled from the piezometers (V-1 to V-5) below the experimental field in Sneberje between October, 2006 and July, 2008.* Vertical lines denote the cabbage growing period.

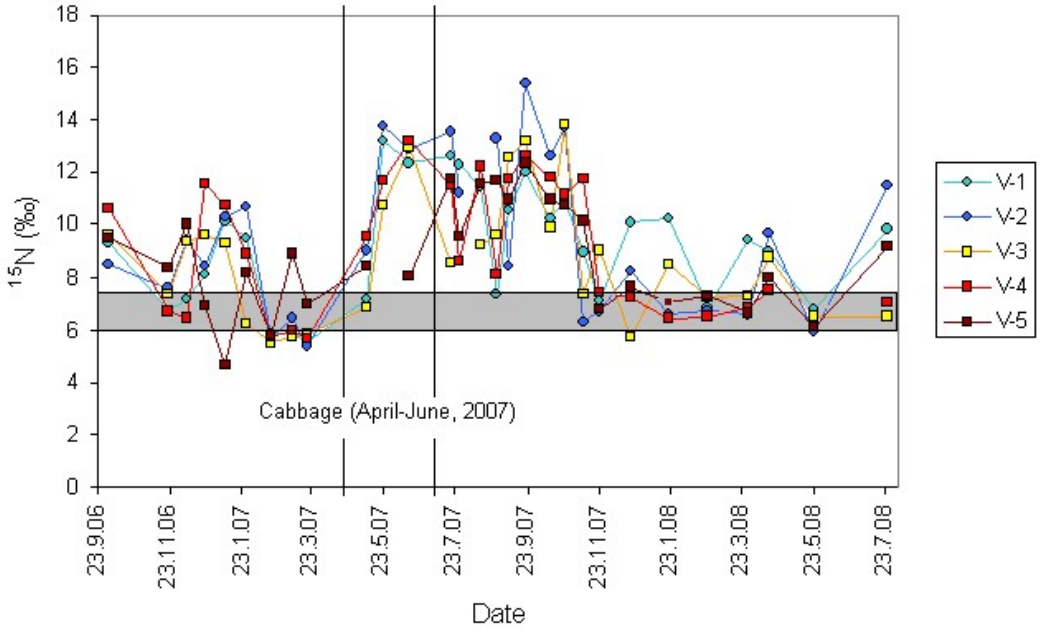


Figure 19: *$\delta^{15}N$ of total N in groundwater indicating no tracers of the labelled fertiliser applied on the experimental field above the aquifer.* Vertical lines denote the cabbage growing period; gray horizontal strip denotes mean ± SD $\delta^{15}N$ of nitrate (n=37).

4.2 The suitability of $\delta^{15}N$ fingerprint as a marker of organic vegetable production

Nitrogen isotopic fingerprint ($\delta^{15}N$) is reported to be a promising indicator to differentiate between organic and conventionally grown vegetables. This is based on the hypothesis that conventionally grown plants have lower $\delta^{15}N$ values compared to plants grown organically due to lower mean $\delta^{15}N$ values of synthetic (inorganic) N fertilisers compared to organic fertilisers. In our study, firstly the $\delta^{15}N$ values of some frequently used organic (farmyard manure, compost, commercially available organic fertilisers: Biogrena and Valentin, naravno organsko gnojilo) and synthetic (N:P:K 7:20:20; N:P:K 15:15:15; KAN; $Ca(NO_3)_2$; $MgNO_3$, KNO_3) fertilisers commonly used in Slovenia were determined.

Secondly, the suitability of $\delta^{15}N$ signature/fingerprint as a marker of the use of synthetic (inorganic) fertiliser in organic production was studied in a greenhouse pot experiment with lettuce (*Lactuca sativa* L.), where the effect of single and split fertiliser as well as the effect of combined usage of synthetic (inorganic) and organic fertilisers on lettuce $\delta^{15}N$ was studied.

The best assessment of whether the N isotope approach is an appropriate tool to discriminate between organic and conventionally grown produce can be made from a survey of the $\delta^{15}N$ values of authentic commercially grown crops from across a wide geographical spread incorporating a broad range in environmental conditions and agricultural practices (Bateman and Kelly, 2007). In this way, in comparison to well controlled cultivation experiments, many other factors which can affect the N isotopic composition of a crop, e.g. soil type, variability in atmospheric N depositions, variations in local agricultural practices, etc., are taken into account. Therefore, thirdly, 14 different types of organically grown vegetables were selected from organic markets and their conventionally grown counterparts from different supermarkets in Slovenia and analyzed for their $\delta^{15}N$ signature in order to asset datasets to ascertain if there are any systematic differences in nitrogen isotope composition due to the method of production.

4.2.1 $\delta^{15}N$ of some organic and synthetic (inorganic) fertilisers

The nitrogen isotopic signatures ($\delta^{15}N$) of synthetic (inorganic) and organic fertilisers determined in this study are presented in Table 19 and are in good agreement with literature data (Table 3). From the two types of fertilisers analyzed (synthetic and organic), considering mean $\delta^{15}N$ values it was possible to discriminate between organic (mean ± SD = 9.5 ± 3.9‰) and synthetic fertilisers (3.0 ± 3.3‰) using N isotopes. However, some values are overlapping (e.g. Biogrena and Farmyard manure with $Ca(NO_3)_2$). Synthetic (inorganic) fertilisers are manufactured by extracting N from air (with $\delta^{15}N$ = 0‰) and since there is only a little fractionation during the production process, $\delta^{15}N$ of samples analyzed is close to zero, with the exception of calcium nitrate with a $\delta^{15}N$ values of 5.7‰ and 5.8‰ and magnesium nitrate with $\delta^{15}N$ value of 7.6‰. These values could reflect the production of these samples as a by– or co–products of some other manufacturing processes where reaction conditions were such that the calcium/magnesium nitrate became enriched with ^{15}N as compared to starting material, i.e. atmospheric N (Bateman and Kelly, 2007). Organic fertilisers have more positive $\delta^{15}N$ values compared to synthetic (inorganic) fertilisers as animals eat plants (with ^{15}N values that reflect soil N; with the exception of N-fixing plants), and their body waste

undergoes further ^{15}N enrichment after extraction with the onset of fractionation processes such as volatilization of ammonia and denitrification or bacterial reworking of the N (Rogers, 2008 and references therein).

Assuming soil $\delta^{15}N$ values near 5‰ (Kendall, 1998; Amundson and Baisden, 2000; Rogers, 2008; $\delta^{15}N$ of the soil used in our field and pot experiment was 6.4 ‰) the use of ^{15}N–depleted synthetic (inorganic) fertilisers would lower the overall soil $\delta^{15}N$ values due to mixing of both synthetic fertiliser N and soil derived N, whereas the use of ^{15}N–enriched organic fertilisers would increase the overall soil $\delta^{15}N$ values (Rogers, 2008), which would reflect in crops that would be grown on these soils.

Table 19: Results of $\delta^{15}N$ determinations in fertiliser samples analysed.

Fertiliser	Type	$\delta^{15}N$ (‰)	Mean ± SD
KAN	synthetic	-0.9	3.0 ± 3.3
KNO_3	synthetic	+0.3	
NPK 15:15:15	synthetic	+0.5	
NPK 7:20:20	synthetic	+1.7	
$Ca(NO_3)_2$	synthetic	+5.7	
$Ca(NO_3)_2$ (Yara Liva)	synthetic	+5.8	
$MgNO_3$	synthetic	+7.6	
Farmyard manure	organic	+6.2	9.5 ± 3.9
Biogrena	organic	+7.2	
Compost	organic	+9.6	
Organsko gnojilo Valentin	organic	+14.8	

4.2.2 Pot experiment with lettuce

So far, the effect on plant $\delta^{15}N$ of split nitrogen fertilisation, which could enable farmers to cover up the use of synthetic (inorganic) fertiliser, is not well studied. Hence, in our study, the use of $\delta^{15}N$ in lettuce as a potential marker for identifying the use of synthetic (inorganic) nitrogen fertilisers was tested on pot grown lettuce (*Lactuca sativa* L.), fertilized with synthetic (inorganic) and organic nitrogen fertiliser applied as single or split application. The effect of combined usage of synthetic and organic fertiliser on $\delta^{15}N$ was also studied.

4.2.2.1 Dry matter and N yield of lettuce

At transplanting, mean dry matter yield, N content and $\delta^{15}N$ of three seedlings (whole plants) were determined, as follows: 0.13 ± 0.05 g plant^{-1}, 49.1 ± 0.5 g kg^{-1} dry matter (DM) basis, and +1.8 ± 0.7‰, respectively (mean ± SD).

Data for dry matter yield and N uptake of lettuce at 20, 30, and 50 days after transplanting for the seven different fertilization regimes are presented in Table 20. Figure 20 presents fresh and dry matter yield at final harvest. Nitrogen fertilisation caused a significant difference in dry matter yield between control and fertilized plants at 20 DAT, with the exception of plants treated with a single application of synthetic (inorganic) fertiliser whose dry matter yield was not significantly different from the

control. The suppression in dry matter accumulation was also observed by Yun et al. (2006) at 40 DAT. However, the difference between treatments was no longer significant at 30 and 50 DAT.

High dry matter yield under unfertilized control treatment indicates that the soil used in the experiment was relatively rich with N. However, in the experiment, practice of local farmers was followed, and since it is not common for local growers (neither conventional nor organic) to perform the soil N analysis before planting and to apply N accordingly, but to apply the amount of N which is expected to be taken up by the particular plant in optimal yield conditions, same was done in the experiment.

Table 20: Fresh weight and dry weight of lettuce at 20, 30 and 50 days after transplant (DAT) in different treatments.*

Treatment§	Fresh weight (g plant^{-1})			Dry weight (g plant^{-1})		
	20 DAT	30 DAT	50 DAT	20 DAT	30 DAT	50 DAT
C	36.3 a	112.0 ab	182.7 a	2.0 a	7.9 a	13.5 a
O	47.2 a	144.0 b	180.7 a	4.4 b	9.5 a	13.4 a
S	41.3 a	138.3 b	198.7 ab	3.1 a	9.8 a	12.5 a
S+S#	41.5 a	139.7 b	199.0 ab	3.7 b	7.7 a	13.7 a
S+O#	40.0 a	130.7 b	212.3 ab	3.7 b	7.7 a	16.2 a
O+S#	44.5 a	125.3 ab	231.8 b	3.7 b	9.1 a	16.1 a
O+O#	37.3 a	92.0 a	200.8 ab	3.7 b	9.1 a	13.2 a

*Data are means of n=3 plants per treatment. Different letters denote significant difference within column ($p<0.05$).

§Treatments: C, control; O, organic; S, synthetic; S+S, synthetic + synthetic; S+O, synthetic + organic; O+S, organic + synthetic; O+O, organic + organic.

#In split treatments (S+S and S+O; O+O and O+S), values at 20 and 30 DAT were calculated based on the same three replications.

Many authors report that the N uptake dynamics is equally or even more important than the total N uptake and are typical for each plant species (Pang and Letey, 2000; del Amor, 2006). On average, about 60 % of all N was accumulated in plants before 30 DAT in treatments with split application, whereas in single fertilisation treatments 72 % and 81 % was accumulated before 30 DAT for O and S treatment, respectively. During the growth stage (20 and 30 DAT), differences in N uptake were observed between treatments (Figure 21, Appendix 5). At final harvest (50 DAT), plants from control pots (C) and plants with split application of organic fertiliser (O+O) showed significantly lower N uptake (308.3 mg plant^{-1} and 352.0 mg plant^{-1}, respectively) compared to other treatments with mean N uptake of 454.3 mg plant^{-1}, indicating lower N availability in these two treatments. In O+O treatment, low N availability could be explained by slow N mineralization from the organic fertiliser, applied after 30 DAT.

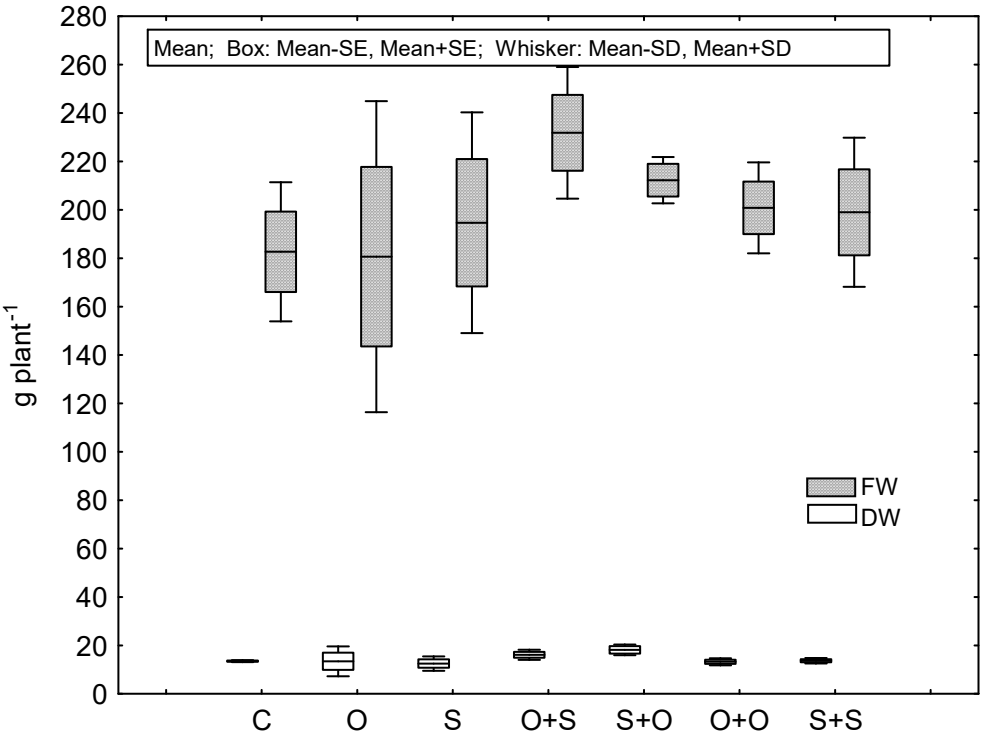


Figure 20: *Whole plant fresh weight (FW) and dry weight (DW) of lettuce at final harvest in different treatments: C, control; O, organic; S, synthetic; O+S, organic + synthetic; S+O, synthetic + organic; O+O, organic + organic; S+S, synthetic + synthetic.*

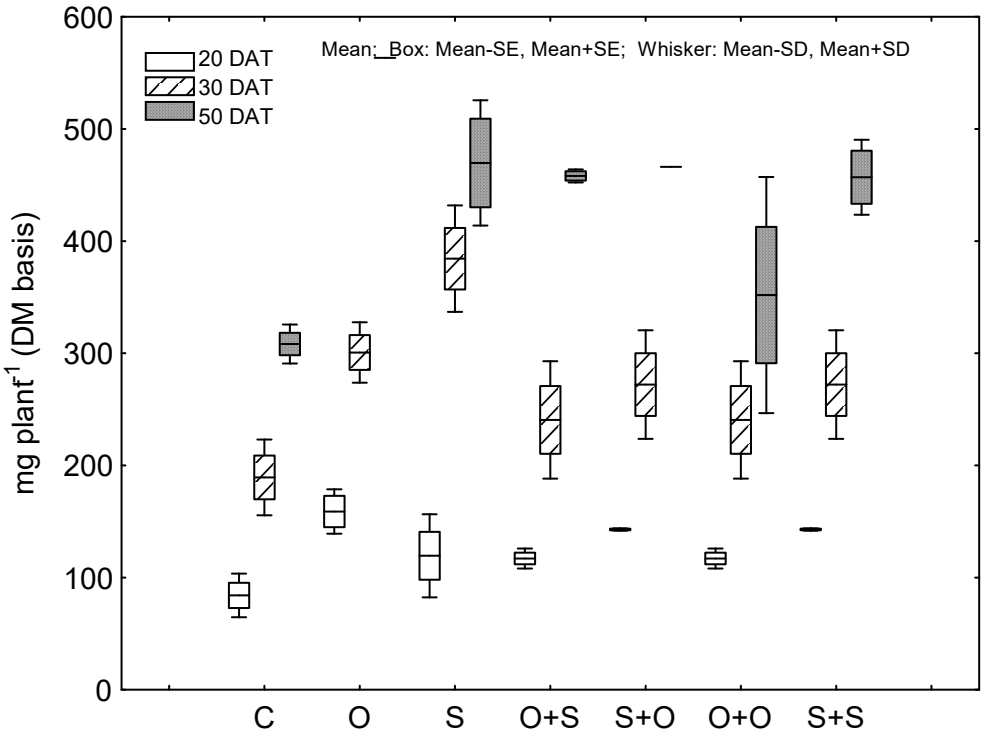


Figure 21: *N uptake of lettuce (mg plant^{-1}) at 20, 30 and 50 days after transplant (DAT) in different treaments: C, control; O, organic; S, synthetic; O+S, organic + synhtetic; S+O, synthetic + organic; O+O, organic + organic; S+S, synthetic + synthetic.*

4.2.2.2 The effect of different N sources and time of application on whole plant $\delta^{15}N$

Results are presented in Table 21 and Figure 22. The $\delta^{15}N$ of whole plants receiving single organic fertiliser (O) were significantly higher compared to the treatments with different N sources (i.e. synthetic fertiliser, soil), reflecting the higher $\delta^{15}N$ values of organic fertiliser (14.8‰) compared to those of synthetic (inorganic) fertiliser (5.7‰) and total soil–N (6.4‰). At final harvest, the mean $\delta^{15}N$ of whole plants receiving a single application of organic fertiliser (O) was 9.6‰, and $\delta^{15}N$ of plants receiving split applications were 8.0‰ and 7.2‰ for O+O and O+S treatments, respectively. The mean $\delta^{15}N$ of whole plants receiving a single application of synthetic fertiliser (S) was 5.3‰, whereas $\delta^{15}N$ of those receiving split applications were 5.2‰ and 6.0‰ for S+S and S+O treatments, respectively. However, lettuce fertilized only with synthetic fertiliser (single or split application) was significantly depleted with ^{15}N compared to unfertilized control plants (C, with $\delta^{15}N$= 7.2‰), strongly indicating the contribution of synthetic fertiliser (with $\delta^{15}N$= 5.7‰).

Table 21: $\delta^{15}N$ (‰) of whole plant lettuce at 20, 30 and 50 days after transplant (DAT) in different treatments.*

Treatment§	20 DAT	30 DAT	50 DAT
C	6.6 a x	7.5 bc x	7.2 b x
O	12.4 c y	10.3 d xy	9.6 c x
S	5.8 a x	5.9 ab x	5.3 a x
S+S#	5.6 a x	5.1 a x	5.2 a x
S+O#	5.6 a x	5.1 a x	6.0 a x
O+S#	10.4 b z	8.8 c y	7.2 b x
O+O#	10.4 b y	8.8 c x	8.0 b x

* Whole plant $\delta^{15}N$ is calculated as integration of three separate plant parts (outer, middle and inner leaves). Different letters denote significant difference ($p<0.05$) within column (a, b, c, d) and within a row (x, y, z).

§Treatments: C, control; O, organic; S, synthetic; S+S, synthetic + synthetic; S+O, synthetic + organic; O+S, organic + synthetic; O+O, organic + organic.

In split treatments (S+S and S+O; O+O and O+S), $\delta^{15}N$ values at 20 and 30 DAT were calculated based on the same three replications.

Significantly higher $\delta^{15}N$ values were found in lettuce receiving a single application of organic fertiliser (O) compared to those receiving split application (O+O), reflecting the proportionately greater contribution of organic fertiliser–N to total plant–N in the single application, which is in accordance with findings of Yun et al. (2006). In contrast, no significant difference in $\delta^{15}N$ was found between lettuces receiving synthetic fertiliser as a single (S) or split application (S+S).

Even though some trends can be observed due to split fertiliser application (e.g. decrease in whole plant $\delta^{15}N$ after the addition of synthetic fertiliser to basal organic fertilisation or increase in $\delta^{15}N$ after the addition of organic fertiliser to basal synthetic fertilisation), split fertilisation did not cause significant alteration of whole plant $\delta^{15}N$, either when isotopically similar (O+O, S+S), nor when isotopically different additional N sources were applied. An exception is the addition of synthetic fertiliser to basal organic

fertilisation (O+S), which caused a significant decrease in whole plant $\delta^{15}N$ from 8.8‰ (30 DAT) to 7.2‰ (50 DAT). However, the whole plant $\delta^{15}N$ (7.2%) of O+S treatment did not significantly differ from O+O treatment ($\delta^{15}N$ = 8.0‰; 50 DAT). Yun et al. (2006) reported significant alteration of cabbage $\delta^{15}N$ when isotopically different (S+O, O+S) additional N sources were applied. This could be explained by the greater difference in $\delta^{15}N$ between applied organic and synthetic (inorganic) N sources in their study ($\Delta\delta^{15}N$ of 17.7‰) as compared to this study ($\Delta\delta^{15}N$ = 9.1‰). Regarding splitting, we observed that fertilisation with additional synthetic (inorganic) fertiliser to basal organic fertilisation (O+S) caused a greater effect in whole plant $\delta^{15}N$ ($\Delta\delta^{15}N_{(30\ DAT–50\ DAT)}$ = -1.6‰) compared to additional organic fertilisation on basal synthetic fertilisation (S+O) ($\Delta\delta^{15}N_{(30\ DAT–50\ DAT)}$ = +0.9‰), indicating greater availability of synthetic fertiliser compared to organic fertiliser (Yun et al., 2006; Pavlou et al., 2007) and soil N.

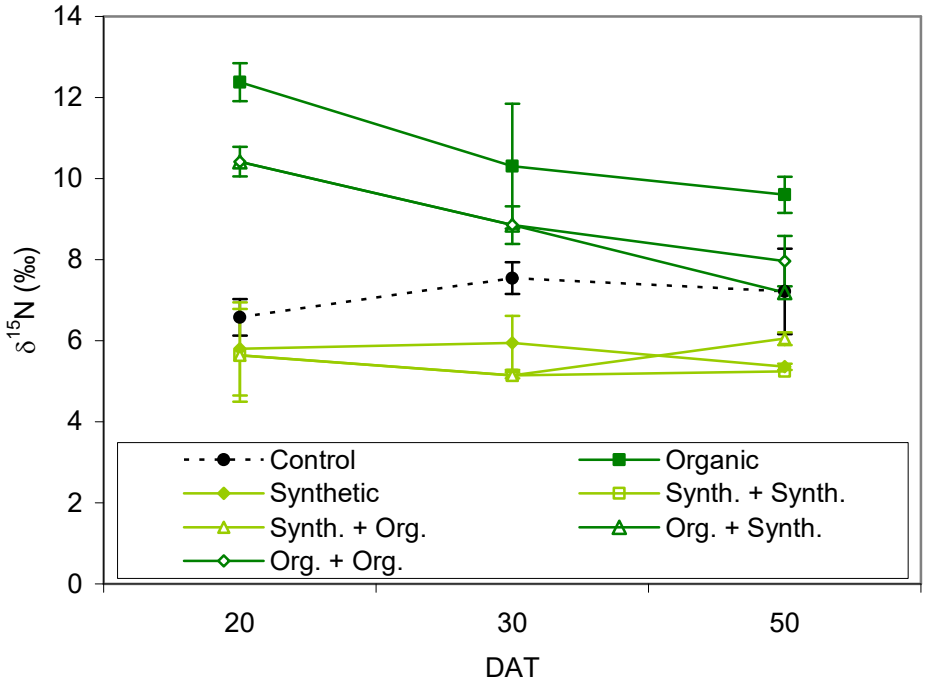


Figure 22: *Lettuce $\delta^{15}N$ under different treatments at 20, 30 and 50 days after transplant (DAT).* Data are means ± SD for n = 3 plants per treatment.

Decreasing in total plant $\delta^{15}N$ with time was found in organically fertilized plants (single and split application with basal organic fertilisation, O, O+O, O+S), which indicates an increased contribution of soil–N to plant–N with time (Yoneyama et al., 1991; Choi et al., 2003; Yun et al., 2006). In addition, the decrease of $\delta^{15}N$ with time in the O+S treatment ($\delta^{15}N$ was lower by 0.8‰ as compared to split O+O fertilisation, not significant) indicates also the contribution of synthetic fertiliser–N (Choi et al., 2003). The $\delta^{15}N$ of plants treated with synthetic fertiliser (S, S+S, S+O), on the other hand, was relatively constant and reflected the $\delta^{15}N$ of synthetic fertiliser–N during the whole of plant growth. The addition of organic fertiliser to basal synthetic fertilisation (S+O) elevated the mean $\delta^{15}N$ value of lettuce tissues by 0.7–0.8‰ as compared to synthetic fertilisation (single and split, S, S+S), but the difference between the treatments was not significant.

Bateman et al. (2005), who studied the effect of irrigation water type (deionised vs. tap water) on plant $\delta^{15}N$, reports that lettuces irrigated with tap water (7 mg $N–NO_3^-$ L^{-1},

$\delta^{15}N = 2–6‰$) assimilate some nitrogen from nitrate present in the water which consistently moderates the $\delta^{15}N$ value of lettuces away from the extreme values observed when the plants were irrigated with deionised water. In our study, the N from the irrigation water presents about 10–15% of the total N applied. $\delta^{15}N$ of the tap water is 4.6‰, which is 1.1‰ and 10.2‰ lower compared to synthetic (inorganic) and organic fertiliser, respectively. This may have an additional effect on the decrease in $\delta^{15}N$ under organic treatments (O, O+O, O+S). The apparent absence of a similar effect on the lettuces grown with synthetic fertiliser may be due to a small difference in $\delta^{15}N$ between synthetic fertiliser and tap water and due to a greater supply of readily available synthetic nitrogen to these plants such that the uptake of tap water derived nitrogen has a negligible effect on lettuce $\delta^{15}N$ values (Bateman et al., 2005).

$\delta^{15}N$ variations within the plant

In the literature, intra–plant variation in $\delta^{15}N$ has been reported for both laboratory and field studies (Shearer and Kohl, 1986; Högberg, 1997). Organ–specific assimilation, reallocation and loss of N, and N source can cause intra–plant variations in $\delta^{15}N$, because most reactions discriminate against ^{15}N (Shearer and Kohl, 1986; Yoneyama and Kaneko, 1989; Choi et al., 2003; Yun et al, 2006), though the global $\delta^{15}N$ value of any plant biomass is primarily determined by that of the actual nitrogen source (Denton et al., 2001; Werner and Schmidt, 2002). Considering that leaves formed at different growth stages should have specific $\delta^{15}N$ values reflecting the isotopic composition of the N assimilated at each stage, a change of available N sources with characteristic isotopic composition (e.g. application of different types of fertiliser) during growth is expected to strongly affect intra–plant $\delta^{15}N$ variations (Yun et al., 2007). Variations in $\delta^{15}N$ within the plant, i.e. between outer, middle and inner leaves, were related to fertiliser type, timing of fertilisation and sampling time (Figure 23, Appendix 6).

During plant growth under treatment with a single synthetic fertilisation (S), inner leaves were depleted with ^{15}N compared to the rest of the plant and most evidently indicated the $\delta^{15}N$ of the source. However, at final harvest the difference between the middle and inner leaves was no longer significant and the $\delta^{15}N$ of all plant parts indicated the $\delta^{15}N$ value of the synthetic fertiliser N. The enrichment with ^{15}N did not change significantly during growth in the inner and middle leaves, whereas the outer leaves were significantly depleted with ^{15}N at 50 DAT, compared to 20 and 30 DAT.

During the growth stage, outer leaves under treatments with split synthetic fertilisation (S+S and S+O) were enriched with ^{15}N compared to the rest of the plant. Additional fertilisation with synthetic fertiliser did not cause any significant difference in $\delta^{15}N$ of lettuce leaves. Throughout the growing period, no significant difference in $\delta^{15}N$ was found between single (S) and split application of the synthetic fertiliser (S+S), with the exception of significantly higher $\delta^{15}N$ in outer leaves in S+S treatment at 50 DAT. Under treatment with basal synthetic and additional organic fertilisation (S+O), the addition of organic fertiliser increased the mean $\delta^{15}N$ of inner leaves by as much as 0.9‰ as compared to synthetic fertilisation (single and split, S, S+S), but the difference was not significant.

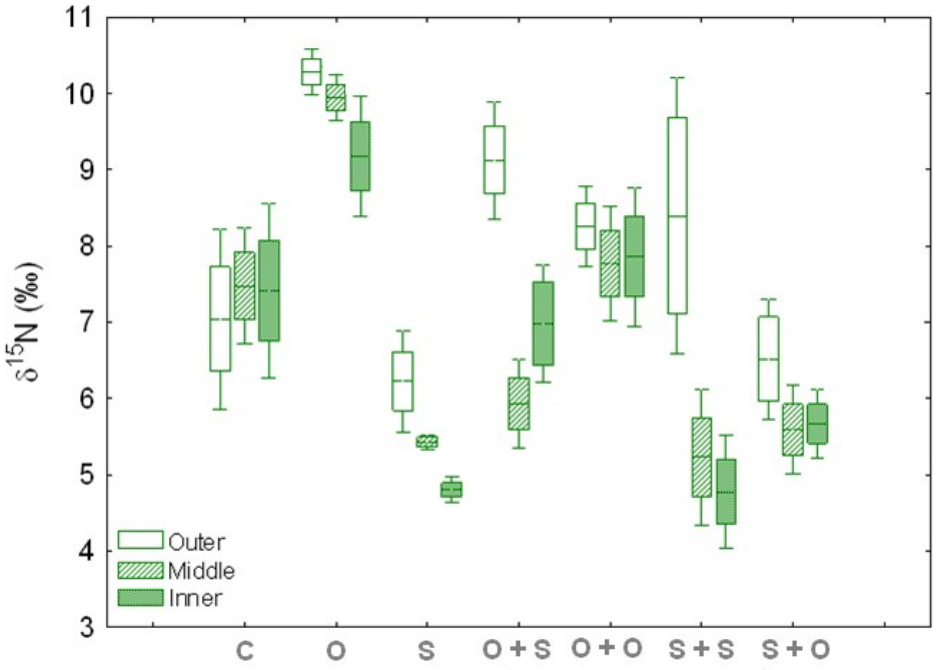


Figure 23: *Intra–plant variations of lettuce $\delta^{15}N$ (‰) at final harvest in different treaments: C, control; O, organic; S, synthetic; O+S, organic + synhtetic; S+O, synthetic + organic; O+O, organic + organic; S+S, synthetic + synthetic.*

At the early growth stage (20 and 30 DAT) inner leaves of plants with basal organic and additional synthetic (O+S) or organic fertilisation (O+O) were significantly depleted with ^{15}N compared to the rest of the plant. However, in O+S treatment at 50 DAT, the middle leaves too were depleted with ^{15}N compared to the rest of the plant. This was also observed by Yun et al. (2006), who studied the interactive effects of N fertiliser source and timing on N isotopic signatures in Chinese cabbage and soil, though in our experiment the difference between middle and inner leaves was not significant. Additional application of synthetic fertiliser to basal organic fertilisation (O+S) significantly lowered the mean $\delta^{15}N$ of middle leaves at 50 DAT by 1.9‰ as compared to split organic fertilisation (O+O) and by 4.2‰ as compared to single application of organic fertiliser (O). However, this difference was not observed in outer leaves, but was observed in inner leaves between O and O+S treatments. Plants from the treatment with split organic fertilisation (O+O) had significantly lower $\delta^{15}N$ values in the inner, younger leaves, compared to the rest of the plant at the early growth stage, whereas at 50 DAT the difference in $\delta^{15}N$ between different leaves was insignificant. Split application of organic fertiliser to basal organic fertilisation (O+O) did not cause significant changes in the $\delta^{15}N$ pattern during the first 30 days of growth, which was also found by Yun et al. (2006). However, it resulted in significantly lower $\delta^{15}N$ values at 50 DAT in outer and middle leaves compared to the single application (O), which can be explained by the higher contribution of soil–N to plant–N in split application, i.e. $\delta^{15}N$ decreased with decreasing contribution of organic fertiliser–N to total plant–N contents (Choi et al., 2003).

Considering the results obtained, $\delta^{15}N$ of lettuce tissues could be used as a marker to reveal the use of synthetic (inorganic) N fertiliser, but only when applied in a single application, since the addition of synthetic fertiliser to the basal organic fertilisation could not be confirmed by this method. For the conditions used in this trial (pot grown lettuce, fertiliser type, growth conditions) it seem not possible to reveal little or moderate rates of synthetic (inorganic) N fertiliser treasonably applied to a lettuce crop in organic production. Even though the use of organic nitrogen fertilisers does not alone prove that

crops were grown in accordance with all the principles of organic farming, the method presented could be used as a quick and relatively cheap tool to reveal the use of prohibited synthetic fertilisers. However, unlike in the study with Chinese cabbage (Yun et al., 2006), in our study, with a difference in $\delta^{15}N$ between organic and synthetic fertiliser of 9.1‰, the method was found to be insufficiently sensitive for identifying the combined use of organic and synthetic fertiliser. This means that growers could cover up the use of synthetic fertiliser when applied in combination with organic fertiliser. If the difference in $\delta^{15}N$ between the applied synthetic and organic nitrogen fertilisers was >9.1‰, detection of the combined usage of the fertilisers would have a greater discriminatory power.

4.2.3 $\delta^{15}N$ in organically and conventionally grown vegetables available on the Slovenian market

The results of isotopic analysis performed on 14 varieties of organically and conventionally grown vegetables from the Slovenian market are presented in Figure 24 and Appendix 7. In general, vegetables, assumed to be grown under organic regimen, had more positive $\delta^{15}N$ values as compared to their conventionally grown counterparts (elevated from 0.8 to 6.3‰). The exceptions were carrot and kohlrabi with lower mean $\delta^{15}N$ values of organic counterparts as compared to conventional ones (1.6 and 0.2‰, respectively).

Large overlapping of $\delta^{15}N$ values, which we observed for carrot, was observed also by Bateman et al. (2007), who suggested that the reasons for this overlap may be the lower N requirement and consequently generally lower levels of fertiliser application and the fact that in an organic rotation system, it is advisable to avoid planting of carrots soon after the application of farmyard manure as this increases the likelihood of mis–shapen roots (Mildura, 2007, Bateman et al., 2007). On the other hand, distinguishable $\delta^{15}N$ values between organically and conventionally grown carrots were obtained in the study of IEH Laboratories and Consulting Group.

Based on the $\delta^{15}N$ values of the samples analysed, it was possible to differentiate between organically and conventionally grown endive, chicory in type “Palla rosa”, leek, potato, and chicory in type “Pan di zucherro”. However, in eight out of fourteen vegetable varieties, despite differences in mean $\delta^{15}N_{org\text{-}conv}$ values, it was not possible to differentiate between organic and conventionally grown counterparts due to widely overlapping ranges. These vegetable varieties were as follows: cauliflower, tomato, garlic, onion, kohlrabi, parsley, sweet pepper, and carrot.

Similar overlapping of $\delta^{15}N$ values was observed in the study obtained by IEH Laboratories and Consulting Group (2010), analysing commercially available vegetables on markets in British Columbia and Canada. They report that based on $\delta^{15}N$ values it was not possible to differentiate between organic and conventionally grown lettuce (green, red), garlic, cauliflower, and onion (yellow), whereas it was possible to differentiate zucchini, tomatoes, spinach, onion (green, red), cabbage (green, red), celery, red pepper and carrot.

Rogers (2008), who studied $\delta^{15}N$ signatures in organically and conventionally grown vegetables available on the markets in New Zealand, on the other hand reports that it was possible to use N isotopes to differentiate between all analysed organic and conventionally grown vegetables (potato, onion, pumpkin, tomato, maize, eggplant, etc.), as long as it was not a N–fixing plant. Same author reports of a very small difference in $\delta^{15}N$ between organic and nonorganic pea (about 0.1‰), which is a N–fixing legume that

removes N from the air ($\delta^{15}N = 0‰$), that is fixed into the soil, and therefore concludes that in the case of N–fixing legumes, it is not possible to determine an organic growing regimen using N isotopes. From this reason, N–fixing legumes, such as peas or beans, were not included in our study. Nevertheless, in the study of Rogers (2008), for all vegetables analysed, only mean $\delta^{15}N$ values of three samples are presented without range of these values, hence the comparison with his data is not completely credible.

In addition, in her study, Rogers (2008) compared faster (tomatoes, cucumber, zucchini, broccoli) *versus* slower growing (pumpkin, eggplant, potatoes, corn) crops to determine the effects of growing time on nitrogen isotopic fractionation. In general, she observed that organic vegetables that are faster to mature (50-80 days to harvest) tend to have more positive $\delta^{15}N$ values and have bigger $\Delta\ ^{15}N_{org.-conv}$ values ranging between 7.9 and 10.2‰ than crops that are slower to mature (90-120 days to harvest) and have smaller $\Delta\ ^{15}N_{org.-conv.}$ values ranging between 2.2 and 4.0‰. In general, plants will preferentially use ammonium over nitrate (Nadelhofer and Fry, 1988; Rogers, 2008). As ammonification is the first step of urea conversion, the relative level of ammonia available for plant uptake is likely to be more abundant in overfertilised soils, and the corresponding ^{15}N signature of organic produce will reflect more positive $\delta^{15}N$ values of the added, enriched manure rather than the soil N pool. In faster growing produce, the uptake of ^{15}N-enriched organic manure and their elevated $\delta^{15}N$ values reflect the available ammonia, which will have had less time to volatilize and return to isotopic equilibrium with the soil N (which has less positive $\delta^{15}N$ value). Therefore, more positive $\delta^{15}N$ values in faster growing organic produce reflect higher $\delta^{15}N$ values of the available nutrients. However, positive $\delta^{15}N$ values in organic produce may also be related to an excess of ^{15}N-enriched ammonia previously accumulated in soils and fresh waters from past fertilizer applications. Volatilization of ^{15}N-depleted ammonia derived from manure would further enrich the residual ammonia in the soil (Rogers, 2008). In our study, however, the difference in $\Delta\ ^{15}N_{org.-conv}$ values between slower (e.g. potato, garlic) and faster growing (e.g. chicory, endive) is not evident.

Rogers (2008) determined also $\delta^{13}C$ values of vegetables and found that vegetables raised in hothouses heated by fossil fuels can be identified by carbon isotopes if they have significantly depleted $\delta^{13}C$ values. However, $\delta^{13}C$ values can not be used to differentiate between organic and conventional produce.

Results obtained in our study confirm finding of Bateman et al. (2007), who asserted that ^{15}N analysis of a “test sample” will not provide unequivocal evidence as to whether synthetic fertilisers have been used on the crop but could, for example, in a situation when there is a suspicion that mislabelling of conventionally grown crops as “organic” is occurring, be used to provide supporting evidence.

It should be pointed out that some additional limitations of using $\delta^{15}N$ in plant tissue to differentiate between organic and conventional production exist which need to be considered but are so far not well studied and should hence be a subject of future studies, such as: the use of organic residues, originating from nitrogen–fixing plants as well as the use of green manures or seaweed based amendments (which are also permitted in organic farming and whose $\delta^{15}N$ values range between 0.6% and 5.4‰) (Bateman et al., 2007), which lower the $\delta^{15}N$ values of plant available N, hence plants are likely to have $\delta^{15}N$ values similar to those fertilized with synthetic fertiliser. On the other hand, conventional growers do not always apply synthetic nitrogen fertilisers to their crops but may use a synthetic N fertiliser or any of the natural fertilisers that may be permitted in organic farming. If organic fertilizer is also used in conventional farming, $\delta^{15}N$ values of crops are expected to be similar to those grown organically, making the distinction based only on $\delta^{15}N$ signature difficult or most likely even impossible.

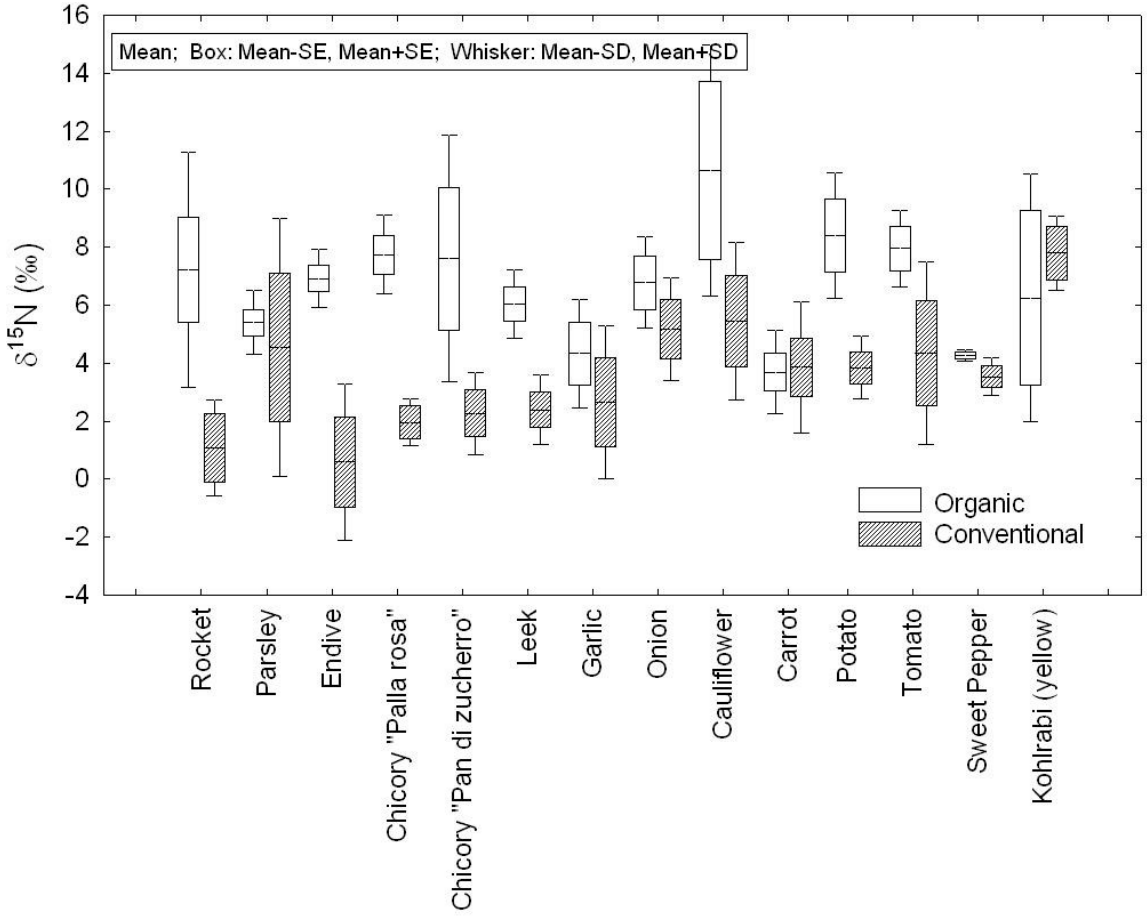


Figure 24: *$\delta^{15}N$ (‰) in organically and conventionally grown vegetables available on the Slovenian market (n=3-6).*

5 Conclusions

Nitrogen, an essential nutrient for plant growth, is recognized as the element with the greatest effect on the yield. However, excessive N fertilisation to obtain maximum levels of crop production leads to (1) contamination of the natural environment due to nitrate leaching below the root zone and hence in groundwater, (2) high nitrate concentrations in plants, as well as (3) unjustified high costs. In order to avoid these problems, an effort was made in our work to find a farming practice which would result in a satisfactory yield at minimal negative effects on the environment.

For this purpose, a field experiment was conducted with white cabbage (*Brassica oleracea* var *capitata* L.) on a sandy loam agricultural soil, located above a shallow groundwater aquifer in Sneberje, near Ljubljana. Four different fertilisation and irrigation treatments were applied and to follow the pathways and distribution of the applied N fertiliser in the plant–soil–groundwater system, ^{15}N labelled KNO_3 fertiliser was used as a tracer. Treatments were compared based on N mass balance calculations and yield quality in terms of nitrate content.

In order to determine the isotopic composition of nitrate nitrogen in groundwater samples, a new method, namely the anion exchange method, was introduced. For determination of the nitrate nitrogen isotopic composition of soil solution samples at natural and enriched ^{15}N levels, the anion exchange method and two different diffusion methods, namely the hook method and the Teflon trap method, were tested and compared for their suitability in routine laboratory use. Based on the results of the comparative study, the following conclusions were drawn:

- The comparative study indicated that all three methods used gave satisfactory and reproducible results at the natural ^{15}N abundance (standard solution), as well as at ^{15}N enriched levels (soil solution; tested with diffusion methods only).
- The anion exchange method was found to be very applicable for samples with low nitrate concentrations, such as groundwater samples, whereas for soil solutions which usually contain high nitrate concentrations, the diffusion methods are more appropriate.
- The Teflon trap method is simple and inexpensive, whereas the anion exchange method is time-consuming, labour intensive, as well as expensive due to the high costs of Ag_2O.
- The hook method is limited by the sample volume, therefore sample pre-concentration to 3 mL is usually required, which elongates the time of sample preparation thereby also increasing the possibility of contamination.
- For the reasons above, the Teflon trap method was chosen as the most suitable method for the isolation of nitrate from soil solution samples and was therefore introduced in our laboratory and applied in this study.
- The results demonstrate that in the Teflon trap method, the use of a correction factor to eliminate the fractionation effect is required. This is due to to incomplete recovery which may occur during diffusion of larger volumes (e.g. 200 mL), resulting in isotope fractionation, thus giving falsely low $\delta^{15}N$ values.

- According to the literature, it is possible to shorten the time of incubation as well as increase the diffusion efficiency by carrying out the incubation at elevated temperature (65 °C) and/or on a horizontal shaker, therefore this should be the subject of future studies.

Based on the results of the field experiment, the following conclusions can be made:

- The results did not confirm our hypothesis that fertigation with drip irrigation, covering 100 % of crop's water requirements, is the best practice for growing white cabbage.
- Farmer's practice, i.e. pre-plant broadcast fertiliser application plus irrigation the day before and after transplanting, resulted in the highest yield and highest N uptake. This suggests that among the tested practices and under the experimental conditions, farmer's practice is the most appropriate for growing white cabbage from the economic (yield) as well as the environmental points of view (lowest N leaching potential).
- Pre–plant broadcast fertiliser application with drip irrigation, covering the 50% of the plant's water requirements was found to result in the lowest yield and N uptake and the highest N leaching (highest N surplus at harvest, highest nitrate concentrations in soil water) and was hence found to be the least appropriate among the tested practices.
- Regarding nitrate content in aboveground cabbage parts, significantly highest nitrate contents were observed in the outer leaves and lowest in inner leaves, which is in accordance with ESFA (2008).
- In future this research should be broadened to include different environmental conditions, crop varieties, as well as different soil types.

In this doctoral work, for the first time in Slovenia, the effect of different fertilizer types and previously poorly studied (only one paper found on Science Direct) split and combined usage of synthetic (inorganic) and organic fertilizers on the isotopic composition of total N ($\delta^{15}N$) were investigated. Thus the suitability of the isotopic $\delta^{15}N$ signature as a tool for distinguishing between organic and conventionally grown vegetables was evaluated, based on the results obtained in greenhouse pot experiment with lettuce (*Lactuca sativa* L.), as well as in a study performed on fourteen varieties of conventionally and organically grown vegetables available on the Slovenian market. The results obtained lead to the following conclusions:

- Considering the mean $\delta^{15}N$ values of the organic and synthetic (inorganic) fertilisers analysed, it was generally possible to differentiate between organic and non-organic synthetic fertilisers. However some values of synthetic fertilisers overlapped with those of organic ones, indicating that it is not always possible to differentiate between different fertiliser types based on their $\delta^{15}N$ value.
- The results of the pot experiments indicate that $\delta^{15}N$ values of lettuce tissues could be used as a rough marker to reveal the use of synthetic fertiliser, but only in the case of a single fertiliser application.
- It is not possible to detect low or moderate rates of synthetic (inorganic) fertiliser illegally applied to a lettuce crop in organic production, which means that growers could cover up the use of prohibited synthetic fertiliser when applied in combination with organic fertiliser.
- In general, the study of $\delta^{15}N$ signatures in fourteen varieties of organic and

conventionally grown vegetables from the Slovenian market confirmed the hypothesis that organically grown vegetables have greater mean $\delta^{15}N$ signatures compared to their conventionally grown counterparts (exceptions were carrot and kohlrabi).

- However, due to widely overlapping ranges of $\delta^{15}N$ values, it was only possible to differentiate between some of the organically and conventionally grown plant varieties. This, however, does not invariably indicate incorrect labelling, but can also be attributed to untypical $\delta^{15}N$ values of the fertilisers used, as well as some other factors which require further study.
- In future the suitability of the use of $\delta^{15}N$ as an authentication tool needs to be tested on a larger sample of commercially available products and should always be tested separately for each plant variety.
- The use of N isotopes to verify organic production was found to have some limitations, but nevertheless the $\delta^{15}N$ signature can be used as a very useful additional, supporting tool to other methods to verify organic vegetable production. Moreover, isotope ratio mass spectrometry is a cheap and fast method, namely $\delta^{15}N$ signature of a vegetable sample can be obtained within a couple of days upon receipt.

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Appendix 1: Soil characteristics of the soil from the Sneberje experimental site: pH, P_2O_5, K_2O, org, matter, C, C/N, N_{tot}, sand, silt, clay content, texture of different profiles, soil layers and depths.

Soil profile	Soil layer	Depth cm	pH $CaCl_2$	P_2O_5 mg 100 g^{-1}	K_2O mg 100 g^{-1}	N_{tot} %	Sand %	Silt Coarse %	Silt Fine %	Silt Total %	Clay %	Texture class
01	Ap	0–20	7.5	34.4	22.0	0.18	39.1	24.8	23.1	47.9	13.0	I
	A12	20–31	7.5	4.4	5.1	0.05	56.9	14.5	18.8	33.3	9.8	PI
	I	31–52	7.6	1.3	3.4		83.6	6.0	7.2	13.2	3.2	IP
	Go1	52–62	7.6				52.5	19.6	20.2	39.8	7.7	PI
	II	62–78	7.7				91.3	1.0	4.3	5.3	3.4	P
	Go2	78–84	7.7				70.3	11.5	12.4	23.9	5.8	PI
02	Ap	0–20	7.4	22.5	9.9	0.13	37.7	21.1	27.7	48.8	13.5	I
	A12	20–30	7.4	15.5	8.8	0.12	40.2	19.5	29.1	48.6	11.2	I
	AGo	15–57	7.7				57.2	19.9	16.3	36.2	6.6	I
	C	57	7.7				87.4	2.8	6.3	9.1	3.5	IP
03	Ap	0–22	7.4	17.4	9.4	0.12	38.2	25.5	22.5	48.0	13.8	I
	A12	22–35	7.4	8.1	5.6	0.09	45.9	18.8	24.7	43.5	10.6	I
	I	35–64	7.8				87.2	2.4	7.8	10.2	2.6	P
	(Go)	64–72	7.7				57.6	13.4	20.4	33.8	8.6	PI

Appendix 2: Soil characteristics of the soil from the Sneberje experimental site: Ca, Mg, K, Na, H content of different profiles, soil layers and depths.

Soil Profile	Soil layer	Depth cm	Ca	Mg	K	Na	H	S	T	V	Ca	Mg	K	Na	H
			mmol C+ 100 g^{-1}							%					
01	Ap	0–20	17.71	1.34	0.46	0.03	1.00	19.5	20.5	95.1	86.4	6.5	2.2	0.1	4.9
	A12	20–31	17.37	0.71	0.11	0.03	0.20	18.2	18.4	98.9	94.4	3.9	0.6	0.2	1.1
	I	31–52	16.11	0.49	0.06	0.02	0.15	16.7	16.8	99.4	95.9	2.9	0.4	0.1	0.9
	Go1	52–62	17.63	0.67	0.09	0.03	0.20	18.4	18.6	98.9	94.8	3.6	0.5	0.2	1.1
	II	62–78	16.49	0.55	0.05	0.02	0.20	17.1	17.3	98.8	95.3	3.2	0.3	0.1	1.2
	Go2	78–84	16.90	0.58	0.08	0.03	0.15	17.6	17.8	98.9	94.9	3.3	0.4	0.2	0.8
02	Ap	0–20	18.53	0.97	0.24	0.04	0.85	19.8	20.7	95.7	89.5	4.7	1.2	0.2	4.1
	A12	20–30	18.85	0.93	0.21	0.04	0.75	20.0	20.8	96.2	90.6	4.5	1.0	0.2	3.6
	AGo	15–57	17.47	0.60	0.09	0.03	0.15	18.2	18.3	99.5	95.5	3.3	0.5	0.2	0.8
	C	57	15.69	0.57	0.07	0.03	0.35	16.4	16.8	97.6	93.4	3.4	0.4	0.2	2.1
03	Ap	0–22	18.09	0.97	0.23	0.05	0.65	19.3	20.0	96.5	90.5	4.9	1.2	0.3	3.3
	A12	22–35	18.43	0.88	0.13	0.04	0.45	19.5	20.0	97.5	92.2	4.4	0.7	0.2	2.3
	I	35–64	16.33	0.62	0.07	0.04	0.10	17.1	17.2	99.4	94.9	3.6	0.4	0.2	0.6
	(Go)	64–72	17.95	0.69	0.08	0.05	0.20	18.8	19.0	98.9	94.5	3.6	0.4	0.3	1.1

Appendix 3: Nitrogen isotopic composition of cabbage at 59, 68 and 78 days after transplanting (DAT).*

Treatment	atom % ^{15}N excess		
	59 DAT	68 DAT	78 DAT
1#	0.005 a	0.009 a	0.011 a
2	2.226 b	1.950 b	1.546 b
3	0.664 c	0.892 c	1.174 c
4	1.676 d	1.602 d	1.187 c

*Different letters denote significant difference between treatments at the $p<0.05$.

#Treatments: 1–control, 2–broadcast fertilisation with 50% irrigation, 3–fertigation with 100% irrigation, 4–broadcast fertilisation with farmer's practice of irrigation.

Appendix 4: Results of the $\delta^{15}N_{tot/nitrate}$ determinations in groundwater samples for the 1. 10. 2006 to 25.7.2008 period.

Sample No.	Well	Sampling date	NO_3^- (mg L^{-1})	$\delta^{15}N_{tot}$ (‰)	$\delta^{15}N_{nitrate}$ (‰)
7220	V-1	1.10.2006	24.0	9.3	n.d.
7221	V-2	1.10.2006	22.8	8.5	n.d.
7222	V-3	1.10.2006	22.6	9.6	n.d.
7223	V-4	1.10.2006	22.6	10.6	n.d.
7224	V-5	1.10.2006	/	9.5	n.d.
7461	V-1	21.11.2006	22.4	6.7	n.d.
7462	V-2	21.11.2006	21.8	7.6	n.d.
7458	V-3	21.11.2006	21.7	74	n.d.
7460	V-4	21.11.2006	21.7	6.7	n.d.
7459	V-5	21.11.2006	21.8	8.4	n.d.
7548	V-1	7.12.2006	23.2	7.2	n.d.
7549	V-2	7.12.2006	22.7	9.4	n.d.
7550	V-3	7.12.2006	22.2	9.4	n.d.
7551	V-4	7.12.2006	22.5	6.4	n.d.
7552	V-5	7.12.2006	22.4	10.1	n.d.
7732	V-1	22.12.2006	21.8	8.1	n.d.
7733	V-2	22.12.2006	20.7	8.5	n.d.
7734	V-3	22.12.2006	22.8	9.6	n.d.
7735	V-4	22.12.2006	22.9	11.6	n.d.
7736	V-5	22.12.2006	20.5	6.9	n.d.
7737	V-1	9.1.2007	22.0	10.1	n.d.
7738	V-2	9.1.2007	21.0	10.3	n.d.
7739	V-3	9.1.2007	21.0	9.3	n.d.
7740	V-4	9.1.2007	20.8	10.7	n.d.
7741	V-5	9.1.2007	20.5	4.7	n.d.
8051	V-1	26.1.2007	24.8	9.5	n.d.
8052	V-2	26.1.2007	22.6	10.7	n.d.
8053	V-3	26.1.2007	22.0	6.3	n.d.
8054	V-4	26.1.2007	20.6	n.d.	n.d.
8055	V-5	26.1.2007	20.8	8.2	n.d.
8079	V-1	16.2.2007	32.9	5.8	n.d.
8080	V-2	16.2.2007	27.3	5.7	n.d.
8081	V-3	16.2.2007	28.2	5.5	n.d.
8082	V-4	16.2.2007	24.4	5.7	n.d.
8083	V-5	16.2.2007	27.8	5.7	n.d.
8175	V-1	7.3.2007	27.5	5.9	n.d.
8176	V-2	7.3.2007	25.7	6.5	n.d.
8177	V-3	7.3.2007	25.9	5.7	n.d.
8178	V-4	7.3.2007	24.8	6.0	n.d.
8179	V-5	7.3.2007	25.7	8.9	n.d.
8333	V-1	19.3.2007	28.0	5.5	6.7
8334	V-2	19.3.2007	26.7	5.4	n.d.
8335	V-3	19.3.2007	26.8	5.8	6.7
8336	V-4	19.3.2007	26.1	5.6	n.d.
8337	V-5	19.3.2007	26.8	7.0	n.d.
8883	V-1	9.5.2007	22.8	7.1	n.d.
8884	V-2	9.5.2007	21.4	9.0	n.d.
8885	V-3	9.5.2007	21.3	6.8	n.d.
8886	V-4	9.5.2007	21.3	9.6	n.d.
8887	V-5	9.5.2007	21.8	8.5	n.d.

Sample No.	Well	Sampling date	NO_3^- (mg L^{-1})	$\delta^{15}N_{tot}$ (‰)	$\delta^{15}N_{nitrate}$ (‰)
8841	V-1	22.5.2007	23.2	13.2	n.d.
8842	V-2	22.5.2007	21.9	13.7	6.8
8843	V-3	22.5.2007	21.7	10.8	6.7
8844	V-4	22.5.2007	21.8	11.7	6.9
8845	V-5	22.5.2007	22.1	7.5	6.7
8990	V-1	4.6.2007	23.0	n.d.	8.3
8991	V-2	4.6.2007	22.5	n.d.	n.d.
8992	V-3	4.6.2007	22.0	n.d.	n.d.
8993	V-4	4.6.2007	22.3	n.d.	n.d.
8994	V-5	4.6.2007	22.5	n.d.	n.d.
9037	V-1	14.6.2007	23.0	12.3	n.d.
9038	V-2	14.6.2007	22.2	12.9	n.d.
9039	V-3	14.6.2007	21.7	12.9	n.d.
9040	V-4	14.6.2007	22.0	13.2	n.d.
9041	V-5	14.6.2007	22.1	8.0	n.d.
9245	V-1	19.7.2007	23.5	12.6	6.9
9246	V-2	19.7.2007	23.2	13.5	6.0
9247	V-3	19.7.2007	22.7	8.6	n.d.
9248	V-4	19.7.2007	22.1	11.5	6.4
9249	V-5	19.7.2007	23.6	11.8	n.d.
9263	V-1	27.7.2007	22.6	12.3	n.d.
9264	V-2	27.7.2007	21.6	11.2	n.d.
9265	V-3	27.7.2007	21.4	n.d.	n.d.
9266	V-4	27.7.2007	21.4	8.6	n.d.
9267	V-5	27.7.2007	21.5	9.6	n.d.
9322	V-1	14.8.2007	22.4	11.4	7.0
9323	V-2	14.8.2007	21.7	n.d.	6.8
9324	V-3	14.8.2007	21.3	9.3	7.1
9325	V-4	14.8.2007	21.3	12.2	7.0
9326	V-5	14.8.2007	21.6	11.6	6.4
9343	V-1	27.8.2007	22.2	7.4	6.8
9344	V-2	27.8.2007	21.7	13.3	6.6
9345	V-3	27.8.2007	21.4	9.6	6.0
9346	V-4	27.8.2007	21.5	8.1	n.d.
9347	V-5	27.8.2007	21.7	11.7	6.8
9418	V-1	7.9.2007	21.7	10.5	6.6
9419	V-2	7.9.2007	21.4	8.4	n.d.
9420	V-3	7.9.2007	21.1	12.6	6.6
9421	V-4	7.9.2007	21.0	11.7	n.d.
9422	V-5	7.9.2007	21.3	11.0	6.5
9538,9	V-1	21.9.2007	23.5	12.0	7.5
9532,3	V-2	21.9.2007	21.9	15.4	7.0
9534,5	V-3	21.9.2007	21.2	13.2	6.9
9540,1	V-4	21.9.2007	20.8	12.6	6.3
9536,7	V-5	21.9.2007	21.5	12.4	n.d.
9648	V-1	12.10.2007	27.2	10.2	6.1
9649	V-2	12.10.2007	26.7	12.6	n.d.
9650	V-3	12.10.2007	26.7	9.9	7.0
9651	V-4	12.10.2007	25.8	11.8	6.8
9652	V-5	12.10.2007	25.9	10.9	n.d.
9666	V-1	24.10.2007	25.4	10.9	6.4
9667	V-2	24.10.2007	23.6	13.7	6.4
9668	V-3	24.10.2007	23.0	13.8	n.d.
9669	V-4	24.10.2007	23.5	11.1	6.1

Sample No.	Well	Sampling date	NO_3^- (mg L^{-1})	$\delta^{15}N_{tot}$ (‰)	$\delta^{15}N_{nitrate}$ (‰)
9670	V-5	24.10.2007	24.7	10.7	6.3
9892	V-1	9.11.2007	25.9	8.9	n.d.
9893	V-2	9.11.2007	25.3	6.3	n.d.
9894	V-3	9.11.2007	24.7	7.3	n.d.
9895	V-4	9.11.2007	24.7	11.7	n.d.
9896	V-5	9.11.2007	25.4	10.2	6.0
10291	V-1	23.11.2007	24.2	7.1	7.6
10292	V-2	23.11.2007	23.4	6.7	6.7
10293	V-3	23.11.2007	22.8	9.0	6.7
10294	V-4	23.11.2007	22.9	7.4	n.d.
10295	V-5	23.11.2007	23.3	6.7	n.d.
10601	V-1	20.12.2007	24.0	10.1	7.7
10602	V-2	20.12.2007	23.9	8.2	6.6
10603	V-3	20.12.2007	23.1	5.7	6.7
10604	V-4	20.12.2007	23.3	7.2	6.8
10605	V-5	20.12.2007	23.4	7.6	6.2
10611	V-1	21.1.2008	24.5	10.2	n.d.
10612	V-2	21.1.2008	23.8	6.5	n.d.
10613	V-3	21.1.2008	23.6	8.5	n.d.
10614	V-4	21.1.2008	23.0	6.5	n.d.
10615	V-5	21.1.2008	23.6	7.0	6.5
10796	V-1	22.2.2008	23.6	6.8	7.3
10797	V-2	22.2.2008	23.3	6.7	7.0
10798	V-3	22.2.2008	22.6	7.3	7.3
10799	V-4	22.2.2008	22.6	6.5	n.d.
10800	V-5	22.2.2008	22.9	7.3	7.0
11241	V-1	28.3.2008	28.8	9.4	n.d.
11242	V-2	28.3.2008	30.8	6.6	n.d.
11243	V-3	28.3.2008	26.0	7.3	n.d.
11244	V-4	28.3.2008	25.4	6.9	n.d.
11245	V-5	28.3.2008	27.6	6.6	n.d.
11288	V-1	14.4.2008	25.8	8.9	n.d.
11289	V-2	14.4.2008	26.2	9.7	n.d.
11290	V-3	14.4.2008	25.5	8.7	n.d.
11291	V-4	14.4.2008	29.1	7.5	n.d.
11292	V-5	14.4.2008	24.5	8.0	n.d.
11391	V-1	23.5.2008	23.9	6.7	6.9
11392	V-2	23.5.2008	23.7	5.9	7.3
11393	V-3	23.5.2008	23.4	6.5	6.4
11394	V-4	23.5.2008	25.5	6.4	6.0
11395	V-5	23.5.2008	23.9	6.1	6.5
11478	V-1	20.6.2008	n.d.	7.0	n.d.
11479	V-2	20.6.2008	n.d.	6.8	n.d.
11480	V-3	20.6.2008	n.d.	7.3	n.d.
11481	V-4	20.6.2008	n.d.	7.0	6.9
11482	V-5	20.6.2008	n.d.	6.6	n.d.
11651	V-1	25.7.2008	n.d.	n.d.	n.d.
11652	V-2	25.7.2008	n.d.	n.d.	6.9
11653	V-3	25.7.2008	n.d.	n.d.	n.d.
11654	V-4	25.7.2008	n.d.	n.d.	n.d.
11655	V-5	25.7.2008	n.d.	n.d.	n.d.

Appendix 5: N uptake (mg plant^{-1} DM basis) of lettuce at 20, 30, and 50 dates after transplant (DAT) in different treatments.*

Treatment§	20 DAT	30 DAT	50 DAT
C	84.1 a	189.3 a	308.3 a
O	158.9 b	300.7 b	420.5 b
S	119.4 a	384.4 b	469.7 b
S+S#	142.9 b	272.1 b	457.0 b
S+ O#	142.9 b	272.1 b	466.0 b
O+S#	117.0 a	240.6 a	458.2 b
O+O#	117.0 a	240.6 a	352.0 a

*Data are means of n=3 plants per treatment. Different letters denote significant difference within column ($p<0.05$).

§Treatments: C, control; O, organic; S, synthetic; S+S, synthetic + synthetic; S+O, synthetic + organic; O+S, organic + synthetic; O+O, organic + organic.

#In split treatments (S+S and S+O; O+O and O+S), values at 20 and 30 DAT were calculated based on the same three replications.

Appendix 6: Intra-plant variations of lettuce $\delta^{15}N$ (‰) at 20, 30 and 50 days after transplant (DAT) in different treatments.*

Treatment§	Plant part	20 DAT	30 DAT	50 DAT
C	Outer	9.1 b y k	9.7 c y kl	7.0 a x kl
	Middle	6.1 a x k	8.2 b y lm	7.5 a xy l
	Inner	5.6 a x k	6.1 a x l	7.4 a y m
O	Outer	14.0 b y l	12.8 b y m	10.3 b x n
	Middle	13.5 b y l	10.5 ab x n	10.1 b x m
	Inner	10.0 a y l	8.5 a x m	9.2 a xy n
S	Outer	8.6 b y k	10.2 c y kl	6.2 b x k
	Middle	6.4 b x k	6.6 b x kl	5.4 a x k
	Inner	3.5 a x k	2.2 a x k	4.8 a x k
S+S#	Outer	9.6 c y k	8.3 c xy k	7.1 b x lm
	Middle	6.8 b x k	6.4 b x k	5.2 a x k
	Inner	3.8 a x k	2.8 a x k	4.8 a x k
S+O#	Outer	9.6 c y k	8.3 c xy k	6.5 a x kl
	Middle	6.8 b x k	6.4 b x k	5.6 a x k
	Inner	3.8 a x k	2.8 a x k	5.7 a y kl
O+S#	Outer	12.2 b y l	11.3 c y lm	9.1 b x mn
	Middle	11.2 b y l	9.4 b y mn	5.9 a x k
	Inner	8.7 a x l	7.4 a x lm	7.0 a x lm
O+O#	Outer	12.2 b y l	11.3 c y lm	8.3 a x lm
	Middle	11.2 b y l	9.4 b xy mn	7.8 a x l
	Inner	8.7 a x l	7.4 a x lm	7.9 a x mn

* Data are means of n = 3 plants. Different letters denote significant difference ($p<0.05$) between outer, middle and inner leaves inside particular treatment at a particular sampling date (a, b, c), difference in a particular plant part inside particular treatment during plant growth between sampling dates within each row (x, y) and difference for each plant part between treatments for each sampling date (k, l, m, n, o).

§Treatments: C, control; O, organic; S, synthetic; S+S, synthetic + synthetic; S+O, synthetic + organic; O+S, organic + synthetic; O+O, organic + organic.

In split treatments (S+S and S+O; O+O and O+S), $\delta^{15}N$ values at 20 and 30 DAT were calculated based on the same three replications.

Appendix 7: $\delta^{15}N$ (‰) in organically and conventionally grown vegetables available on the Slovenian market (n = 3–6).

Vegetable variety	Farming practice								
	Conventional				Organic				
	Mean	SD	Min	Max	Mean	SD	Min	Max	Δ conv. - org.
Distinguishable									
Endive	0.6	2.7	-2.0	3.4	6.9	1.1	5.6	8.3	6.3
Rocket	1.1	1.6	-0.1	2.3	7.2	4.1	3.0	13.6	6.1
Chichory in type "Palla rosa"	2.0	0.8	1.4	2.5	7.7	1.4	5.6	8.3	5.7
Leek	1.9	1.0	1.1	3.1	7.3	3.0	5.0	12.3	5.4
Potato	3.8	1.1	2.5	5.0	8.4	2.2	6.6	10.8	4.6
Chichory in type "Pan di zucherro"	2.3	1.4	0.8	3.6	5.3	1.7	4.0	6.5	3.0
Not distinguishable									
Cauliflower	5.5	2.7	2.9	8.3	10.7	4.3	7.6	13.7	5.2
Tomato	4.4	3.2	2.5	8.0	8.0	1.3	6.5	9.0	3.6
Garlic	2.6	2.6	1.0	5.7	4.3	1.9	2.2	5.6	1.7
Onion (yellow)	5.2	1.8	3.6	7.1	6.8	1.6	5.7	8.6	1.6
Kohlrabi (yellow)	7.8	1.3	6.9	8.7	6.2	4.3	9.3	3.2	1.6
Parsley	4.5	4.5	0.4	8.2	5.4	1.1	4.0	7.2	0.9
Sweet pepper	3.5	0.7	3.2	4.3	4.3	0.2	4.1	4.4	0.8
Carrot	3.9	2.3	1.9	7.8	3.7	1.4	2.2	5.8	0.2

*standard deviation.

Appendix 8: Personal bibliography for the period 2006–2011.

MARTINA ŠTURM [28487]

ARTICLES AND OTHER COMPONENT PARTS

1.01 Original scientific article

1. ŠTURM, Martina, LOJEN, Sonja, MARKIČ, Miloš, PEZDIČ, Jože. Speciation and isotopic composition of sulphur in low-rank coals from four Slovenian coal seams. *Acta chim. slov.*. [Tiskana izd.], 2009, vol. 56, str. 989-996. [COBISS.SI-ID 23185191]

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3. ŠTURM, Martina, KACJAN-MARŠIĆ, Nina, ZUPANC, Vesna, BRAČIČ-ŽELEZNIK, Branka, LOJEN, Sonja, PINTAR, Marina. Effect of different fertilisation and irrigation practices on yield, nitrogen uptake anf fertiliser use efficiency of white cabbage. *Sci. hortic.*. [Print ed.], 2010, vol. 125, str. 103-109. [COBISS.SI-ID 23519015]

Submitted articles:

ŠTURM, Martina, LOJEN, Sonja. Comparison of different methods for isolation of nitrate-N from soil solution for determination of its isotopic composition. *Acta chim. slov.*

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1.03 Short scientific article

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1.06 Published scientific conference contribution (invited lecture)

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1.08 Published scientific conference contribution

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7. ZUPANC, Vesna, KORPAR, Peter, KACJAN-MARŠIĆ, Nina, URBANC, Janko, BRAČIČ-ŽELEZNIK, Branka, LOJEN, Sonja, ŠTURM, Martina, PINTAR, Marina. Dinamika gibanja vode v tleh pri pridelavi vrtnin na Ljubljanskem polju = Influence of irrigation method on soil moisture dynamics on Ljubljana field. V: TAJNŠEK, Anton (ur.). *Novi izzivi v poljedelstvu 2008 : zbornik simpozija : proceedings of symposium, Rogaška Slatina, [4. in 5. december] 2008*. Ljubljana: Slovensko agronomsko društvo, 2008, str. 184-190. [COBISS.SI-ID 5789305]

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1.09 Published professional conference contribution

12. COLLADO, Mario, ŠTURM, Martina, KORPAR, Peter, TAPARAUSKIENE, Laima. Precipitation - groundwater-stream interaction. V: Lectures & Presentation on FAO/IAEA Interregional Training Course "Use of Nuclear and Related Techniques to Measure Storage, Flows and Balance of Water in Cropping Systems", 1-25 October 2007, Seibersdorf. *Lectures & Presentations*. Seibersdorf: FAO/IAEA, 2007, 8 str. [COBISS.SI-ID 22106407]

1.12 Published scientific conference contribution abstract

13. ŠTURM, Martina, LOJEN, Sonja, PEZDIČ, Jože. Izotopska frakcionacija žvepla v rjavih premogih = Isotopic fractionation of sulphur in brown coals. V: REŽUN, Bojan (ur.). 2. slovenski geološki kongres, Idrija, 26.-28. september 2006. *Zbornik povzetkov*. Idrija: Rudnik živega srebra v zapiranju, 2006, str. 96-97. [COBISS.SI-ID 20196903]

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18. ZUPANC, Vesna, ŠTURM, Martina, KACJAN-MARŠIĆ, Nina, LOJEN, Sonja, KORPAR, Peter, BRAČIČ-ŽELEZNIK, Branka, URBANC, Janko, PINTAR, Marina. Complex field experiment for nitrate migration processes studies in the plant-soil-groundwater system of Ljubljana field. V: OLLESCH, G. (ur.). *International conference on land and water degradation : processes and management, 7th - 9th September 2009, Magdeburg, germany*. [S. l.: s. n., 2009], str. 56. [COBISS.SI-ID 6082937]

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Appendix 9: Article: Can $\delta^{15}N$ in lettuces tissues reveal the use of synthetic nitrogen fertiliser in organic production? *Journal of the Science of Food and Agriculture*, 91, 262-267, 2011.

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Can $\delta^{15}N$ in lettuce tissues reveal the use of synthetic nitrogen fertiliser in organic production?

Martina Šturm,[a*] Nina Kacjan-Maršić[b] and Sonja Lojen[a]

Abstract

BACKGROUND: The nitrogen isotopic fingerprint ($\delta^{15}N$) is reported to be a promising indicator for differentiating between organically and conventionally grown vegetables. However, the effect on plant $\delta^{15}N$ of split nitrogen fertilisation, which could enable farmers to cover up the use of synthetic fertiliser, is not well studied. In this study the use of $\delta^{15}N$ in lettuce as a potential marker for identifying the use of synthetic nitrogen fertiliser was tested on pot-grown lettuce (*Lactuca sativa* L.) treated with synthetic and organic nitrogen fertilisers (single or split application). The effect of combined usage of synthetic and organic fertilisers on $\delta^{15}N$ was also investigated.

RESULTS: The $\delta^{15}N$ values of whole plants treated with different fertilisers differed significantly when the fertiliser was applied in a single treatement. However, additional fertilisation (with isotopically the same or different fertiliser) did not cause a significant alteration of plant $\delta^{15}N$.

CONCLUSION: The findings of the study suggest that the $\delta^{15}N$ value of lettuce tissues could be used as a rough marker to reveal the history of nitrogen fertilisation, but only in the case of single fertiliser application. However, if the difference in $\delta^{15}N$ between the applied synthetic and organic nitrogen fertilisers was >9.1‰, the detection of split and combined usage of the fertilisers would have greater discriminatory power.

Keywords: nitrogen; stable isotopes; ^{15}N; conventional farming; organic farming; *Lactuca sativa* L.

INTRODUCTION

Organic farming has experienced rapid growth over recent years. Since organic products attain higher prices on the market, mainly owing to higher production costs and costs connected with the certification process, there is concern among users over mislabelling conventionally grown crops as 'organic'. As a result, studies have begun to look for ways to test organic products for authenticity.[1] Some of these tests are oriented towards using stable nitrogen isotopes as indicators of organic produce,[2–9] while other researchers are investigating whether concentrations of trace elements could be used to differentiate between organic and conventional produce.[10,11]

The possible use of nitrogen isotopes to differentiate between crops grown with and without inputs of synthetic nitrogen (N), which are prohibited in organic agriculture, is based on the hypothesis that the application of synthetic N fertilisers with $\delta^{15}N$ values close to zero will result in the $\delta^{15}N$ values of plants grown in conventional regimes being lower than those of plants grown in organic regimes owing to the different fertiliser production processes.[5] Synthetic N fertilisers tend to have $\delta^{15}N$ values close to zero, since their nitrogen is derived from atmospheric nitrogen (with $\delta^{15}N = 0‰$) and there tends to be little fractionation during the production process.[5,12] Animal manures with $\delta^{15}N$ values around 5‰, on the other hand, have been reported to produce nitrate with $\delta^{15}N$ values in the range 10–22‰[5,13] owing to the preferential volatilisation of ^{14}N ammonia from the manure.

In recent years, many studies have been conducted to test this hypothesis on various pot/greenhouse-grown[5,7,9] and commercially available[8,11] crops such as maize, cabbage, lettuce, tomato and carrot. As summarised by Kelly and Bateman,[11] previous studies have demonstrated that the nitrogen isotopic composition of a crop may be used to distinguish between crops grown using conventional synthetic fertilisers and crops grown under organic conditions,[4] as long as the crops are not nitrogen-fixing plants that remove nitrogen from the air ($\delta^{15}N_{air} = 0‰$) rather than using nitrogen reserves from the soil[14] and that the timing of application,[3] irrigation water[5] and the chemical form of synthetic fertiliser[2] are, along with some other factors (i.e. soil type, variations in local agricultural practices, etc.), also important in determining how fertiliser $\delta^{15}N$ impacts crop $\delta^{15}N$. However, with the exception of Yun *et al.*,[7] who studied the interactive effects of N fertiliser source and timing of fertilisation on $\delta^{15}N$

* Correspondence to: Martina Šturm, Department of Environmental Sciences, 'Jožef Stefan' Institute, Jamova cesta 39, SI-1000 Ljubljana, Slovenia.
E-mail: Martina.Sturm@ijs.si

a Department of Environmental Sciences, 'Jožef Stefan' Institute, Jamova cesta 39, SI-1000 Ljubljana, Slovenia

b Biotechnical Faculty, University of Ljubljana, Jamnikarjeva 101, SI-1000 Ljubljana, Slovenia

signatures in *Brassica campestris* L. cv. Maeryok (Chinese cabbage), the most popular vegetable in Asia, most studies[3–5,8] have dealt only with $\delta^{15}N$ variations in plants after a single application of organic or synthetic N fertiliser. Hence the effect on plant $\delta^{15}N$ of split N fertilisation, which might enable growers to cover up the use of synthetic fertiliser, is not well studied and should be extended to other plant species that are popular in Europe, as we know that almost a quarter of the world's organic land is in Europe (i.e. 8.2 Mha, which is 1.7% of the European agricultural area) and the sales of organic products achieved in 2008 were approximately 18 000 M€, with the vegetable crop market constituting an important part.[15]

The aim of the present study was to test whether N fertiliser type and time of its application (single or split application) leave a specific $\delta^{15}N$ fingerprint in lettuce (*Lactuca sativa* L.) tissues that could be used as a potential marker to reveal the use of synthetic N fertiliser in organic production. The suitability of the $\delta^{15}N$ fingerprint was studied by using synthetic and organic fertilisers with a small difference in $\delta^{15}N$ (9.1‰), making identification of the fertiliser type more difficult owing to various factors that might blur the $\delta^{15}N$ of the initial fertiliser source.

MATERIALS AND METHODS

Soil and N fertilisers

A sandy loam soil was collected from an experimental field in Sneberje, near Ljubljana, Slovenia. The soil was air dried, passed through a 20 mm sieve, mixed and used for a pot experiment. All chemical analyses were performed on air-dried pulverised (to pass a 2 mm sieve) and homogenised soil samples. The soil nitrogen contents were 1700 mg kg^{-1} for total N, 6.8 mg kg^{-1} for NO_3^--N, 1.6 mg kg^{-1} for NH_4^+-N and 1691.6 mg kg^{-1} for organic N. The $\delta^{15}N$ value for total N was 6.4‰. The C_{org}/N_{tot} ratio of the soil was 12 and the pH was 7.4 ($CaCl_2$, 1 : 5). Commercial organic fertiliser earmarked for organic farming and gardening (Valentin naravno organsko gnojilo, Semenarna Ljubljana d.d., Ljubljana, Slovenia) with a total N content of 63 g kg^{-1} and a $\delta^{15}N$ value of 14.8‰ was used as organic input, while $Ca(NO_3)_2$ with a $\delta^{15}N$ value of 5.7‰ was used as synthetic fertiliser. A description of the methods used for N content and $\delta^{15}N$ determination is given below.

Pot experiment

A pot experiment with lettuce (*L. sativa* L.) was performed in a greenhouse (15–25 °C, 80% transparency to visible radiation, solar radiation duration of 173, 266 and 210 h in April, May and June respectively) at the Biotechnical Faculty of Ljubljana, Slovenia. Lettuce seeds were sown in plug trays containing Klasmann tray substrate, and seedlings were individually transplanted into pots 35 days later at the five to seven true leaf stage. Plants were grown in pots for 50 days from 24 April to 10 June 2009. Seven treatments were applied in a completely randomised design with three replications, as follows: an unfertilised control (C); a single basal organic fertilisation of 40 mg N kg^{-1} soil (O); a single basal synthetic fertilisation of 40 mg N kg^{-1} soil (S); a basal synthetic fertilisation of 20 mg N kg^{-1} soil followed by an additional organic fertilisation of 20 mg N kg^{-1} soil (S + O); a basal organic fertilisation of 20 mg N kg^{-1} soil followed by an additional synthetic fertilisation of 20 mg N kg^{-1} soil (O + S); a basal synthetic fertilisation of 20 mg N kg^{-1} soil followed by an additional synthetic fertilisation of 20 mg N kg^{-1} soil (S + S); a basal organic fertilisation of 20 mg N kg^{-1} soil followed by an additional organic fertilisation of 20 mg N kg^{-1} soil (O + O). The levels of applied N are typical of those used in lettuce cultivation by local farmers. As the basal application, N-based fertilisers ($Ca(NO_3)_2$ and organic fertiliser) were mixed thoroughly with 7.5 kg of soil per pot. Organic fertilisation with pulverised (to pass a 2 mm sieve) fertiliser was performed 2 days before transplanting, whereas synthetic fertiliser was applied on the day of transplanting. Additional application of N fertilisers was performed immediately after sampling at 30 days after transplanting (DAT). During the experiment, plants were irrigated manually with tap water (3.5 mg N-NO_3^- L^{-1}, $NH_4^+ + NO_2^- < 0.02$ mg N L^{-1}, $\delta^{15}N$ = 4.6‰) every 2 days at the beginning, then, as the plants grew bigger, watering was performed daily. All treatments received the same amount of irrigation. Watering was adjusted by weighing and kept near to field capacity.

Sampling and sample preparation

During the experiment, above-ground lettuce samples were taken at 0, 20, 30 and 50 DAT. At each sampling event, three plants were destructively sampled for each treatment. Each plant was divided into three parts, i.e. inner, middle and outer leaves, and fresh weight was measured for each part. Dry weight was determined after drying in a drying chamber at 60 °C until constant weight. After drying, plant samples were homogenised by grinding to a fine powder using an agate mortar and pestle. For the determination of N content and ^{15}N abundance, about 5.5–6.5 mg of dried and homogenised plant sample or 50–60 mg of soil sample was weighed into a tin cup.

Sample analysis

N content and $\delta^{15}N$ were determined simultaneously using a PDZ Europa Scientific (PDZ Europa Scientific Ltd., Crewe, UK) ANCA-SL elemental analyser linked to a Europa 20-20 continuous flow isotope ratio mass spectrometer. All samples were analysed in duplicate. The estimated measuring uncertainty of N content determinations was 7%, based on long-term measurements of an in-house sunflower reference material (7.97 g N kg^{-1}). Results of nitrogen isotopic composition are reported in δ notation in units of per mil (‰) with respect to atmospheric nitrogen (air) according to the equation

$$\delta^{15}N_{sample}(‰) = [(R_{sample}/R_{standard}) - 1] \times 1000 \quad (1)$$

where R denotes $^{15}N/^{14}N$ and the standard is atmospheric nitrogen with a $^{15}N/^{14}N$ ratio of 0.00368 and a $\delta^{15}N$ value of 0‰. $\delta^{15}N$ values were accepted when the sample standard deviation was ≤0.2‰. The accuracy of the isotopic analysis was checked with the certified reference materials USGS 34 (−1.8‰) and IAEA-N-2 (20.3‰) and the in-house reference materials ammonium sulfate (2.5‰) and sunflower (7.5‰) and was better than 0.2‰. The accuracy of $\delta^{15}N$ measurements was verified by successful participation in an international interlaboratory study (WEPAL International Plant-analytical Exchange Programme, IPE 2009.2).

N uptake of each plant part was calculated as

$$\text{N uptake} = \text{dry matter yield} \times \%N/100 \quad (2)$$

N uptake of whole plants was calculated as the sum of N uptakes of above-ground plant parts. Integrated whole-plant $\delta^{15}N_w$ values were calculated from the $\delta^{15}N$ values of different parts (i.e. inner,

Table 1. Dry matter (DM) yield and nitrogen (N) uptake of lettuce at 20, 30 and 50 days after transplanting (DAT)[a]

	DM yield (g per plant)			N uptake (mg per plant, DM basis)		
Treatment	20 DAT	30 DAT	50 DAT	20 DAT	30 DAT	50 DAT
C	2.0a	7.9a	13.5a	84.1a	189.3a	308.3a
O	4.4b	9.5a	13.4a	158.9b	300.7b	420.5b
S	3.1a	9.8a	12.5a	119.4a	384.4b	469.7b
S + S[b]	3.7b	7.7a	13.7a	142.9b	272.1b	457.0b
S + O[b]	3.7b	7.7a	16.2a	142.9b	272.1b	466.0b
O + S[b]	3.7b	9.1a	16.1a	117.0a	240.6a	458.2b
O + O[b]	3.7b	9.1a	13.2a	117.0a	240.6a	352.0a

[a] Data are mean of $n = 3$ plants per treatment. Different letters denote significant difference within a column ($P < 0.05$).
[b] In split treatments (S + S and S + O; O + O and O + S), $\delta^{15}N$ values at 20 and 30 DAT were calculated based on the same three replications.

Table 2. $\delta^{15}N$ of whole-plant lettuce at 20, 30 and 50 days after transplanting (DAT)[a]

	$\delta^{15}N$ (‰)		
Treatment	20 DAT	30 DAT	50 DAT
C	6.6a,x	7.5bc,x	7.2b,x
O	12.4c,y	10.3d,xy	9.6c,x
S	5.8a,x	5.9ab,x	5.3a,x
S + S[b]	5.6a,x	5.1a,x	5.2a,x
S + O[b]	5.6a,x	5.1a,x	6.0a,x
O + S[b]	10.4b,z	8.8c,y	7.2b,x
O + O[b]	10.4b,y	8.8c,x	8.0b,x

[a] Whole-plant $\delta^{15}N$ was calculated as integration of three separate plant parts (outer, middle and inner leaves). Different letters denote significant difference ($P < 0.05$) within a column (a, b, c, d) or within a row (x, y, z).
[b] In split treatments (S + S and S + O; O + O and O + S), $\delta^{15}N$ values at 20 and 30 DAT were calculated based on the same three replications.

middle and outer leaves) as follows:[7,16]

$$\delta^{15}N_w = (\delta^{15}N_iM_i + \delta^{15}N_mM_m + \delta^{15}N_oM_o) / (M_i + M_m + M_o) \quad (3)$$

where M denotes the mass of total N and the subscripts w, i, m and o denote whole, inner, middle and outer leaves, respectively.

Statistical analysis

Data were verified statistically by analysis of variance using Statistica 6.0 (StatSoft, Tulsa, OK, USA). Means were separated by the *post hoc* least significant difference test. Differences were considered to be significant at $P < 0.05$.

RESULTS AND DISCUSSION

Dry matter and N yield of lettuce

At transplanting, mean dry matter (DM) yield, N content and $\delta^{15}N$ of three seedlings (whole plants) were determined as follows (mean ± standard deviation) 0.13 ± 0.05 g per plant, 49.1 ± 0.5 g kg^{-1} DM basis and 1.8 ± 0.7‰, respectively.

Data for DM yield and N uptake of lettuce at 20, 30 and 50 DAT are presented in Table 1. N fertilisation caused a significant difference in DM yield between control and fertilised plants at 20 DAT, with the exception of plants treated with a single application of synthetic fertiliser, whose DM yield was not significantly different from that of control plants. The suppression of DM accumulation was also observed by Yun *et al.*[7] at 40 DAT. However, the difference between treatments was no longer significant at 30 and 50 DAT. High DM yield under unfertilised control treatment indicates that the soil used in the experiment was relatively rich in N. However, since it is not common for local farmers (either conventional or organic) to perform soil N analysis before planting and apply N accordingly, but to apply the amount of N that is expected to be taken up by the particular plant in optimal yield conditions, the same practice was followed in the present experiment.

Many authors report that the dynamics of N uptake is equally or even more important than the total N uptake and is typical for each plant species.[17,18] On average, about 60% of all N was accumulated in plants before 30 DAT in treatments with split application, whereas, in single fertilisation treatments, 72 and 81% were accumulated before 30 DAT in the O and S treatments respectively. During the growth stage (20 and 30 DAT), differences in N uptake were observed between treatments (Table 1). At final harvest (50 DAT), plants from control pots (C) and plants with split application of organic fertiliser (O + O) showed significantly lower N uptake (308.3 and 352.0 mg per plant, respectively) compared with other treatments (mean N uptake 454.3 mg per plant), indicating lower N availability in these two treatments. In the O + O treatment, low N availability could be explained by slow mineralisation of the organic N applied at 30 DAT.

Effect of different N sources and time of application on whole-plant $\delta^{15}N$

The $\delta^{15}N$ values of whole plants receiving single organic fertiliser (O) were significantly higher compared with the treatments with different N sources (i.e. synthetic fertiliser, soil), reflecting the higher $\delta^{15}N$ value of organic fertiliser (14.8‰) compared with synthetic fertiliser (5.7‰) and total soil N (6.4‰) (Table 2). At final harvest the mean $\delta^{15}N$ of whole plants receiving a single application of organic fertiliser (O) was 9.6‰, while the $\delta^{15}N$ values of plants receiving split applications were 8.0 and 7.2‰ for the O + O and O + S treatments, respectively. The mean $\delta^{15}N$ of whole plants receiving a single application of synthetic fertiliser (S) was 5.3‰, while the $\delta^{15}N$ values of plants receiving split applications were 5.2 and 6.0‰ for the S + S and S + O treatments respectively. However, lettuces fertilised only with synthetic fertiliser (single or split application) were significantly depleted in ^{15}N compared with unfertilised control plants (C, with $\delta^{15}N = 7.2$‰), strongly indicating the contribution of synthetic fertiliser (with $\delta^{15}N = 5.7$‰).

Significantly higher $\delta^{15}N$ values were found in lettuces receiving a single application of organic fertiliser (O) compared with plants receiving a split application (O + O), reflecting the proportionately greater contribution of organic fertiliser N to total plant N in the single application, which is in accordance with the findings of Yun *et al.*[7] In contrast, no significant difference in $\delta^{15}N$ was found between lettuces receiving synthetic fertiliser as a single (S) or split (S + S) application.

Although some trends due to split fertiliser application can be observed (e.g. decrease in whole-plant $\delta^{15}N$ after addition of

synthetic fertiliser to basal organic fertilisation or increase in $\delta^{15}N$ after addition of organic fertiliser to basal synthetic fertilisation) (Table 2), split fertilisation did not cause a significant alteration in whole-plant $\delta^{15}N$ when either isotopically similar (O + O, S + S) or different additional N sources were applied. An exception is the addition of synthetic fertiliser to basal organic fertilisation (O + S), which caused a significant decrease in whole-plant $\delta^{15}N$ from 8.8‰ (30 DAT) to 7.2‰ (50 DAT). However, whole-plant $\delta^{15}N$ of the O + S treatment (7.2‰) did not differ significantly from that of the O + O treatment (8.0‰) at 50 DAT. Yun *et al.*[7] reported a significant alteration in cabbage $\delta^{15}N$ when isotopically different (S + O, O + S) additional N sources were applied. This could be explained by the greater difference in $\delta^{15}N$ between applied organic and synthetic N sources in their study ($\Delta\delta^{15}N = 17.7$‰) compared with the present study ($\Delta\delta^{15}N = 9.1$‰). Concerning splitting, we observed that the addition of synthetic fertiliser to basal organic fertilisation (O + S) had a greater effect on whole-plant $\delta^{15}N$ ($\Delta\delta^{15}N_{(30-50\ DAT)} = -1.6$‰) compared with the addition of organic fertiliser to basal synthetic fertilisation (S + O) ($\Delta\delta^{15}N_{(30-50\ DAT)} = 0.9$‰), indicating greater availability of synthetic fertiliser N compared with organic fertiliser N[7,19] and soil N.

A decrease in whole-plant $\delta^{15}N$ with time was found in organically fertilised plants (O, O + O, O + S), which indicates an increased contribution of soil N to plant N with time.[4,7,20] In addition, the decrease in $\delta^{15}N$ with time in the O + S treatment ($\delta^{15}N$ was lower by 0.8‰ compared with O + O fertilisation, not significant) indicates also the contribution of synthetic fertiliser N.[4] The $\delta^{15}N$ of plants treated with synthetic fertiliser (S, S + S, S + O), on the other hand, was relatively constant and reflected the $\delta^{15}N$ of synthetic fertiliser N during the whole of plant growth. The addition of organic fertiliser to basal synthetic fertilisation (S + O) elevated the mean $\delta^{15}N$ value of lettuce tissues by 0.7–0.8‰ compared with synthetic fertilisation (S, S + S), but the difference between treatments was not significant.

Bateman *et al.*[5] who studied the effect of irrigation water type (deionised *versus* tap water) on plant $\delta^{15}N$, reported that lettuces irrigated with tap water (7 mg $N\text{-}NO_3^-$ L^{-1}, $\delta^{15}N = 2$–6‰) assimilated some N from nitrate present in the water, which consistently moderated the $\delta^{15}N$ value of lettuces away from the extreme values observed when the plants were irrigated with deionised water. In our study the N from the irrigation water represents about 10–15% of the total N applied. The $\delta^{15}N$ of tap water is 4.6‰, which is 1.1 and 10.2‰ lower compared with synthetic and organic fertilisers, respectively. This may have an additional effect on the decrease in $\delta^{15}N$ under organic treatments (O, O + O, O + S). The apparent absence of a similar effect on the lettuces grown with synthetic fertiliser may be due to a small difference in $\delta^{15}N$ between synthetic fertiliser and tap water and to a greater supply of readily available synthetic N to these plants such that the uptake of tap water-derived N has a negligible effect on lettuce $\delta^{15}N$ values.[5]

Table 3. Intra-plant $\delta^{15}N$ variations of lettuce at 20, 30 and 50 days after transplanting (DAT)[a]

		$\delta^{15}N$ (‰)		
Treatment	Plant part	20 DAT	30 DAT	50 DAT
C	Outer	9.1b,y,k	9.7c,y,kl	7.0a,x,kl
	Middle	6.1a,x,k	8.2b,y,lm	7.5a,xy,l
	Inner	5.6a,x,k	6.1a,x,l	7.4a,y,m
O	Outer	14.0b,y,l	12.8b,y,m	10.3b,x,n
	Middle	13.5b,y,l	10.5ab,x,n	10.1b,x,m
	Inner	10.0a,y,l	8.5a,x,m	9.2a,xy,n
S	Outer	8.6b,y,k	10.2c,y,kl	6.2b,x,k
	Middle	6.4b,x,k	6.6b,x,kl	5.4a,x,k
	Inner	3.5a,x,k	2.2a,x,k	4.8a,x,k
S + S[b]	Outer	9.6c,y,k	8.3c,xy,k	7.1b,x,lm
	Middle	6.8b,x,k	6.4b,x,k	5.2a,x,k
	Inner	3.8a,x,k	2.8a,x,k	4.8a,x,k
S + O[b]	Outer	9.6c,y,k	8.3c,xy,k	6.5a,x,kl
	Middle	6.8b,x,k	6.4b,x,k	5.6a,x,k
	Inner	3.8a,x,k	2.8a,x,k	5.7a,y,kl
O + S[b]	Outer	12.2b,y,l	11.3c,y,lm	9.1b,x,mn
	Middle	11.2b,y,l	9.4b,y,mn	5.9a,x,k
	Inner	8.7a,x,l	7.4a,x,lm	7.0a,x,lm
O + O[b]	Outer	12.2b,y,l	11.3c,y,lm	8.3a,x,lm
	Middle	11.2b,y,l	9.4b,xy,mn	7.8a,x,l
	Inner	8.7a,x,l	7.4a,x,lm	7.9a,x,mn

[a] Data are mean of $n = 3$ plants per treatment. Different letters denote significant difference ($P < 0.05$) between outer, middle and inner leaves within a particular treatment at a particular sampling date (a, b, c), in a particular plant part within a particular treatment during plant growth between sampling dates within each row (x, y) and for each plant part between treatments for each sampling date (k, l, m, n).
[b] In split treatments (S + S and S + O; O + O and O + S), $\delta^{15}N$ values at 20 and 30 DAT were calculated based on the same three replications.

Variations in $\delta^{15}N$ within plant

Variations in $\delta^{15}N$ within the plant, i.e. among the outer, middle and inner leaves, were related to fertiliser type, timing of fertilisation and sampling time (Table 3). In the literature, intra-plant variation in $\delta^{15}N$ has been reported for both laboratory and field studies.[21,22] Organ-specific assimilation, reallocation and loss of N as well as N source can cause intra-plant variations in $\delta^{15}N$, because most reactions discriminate against ^{15}N,[4,7,21,23] though the global $\delta^{15}N$ value of any plant biomass is primarily determined by that of the actual N source.[24,25] Considering that leaves formed at different growth stages should have specific $\delta^{15}N$ values reflecting the isotopic composition of the N assimilated at each stage, a change in available N sources with characteristic isotopic composition (e.g. application of different types of fertiliser) during growth is expected to strongly affect intra-plant $\delta^{15}N$ variations.[7]

In all leaves under treatment with single organic fertilisation (O) the $\delta^{15}N$ values decreased significantly during plant growth (with the exception of the inner leaves at 50 DAT), indicating an increased contribution of soil N and irrigation water N to plant N with time.[3,7,20] During growth the inner, younger leaves were significantly depleted in ^{15}N compared with the rest of the plant, with the exception of an insignificant difference between the inner and middle leaves at 30 DAT.

During plant growth under treatment with single synthetic fertilisation (S) the inner leaves were depleted in ^{15}N compared with the rest of the plant and most evidently indicated the $\delta^{15}N$ of the source. However, at final harvest the difference between the middle and inner leaves was no longer significant and the $\delta^{15}N$ values of all plant parts indicated the $\delta^{15}N$ of the synthetic fertiliser N. The enrichment with ^{15}N did not change significantly during growth in the inner and middle leaves, whereas the outer leaves were significantly depleted in ^{15}N at 50 DAT compared with 20 and 30 DAT.

During the growth stage, the outer leaves under treatments with split synthetic fertilisation (S + S and S + O) were enriched in ^{15}N compared with the rest of the plant. Additional treatment with synthetic fertiliser did not cause any significant difference in δ^{15}N of lettuce leaves. Throughout the growing period, no significant difference in δ^{15}N was found between single (S) and split (S + S) applications of synthetic fertiliser, with the exception of significantly higher δ^{15}N in the outer leaves in the S + S treatment at 50 DAT. Under treatment with basal synthetic and additional organic fertilisation (S + O) the addition of organic fertiliser increased the mean δ^{15}N of the inner leaves by as much as 0.9‰ compared with synthetic fertilisation (S, S + S), but the difference was not significant.

At the early growth stage (20 and 30 DAT) the inner leaves of plants with basal organic and additional synthetic (O + S) or organic (O + O) fertilisation were significantly depleted in ^{15}N compared with the rest of the plant. However, in the O + S treatment at 50 DAT the middle leaves too were depleted in ^{15}N compared with the rest of the plant. This was also observed by Yun *et al.*[7] who studied the interactive effects of N fertiliser source and timing on N isotopic signatures in Chinese cabbage and soil, though in our experiment the difference between the middle and inner leaves was not significant. Additional application of synthetic fertiliser to basal organic fertilisation (O + S) significantly lowered the mean δ^{15}N of the middle leaves at 50 DAT by 1.9‰ compared with split organic fertilisation (O + O) and by 4.2‰ compared with single organic fertilization (O). This difference was not observed in the outer leaves, but it was observed in the inner leaves between the O and O + S treatments. Plants from the treatment with split organic fertilisation (O + O) had significantly lower δ^{15}N values in the inner, younger leaves compared with the rest of the plant at the early growth stage, whereas at 50 DAT the difference in δ^{15}N between different leaves was non-significant. Split application of organic fertiliser to basal organic fertilisation (O + O) did not cause significant changes in the δ^{15}N pattern during the first 30 days of growth, which was also found by Yun *et al.*[7] However, it resulted in significantly lower δ^{15}N values at 50 DAT in the outer and middle leaves compared with the single application (O), which can be explained by the higher contribution of soil N to plant N in split application, i.e. δ^{15}N decreased with decreasing contribution of organic fertiliser N to total plant N.[4]

CONCLUSION

Considering the results obtained, δ^{15}N of lettuce tissues could be used as a marker to reveal the use of synthetic N fertiliser, but only when applied in a single dose, since the addition of synthetic fertiliser to basal organic fertilisation could not be confirmed by this method. Under the conditions used in this trial (pot-grown lettuce, fertiliser type, growth conditions), it seems not possible to detect low or moderate rates of synthetic N fertiliser illegally applied to a lettuce crop in organic production. Although the use of organic N fertilisers does not alone prove that crops were grown in accordance with all principles of organic farming, the method presented could be used as a quick and relatively cheap (5–45 € per sample) tool to reveal the use of prohibited synthetic fertilisers. However, unlike in the study with Chinese cabbage,[7] in our study, with a difference in δ^{15}N between organic and synthetic fertilisers of 9.1‰, the method was found to be insufficiently sensitive for identifying the combined use of organic and synthetic fertilisers. This means that growers could cover up the use of synthetic fertiliser when applied in combination with organic fertiliser. If the difference in δ^{15}N between the applied synthetic and organic N fertilisers was >9.1‰, detection of the combined usage of the fertilisers would have a greater discriminatory power.

However, there are some additional limitations of using δ^{15}N in plant tissues to differentiate between organic and conventional production that need to be considered but are so far not well studied. The use of organic residues originating from nitrogen-fixing plants or the use of green manures and seaweed-based amendments (which are also permitted in organic farming and whose δ^{15}N values range between 0.6 and 5.4‰)[8] lowers the δ^{15}N values of plant available N, so plants are likely to have δ^{15}N values similar to those of plants treated with synthetic fertiliser. On the other hand, conventional growers do not always apply synthetic N fertilisers to their crops but may use a synthetic N fertiliser or any of the natural fertilisers that are permitted in organic farming.[5] Lettuce, however, is a crop with high N requirements, so it is usual for conventional growers to apply synthetic fertiliser throughout the growing period.[5]

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Appendix 10: Article: Effect of different fertilisation and irrigation practices on yield, nitrogen uptake and fertiliser use efficiency of white cabbage (Brassica oleracea var capitata L.), *Scientia Horticulturae*, 125, 103-109, 2010.

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Effect of different fertilisation and irrigation practices on yield, nitrogen uptake and fertiliser use efficiency of white cabbage (*Brassica oleracea* var. *capitata* L.)

Martina Šturm[a,*], Nina Kacjan-Maršić[b], Vesna Zupanc[b], Branka Bračič-Železnik[c], Sonja Lojen[a], Marina Pintar[b]

[a] Department of Environmental Sciences, Jožef Stefan Institute, Jamova cesta 39, 1000 Ljubljana, Slovenia
[b] Agronomy Department, Biotechnical Faculty, University of Ljubljana, Jamnikarjeva 101, 1000 Ljubljana, Slovenia
[c] Drinking Water and Sewerage System Public Utility, Vodovodna cesta 90, 1000 Ljubljana, Slovenia

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ABSTRACT

The effect of different fertilisation (i.e. broadcast application and fertigation) and irrigation practices (tank sprinkler and drip irrigation) on yield, yield quality (nitrate content), nitrogen uptake of white cabbage (*Brassica oleracea* var. *capitata* L.) and the potential for N losses was assessed on sandy-loam agricultural soil. ^{15}N-labelled fertiliser was used as a tracer. It was found that different practices significantly affected yield, nitrate content in plants, N uptake, as well as fertiliser use efficiency. The highest yield (93 t ha^{-1}), plant N uptake (246 kg ha^{-1}), and fertiliser use efficiency (42%) were obtained under treatment with broadcast fertilisation with farmer's practice of irrigation (tank sprinkler). The N surplus after harvest was −41 kg N ha^{-1}, indicating the lowest potential for N losses. Treatment by fertigation and drip irrigation covering 100% of the crop's water requirements did not result in the highest yield as expected (72 t ha^{-1}), the N surplus after harvest was about +38 kg ha^{-1}. The lowest yield (58 t ha^{-1}), fertiliser use efficiency (30%) and hence the highest potential for N losses (N surplus after harvest +68 kg ha^{-1}) were found in treatment with broadcast fertilisation and drip irrigation covering 50% of the crop's water requirements.

1. Introduction

Nitrogen (N) is an essential nutrient for plant growth and in the desire to produce more food, farmers apply it intensively, and often in excessive quantities, in the form of nitrogen-based fertilisers. If fertiliser application exceeds plant demands and the denitrification capacity of the soil, nitrate not taken up by the crop may potentially contribute to ground and surface water pollution through nitrate leaching and soil erosion (Gastal and Lemaire, 2002; Wang et al., 2002; Chen et al., 2004; Almasri and Kaluarachchi, 2007 and references therein), possibly raising the nitrate concentrations in groundwater above the maximum allowed level of 50 mg L^{-1}, set by the European Commission Nitrate Directive (91/676/EEC).

Nitrogen uptake by the aboveground biomass of plants is a very important item of information. This parameter shows variations in yield as well as variations in N concentration in plants (Balík et al., 2003). Nitrate is often the major source of nitrogen available to higher plants (Marschner, 1995). Its uptake and distribution in plants is of major importance with respect to both environmental concerns and the quality of plant products (Gastal and Lemaire, 2002). Fertiliser N taken up by the plant affects not only the yield but also the quality of the plant (Turan and Sevimli, 2005). It can cause high nitrate accumulation in plants, especially in most leafy vegetables (Chen et al., 2004), and as reported in Commission Regulation (EC) No. 1881/2006, vegetables are the major source of nitrate in the human intake. Nitrate is relatively non-toxic but its metabolites (nitrite) may produce a number of deleterious health effects (e.g. methaemoglobinaemia, carcinogenesis) (Santamaria, 2006), so care should be taken, especially for pregnant women and babies, not to exceed the acceptable daily intake of 3.65 mg nitrate per kg body weight.

Hence, in order to reduce the burden to the natural environment caused by excessive N fertilisation, optimal crop cultivation methods are sought to diminish the negative effect of N compounds on the environment, and ensure high and quality yields at the same time (Rahn, 2002). While it is not possible to prevent nitrate leaching, improved management practices leading to increased fertiliser N use efficiency can reduce the potential for nitrate contamination of groundwater (Bijay-Sighn et al., 1995; Cassman et al., 2002). The utilization of N can be increased by balanced application of N, P and K and lighter and more frequent irrigation (Bijay-Singh et al., 1995; Bijay-Singh and Sekhon, 1979; Bijay-Singh and Sekhon, 1997).

* Corresponding author. Tel.: +386 1 5885 452; fax: +386 1 5885 346.
E-mail address: martina.sturm@ijs.si (M. Šturm).

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Table 1
Chemical and physical properties of soil on the Ljubljansko polje experimental area.

Parameters	
pH ($CaCl_2$, 1:5)	7.4
Total organic C	
Content (mg/kg)	14600
Total N	
Content (mg/kg)	1400
at.% ^{15}N excess	0.003
2 M $CaCl_2$ extractable NH_4^+-N	
Content (mg/kg)	1.6
2 M $CaCl_2$ extractable NO_3^--N	
Content (mg/kg)	6.8
Organic N[a]	
Content (mg/kg)	1391.6
C/N weight ratio	12.2
Texture	Loam and sandy loam

[a] The content of organic N was determined as the difference in N between total N and inorganic N.

The increasing concern for maximizing the efficiency of fertiliser nitrogen use in crop production is reflected in the experimental increase in the use of ^{15}N to study the uptake of applied nitrogen (Hauck and Bremner, 1976). There is a persisting and perhaps widespread view that the behaviour of ^{15}N in soils and plants is too complex to permit variations in its natural abundance to be used as a tracer or even as a probe to explore plant–soil relationships in natural ecosystem (Hauck et al., 1972; Stewart, 2001). However, with the ^{15}N labelling method, the isotopic signature of the enriched tracer can be pre-determined to ensure significant differences in at.% ^{15}N between the source and background level, even when fractionation occurs. This technique has been used extensively for a number of years (Broadbent and Nakashima, 1974; Fried et al., 1975; Hardarson et al., 1984; Mulvaney and Boast, 1986; Bronson et al., 2000; Bedard-Haughn et al., 2003; Zamora et al., 2009; and many others), and has been accepted by the scientific community at large as the most reliable way to follow the flow and fate of N in systems (Bedard-Haughn et al., 2003). When ^{15}N enriched material is applied it becomes part of the overall N-cycle and the path of ^{15}N through the various soil and plant N pools can be followed (Bedard-Haughn et al., 2003). The isotopic method is the only direct means of measuring N uptake from applied fertiliser. The recovery data are known to be the "real coefficient of utilization" (van Cleemput et al., 2008).

White cabbage (*Brassica oleracea* var. *capitata* L.) is an important vegetable in Slovenia. It is frequently grown in fields located above shallow groundwater bodies which are very susceptible to nitrogen leaching and prone to pollution of groundwater, which in Slovenia represents the most important source of drinking water. Therefore, a field experiment was conducted with white cabbage to study the effect of different fertilisation and irrigation practices on yield, N-fertiliser use efficiency and consequently yield quality (i.e. nitrate content) and the potential for N losses (i.e. N surplus after harvest) in order to obtain data which could lead to recommendations for farmers growing white cabbage on sandy-loam soils inside groundwater protection areas. The study was preformed between April and June, 2007, with ^{15}N enriched potassium nitrate fertiliser as a tracer.

2. Materials and methods

The experimental field (40°4′42″ N, 14°35′46″ E, 30 m wide and 70 m long) was located above the sandy-gravel aquifer of Ljubljansko polje, east of Ljubljana, the capital of Slovenia. The agricultural soil was classified as gleyic fluvisol and endogleyic fluvisol (World Reference Base for Soil Resources, 2006). The chemical and physical properties of the soil are presented in Table 1. The mean annual precipitation in the study area for the 1971–2000 reference period was 1368 mm, and the average annual air temperature 10.2 °C, measured at the meteorological station Ljubljana-Bežigrad (299 m.a.s.l., 46°3′57″ N, 14°31′2″ E). In addition, data for precipitation, air temperature, wet deposition of nitrate and ammonium for the growing season were obtained from the Environmental Agency of the Republic of Slovenia and are presented in Table 2. During the growing period, the mean temperature was above the 30 year average by 4.6 °C in April and by about 2 °C in May, June and July. Precipitation was below the 30 year average in all by 140 mm, with the greatest shortfall in April (97 mm below average) and June (74 mm below average), whereas in July precipitation was 31 mm above the 30 year average.

In the experiment, four different fertilisation and irrigation treatments were applied, as follows: (1) Unfertilised control plots with the farmer's practice of irrigation; (2) treatment with the farmer's practice of fertilisation and drip irrigation covering 50% of the crop's water requirements; (3) treatment by fertigation with drip irrigation covering 100% of crop's water requirements; and (4) treatment with farmer's practice of fertilisation and irrigation. Farmer's practice consisted of broadcast fertiliser application the day before transplanting plus irrigation the day before and the day after transplanting (DAT), using a tank sprinkler. Tap water was used for irrigation (1.9 mg N L^{-1}, 0.369 at.% ^{15}N; most of the N was present in nitrate form). The two treatments with drip irrigation covered 100% and 50% of crop water requirements (CWR), determined by the Penman-Monteith method (Allen et al., 1998). Each treatment was replicated three times. Fertilised plots (6.5 m^2) were divided into three subplots (2.6 m^2), and ^{15}N-labelled fertiliser was applied on the middle subplot.

The following fertiliser norm was followed: 200 kg N ha^{-1}, 80 kg P ha^{-1}, 300 kg K ha^{-1}, 280 kg Ca ha^{-1} and 30 kg Mg ha^{-1}. The amount of fertiliser applied was determined according to the Regulations on Integrated Production of Vegetables (Official Gazette of the Republic of Slovenia (RS) 63/2002) and Technological Instructions for Vegetable Production (Ministry of Agriculture, Food and Forestry (MAFF), 2003). ^{15}N-labelled KNO_3 fertiliser (Shanghai Research Institute, Shanghai, China) was used as a tracer. In fertigation treatment, the total amount of P and K and 30% of the total N rate were applied as unlabelled granular fertiliser as a pre-

Table 2
Weather data for the growing season (April–July, 2007) and for the 1971–2000 reference period.

Month	Mean T (°C)		Precipitation (mm)		Wet deposition (g m^{-2})	
					$N\text{-}NH_4^+$	$N\text{-}NO_3^-$
	2007	1971–2000	2007	1971–2000	2007	
April	14.6	10.0	6	103	0.007	0.008
May	17.2	15.0	113	113	0.04	0.032
June	20.9	18.1	80	154	0.053	0.038
July	22	20.4	148	117	0.055	0.043
Sum			347	487	0.155	0.121

Data obtained from the Environmental Agency of the Republic of Slovenia.

Table 3
Yield, dry matter, N content, total aboveground N uptake, fertiliser N and soil N yield at final harvest.

Treatment	Yield (t ha^{-1})	Dry matter (%)	N content (% DM)	N uptake by crops (kg ha^{-1})	Fert. N yield (kg ha^{-1})	Soil N yield (kg ha^{-1})
1	47^{a}	11.03^{a}	1.62^{a}	84.2^{a}	–	84.2
2	58^{ab}	8.79^{b}	2.70^{b}	137.7^{b}	60.6	77.1
3	72^{b}	9.32^{b}	2.52^{b}	168.8^{b}	–	–
4	93^{c}	9.02^{b}	2.86^{c}	246.0^{c}	83.6	162.4

DM: dry matter.
N content represents weighted average content in aboveground cabbage at final harvest.
Different letters denote significant difference between treatments at the $p < 0.05$.

plant broadcast application and the remaining N as ^{15}N-labelled solution via fertigation. During the growing season, fertigation was performed three times, i.e. at 57, 66, and 75 DAT. The labelled KNO_3 + unlabelled water soluble $Ca(NO_3)_2$ was dissolved in tap water and applied as solution with 3.52 ± 0.04 at.% ^{15}N. On plots with farmer's practice of fertilisation, unlabelled $Ca(NO_3)_2$ (0.365 at.% ^{15}N) was applied as a broadcast application, followed by the application of the labelled fertiliser, which was applied as a solution. We assumed that $Ca(NO_3)_2$, which was applied as a broadcast application the day before transplanting, was dissolved after the irrigation within a few hours and mixed with the labelled fertiliser in the soil. In all treatments, for unlabelled subplots only unlabelled $Ca(NO_3)_2$ was applied.

Cabbage (*B. oleracea* var. *capitata* L.) was sown in plug trays, containing Klasman tray substrate, and transplanted to the field 48 days later. It was grown for 126 days, from April 11 to July 27, 2007. Samples were taken at 59, 68 and 78 DAT. At each sampling event, three plants were destructively sampled from each subplot of each replicate. For determination of isotopic composition, only the samples from ^{15}N-labelled subplots were used. Cabbage heads were divided into three equal parts, inner, middle and outer parts. Fresh weight was determined for all aboveground plant parts. Samples were dried at 60 °C, weighed for determination of dry mass, ground to a fine powder and homogenized. Total N was determined after incineration at 900 °C in a VarioMAX CN analyser and determined by a Thermal Conductivity Detector (TCD) (ISO 13878). Measurement uncertainty was 9%. Nitrate content in fresh samples was determined after in water extracts with UV/VIS spectrometer, Perkin-Elmer, Lambda 2, with a FIA-system (Neumann and Bassler, 1976). Measurement uncertainty was 19%. For isotopic composition, dry samples were weighed into tin cups and the at.% ^{15}N was determined using a continuous flow Europa 20-20 IRMS with an ANCA-SL preparation module for solid and liquid samples (PZD Europa Ltd., UK). All samples were analysed in duplicate. Results of nitrogen isotopic composition are reported with respect to international standard atmospheric nitrogen (air) in units of at.% which is the absolute abundance of an isotope, i.e. the percentage of atoms which occur as the various isotopes. The reproducibility and accuracy of the isotopic measurements depended on the enrichment, but it was always better than ±0.012 at.% ^{15}N, based on replicate measurements of samples and the reference materials: IAEA 305-B (0.050 at.% ^{15}N), IAEA PLANT RM (1.187 at.% ^{15}N), and IAEA-311 (2.05 at.% ^{15}N). The accuracy was verified also with successful participation in the international interlaboratory study (WEPAL International Plant-analytical Exchange Program, IPE 2009.2).

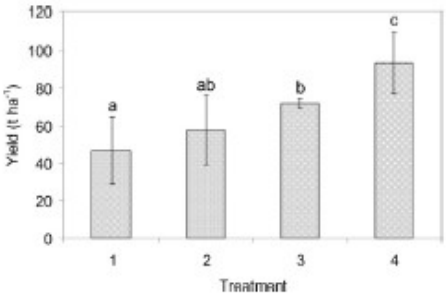

Fig. 1. Cabbage yield under different treatments: 1—control, 2—broadcast fertilisation with 50% irrigation, 3—fertigation with 100% irrigation, 4—broadcast fertilisation with farmer's practice of irrigation. Data are means ± SD (n = 3). Bars designated by different letters are significantly different at the $p < 0.05$.

Dry matter yield, nitrogen uptake (i.e. N yield), the portion of N derived from the fertiliser (%Ndff) and from soil (%Ndfs), fertiliser N yield and % fertiliser utilization were calculated, using the following equations (IAEA, Training Course Series No. 14, 2001):

$$\text{Dry matter yield (kg ha}^{-1}) = \text{FW (kg)} \times \frac{10000\ (\text{m}^2\,\text{ha}^{-1})}{\text{area harvested (m}^2)} \times \frac{\text{SDW (kg)}}{\text{SFW (kg)}},$$

$$\text{N yield (kg ha}^{-1}) = \text{dry matter yield (kg ha}^{-1}) \times \frac{\%\text{N}}{100},$$

$$\%\text{Ndff} = \frac{\text{at.}\%\,^{15}\text{N excess}_{\text{plant}}}{\text{at.}\%\,^{15}\text{N excess}_{\text{fertilizer}}} \times 100,$$

$$\%\text{Ndfs} = 100 - \%\text{Ndff},$$

$$\text{Fertilizer N yield (kg ha}^{-1}) = \text{N yield (kg ha}^{-1}) \times \frac{\%\text{Ndff}}{100},$$

$$\%\text{Fertilizer N utilization} = \frac{\text{fertilizer N yield}}{\text{rate of N application}} \times 100,$$

where FW is sample fresh weight per area harvested and SDW and SFW are subsample dry and fresh weight, respectively. In treatment by fertigation the pre-plant broadcast application was made with unlabelled fertiliser, and ^{15}N was applied by fertigation only, hence the % Ndff, % Ndfs, fertiliser N yield and % fertiliser N utilization were not calculated for this treatment but for the two treatments with a single broadcast application only. Total N accumulation (kg ha^{-1}) was calculated by multiplying the dry matter yield of plant parts and the mean N concentration in the plant parts.

The results obtained were verified statistically with the ANOVA module, the post hoc LSD Fisher test for yield, dry matter, total N and nitrate concentrations and the nonparametric Mann–Whitney U test for at.% ^{15}N excess, using the Statistica 6.0 package. Significant differences are given at the 95% level.

3. Results

3.1. Yield and dry matter

The study revealed that the treatment used significantly affected cabbage yield (Table 3 and Fig. 1). The highest yield was found for treatment with farmer's practice (93 t ha^{-1}), lower for fertigation

Table 4
Nitrate content in different leaves of cabbage at final harvest.

Treatment	NO_3^- (mg kg^{-1} fresh weight)		
	I	M	O
1	344.0^a	228.2^a	324.4^a
2	544.5^a	845.1^a	1222.4^b
3	819.9^a	753.3^b	1478.7^c
4	775.2^a	1305.6^b	1685.7^b

I, M, O: plant leaves, inner, middle, outer.
Data are means for $n = 9$ plants per treatment.
Different letters denote significant difference between different leaves at the $p < 0.05$.

(72 t ha^{-1}) and the lowest for treatments with broadcast fertilisation and irrigation covering 50% of the crop's water requirements (58 t ha^{-1}) and for the control (47 t ha^{-1}). We found a significant effect of nitrogen fertilisation on dry matter content in cabbage (Table 3). The average dry matter content was 9.04% in fertilised plants and 11.03% in unfertilised plants.

3.2. Total N and nitrate content in cabbage

Fertilisation caused a significant increase in total nitrogen and nitrate content in cabbage heads, compared to the control (Tables 3 and 4). Under fertilised conditions, a significantly higher N content was determined in crops under farmer's practice of irrigation, whereas the difference between the two treatments with drip irrigation (covering 50% and 100% of the crop's water requirements), was nonsignificant. Under all treatments, a decline in N content was observed towards the final harvest in inner, middle and outer leaves of cabbage (Table 5 and Fig. 2) due to the dilution effect during plant growth.

At final harvest, a statistically significant difference in N content was found between different leaves under each treatment (Table 5 and Fig. 3). On fertilised plots as well as under control, most of the N was accumulated in the outer leaves and least in the middle leaves of the plants, indicating the highest uptake of N at early growth stage (Gastal and Lemaire, 2002).

At final harvest, the highest mean nitrate content was found for farmer's practice (1326 mg kg^{-1} fresh weight) and for treatment with fertigation (1132 mg kg^{-1}), lower for treatment with broadcast fertilisation and irrigation covering 50% of the plant's water requirements, and the lowest for the control (345 mg kg^{-1}). Table 5 and Fig. 4 show nitrate contents in the inner, middle and outer leaves of cabbage at final harvest (78 DAT).

No significant intra-plant variations were found in the control, whereas for fertilised treatments with broadcast application, a significantly lower content was found in the inner, younger parts and the highest in the outer, older leaves of the plants. For treatment with fertigation, the lowest nitrate content was found in the middle parts and the highest in the outer, older leaves of cabbage.

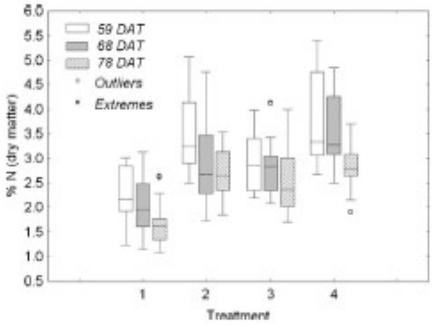

Fig. 2. % N in dry matter of cabbage leaves at 59, 68 and 79 DAT. Treatments: 1—control, 2—broadcast fertilisation with 50% irrigation, 3—fertigation with 100% irrigation, 4—broadcast fertilisation with farmer's practice of irrigation. DAT—days after transplanting. Box gives lower and upper quartiles and median; whiskers show 25th and 75th percentiles; bars represent the nonoutlier range.

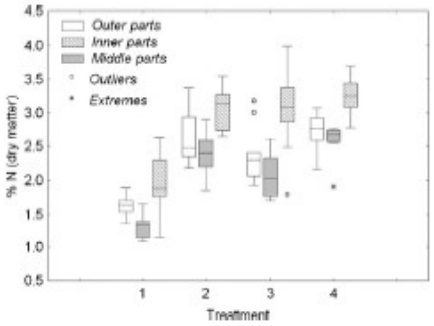

Fig. 3. Intra-plant variation of N content in inner, middle and outer cabbage leaves at final harvest. Treatments: 1—control, 2—broadcast fertilisation with 50% irrigation, 3—fertigation with 100% irrigation, 4—broadcast fertilisation with farmer's practice of irrigation. Box gives lower and upper quartiles and median; whiskers show 25th and 75th percentiles; bars represent the nonoutlier range.

3.3. ^{15}N of cabbage

Plants from control plots were significantly depleted with ^{15}N compared to crops from fertilised plots, as they reflected the isotopic composition of soil N (Table 6 and Fig. 5). On fertilised plots, the lowest ^{15}N values were observed in plants from treatment by fertigation. At final harvest, the difference in at.% ^{15}N excess between treatments by fertigation and farmer's practice

Table 5
N content of different leaves of cabbage at 59, 68 and 78 days after transplanting.

Treatment	% N								
	59 DAT			68 DAT			78 DAT		
	I	M	O	I	M	O	I	M	O
1	2.59^a	1.85^b	2.40^a	2.02^{ab}	1.67^a	2.23^b	1.62^b	1.30^a	1.93^b
2	3.28^a	2.94^a	4.29^b	2.62^a	2.58^a	3.49^b	2.65^{ab}	2.37^a	3.08^b
3	3.14^a	2.42^b	3.64^c	2.67^a	2.45^a	3.31^b	2.38^a	2.11^a	3.06^b
4	3.25^a	3.11^a	4.89^b	3.17^a	3.03^a	4.34^b	2.71^a	2.60^a	3.26^b

DAT: days after transplanting.
Different plant leaves: I, inner; M, middle; O-outer.
Data are means for $n = 9$ plants per treatment.
Different letters denote significant difference between different leaves at the $p < 0.05$.

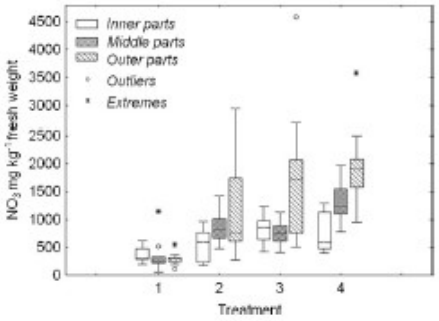

Fig. 4. Nitrate content in inner, middle and outer fresh cabbage leaves at final harvest. Treatments: 1—control, 2—broadcast fertilisation with 50% irrigation, 3—fertigation with 100% irrigation, 4—broadcast fertilisation with farmer's practice of irrigation. Box gives lower and upper quartiles and median; whiskers show 25th and 75th percentiles; bars represent the nonoutlier range.

Table 6
At.% ^{15}N excess in cabbage at 59, 68 and 78 days after transplanting.

Treatment	at.% ^{15}N excess		
	59 DAT	68 DAT	78 DAT
1	0.005[a]	0.009[a]	0.011[a]
2	2.226[b]	1.950[b]	1.546[b]
3	0.664[c]	0.892[c]	1.174[c]
4	1.676[d]	1.602[d]	1.187[c]

DAT: days after transplanting.
Different letters denote significant difference between treatments at the $p<0.05$.

became nonsignificant (1.17 and 1.19 at.% ^{15}N excess, respectively), whereas cabbage under treatment by broadcast fertilisation with irrigation covering 50% of the crop's water requirements was still significantly more enriched in ^{15}N (1.55 at.% ^{15}N). No significant differences in at.% ^{15}N were observed between outer, middle and inner leaves of cabbage (data not shown).

3.4. Nitrogen uptake

Under the field experimental conditions, N uptake was 84 kg N ha^{-1} for the control and 138–246 kg ha^{-1} for fertilised

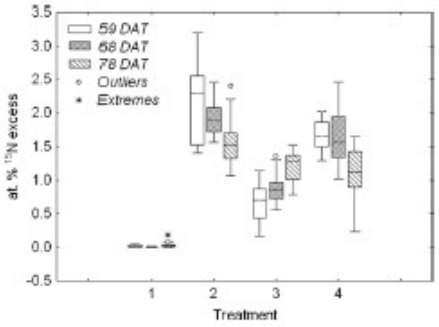

Fig. 5. at.% ^{15}N excess in inner, middle and outer cabbage leaves at 59, 68 and 78 DAT. Treatments: 1—control, 2—broadcast fertilisation with 50% irrigation, 3—fertigation with 100% irrigation, 4—broadcast fertilisation with farmer's practice of irrigation. DAT—days after transplanting. Box gives lower and upper quartiles and median; whiskers show 25th and 75th percentiles; bars represent the nonoutlier range.

Table 7
N balance of N inputs and outputs and fertiliser use efficiency.

Treatment	Inputs	Outputs	N surplus (inputs–outputs)	Fertiliser use efficiency
	(kg N ha^{-1})			(%)
1	4.7	84.2	−79.5	–
2	205.7	137.7	+68.0	30.3
3	206.6	168.8	+37.8	–
4	204.7	246.0	−41.3	41.8

Inputs comprise fertiliser N, irrigation N and N from wet deposition. Outputs comprise only the uptake by the aboveground biomass of crops. N surplus represents N that was lost by ammonia volatilization, denitrification or leaching, or stored in various soil fractions.

treatments (Table 3). The proportion of N taken up from soil or fertiliser varied between treatments. The highest uptake of soil N was calculated for farmer's practice (162.4 kg ha^{-1}), lower for the control (84.2 kg N ha^{-1}) and the lowest for treatment with broadcast fertilisation with irrigation covering 50% of the crop's water requirements (77.1 kg N ha^{-1}), whereas higher fertiliser N utilization was determined under farmer's practice (41.8%), compared to treatment with broadcast fertilisation with irrigation, covering 50% of crop's water requirements (30.3%) (Table 7).

Table 7 presents the balance of N inputs and outputs for the experiment. The inputs involve N in fertilisers, N from wet deposition and irrigation N, and the outputs comprise only N uptake by the aboveground biomass of plants (van Eerdt and Fong, 1998; Oenema et al., 2003; Ju et al., 2006). The N surplus, i.e. the difference between inputs and outputs, represents the N that was lost by volatilization of ammonia, denitrification or leaching, or stored in various soil fractions (Ju et al., 2006). The control and farmer's practice had a negative balance (−79.5 and −41.3 kg N ha^{-1}, respectively), whereas treatment via broadcast application and irrigation covering 50% of the crop's water requirements and treatment with fertigation with irrigation covering 100% of the crop's water requirements had positive balances (+68.0 and +37.8 kg N ha^{-1}, respectively) of N inputs and outputs.

4. Discussion

Fertigation did not increase the yield compared to broadcast fertiliser application, as was also found by Hagin and Lowengart-Aycicegi (1999) and Kacjan-Maršić (2004). In comparison to the control, fertilisation caused a considerable decline in dry matter content (Table 3). A similar observation was made by Smolen and Sady (2009), who studied the effects of various nitrogen fertilisation and foliar nutrition regimes on the dry matter content in carrot roots (*Daucas carota* L.). Sørensen (1999), who studied the effects of nitrogen on vegetable crop production and chemical composition, also reported that increased N supply decreased the dry matter content. Fertilisation increased the nitrate content in crops compared to the control, as also found by various authors, who studied the NO_3^- contents in cabbage (Turan and Sevimli, 2005), carrot (Smolen and Sady, 2009 and references therein), and in rape, Chinese cabbage and spinach (Chen et al., 2004).

As also reported by van Cleemput et al. (2008), the use of ^{15}N-labelled fertiliser revealed differences in the uptake of soil N between plants fertilised with N and those unfertilised. The portion of N derived from the soil exceeded the portion of N derived from the fertiliser (Table 3). In experiments with ^{15}N-labelled fertiliser, it is often observed that the uptake of unlabelled N is greater by plants receiving labelled fertiliser than in the unfertilised control (Powlson and Barraclough, 1993). This can occur entirely as a

result of "pool substitution", i.e. labelled inorganic N from the added fertiliser taking the place of unlabelled inorganic N that would otherwise have been immobilized. The greater the amount of labelled N that is added, the smaller will be the proportion of unlabelled N that is immobilized and the more unlabelled N will remain in the inorganic pool available for uptake by plants (Powlson and Barraclough, 1993).

During the growing season, two different trends and a significant difference in the at.% ^{15}N excess were observed between fertilised treatments. With time, enrichment with ^{15}N in cabbage increased in treatment by fertigation and decreased in both treatments with broadcast application. The variation in at.% ^{15}N excess in cabbage could be attributed to the different availability of soil N and fertiliser N with time of growth (Choi et al., 2002). Decreasing enrichment with ^{15}N with time in cabbage under the two treatments with broadcast application indicates an increased accumulation of N from soil (Choi et al., 2002) and the increasing enrichment under treatment with fertigation, on the other hand, indicates increased accumulation of the enriched fertiliser N with growth time. When available fertiliser N was low in the soil, the relative contribution of soil N to plant N increased with time (Yun and Ro, 2009).

Fertiliser use efficiency deserves careful attention if agriculturists are to produce maximum crop yields and prevent pollution of natural waters with plant nutrients (Bijay-Singh et al., 1995). This depends largely on the synchrony between plant N demand and the quantities of N supplied by the fertiliser and by the soil. Consequently, it is strongly affected by N management methods, as well as by crop management practices (van Cleemput et al., 2008). Since only a fraction of the applied fertiliser N (on average less than 50%) is taken up by the crop, the remainder is subjected to loss, representing both an economic cost and an environmental risk (van Cleemput et al., 2008, references therein). Even though low fertiliser use efficiency does not always imply that unused N will leach into groundwater and does not necessarily pose a hazard to the environment (as long as excess water does not leach it beneath the root zone (Bijay-Singh et al., 1995)), maximizing the efficiency of fertiliser nitrogen can reduce the risk of nitrate pollution from leaching (Bijay-Singh and Sekhon, 1979). Under our experimental conditions, calculation of the N budget (Table 7) indicates a higher potential for N losses in treatments with broadcast fertiliser application with 50% irrigation (highest N surplus) and fertigation with 100% irrigation, compared to farmer's practice of fertilisation and irrigation, where crop N uptake exceeded N inputs by over 40 kg N ha^{-1}, thus resulting in soil N depletion to that extent. Regarding the results obtained, under these environmental conditions and soil type the application of N-fertiliser should be increased by about 41 kg ha^{-1} under farmer's practice of fertilisation and irrigation, decreased by about 68 kg ha^{-1} under treatment with broadcast fertiliser application with 50% irrigation, and decreased by 38 kg ha^{-1} or apply N in more frequent fertigation events under treatment with fertigation with 100% irrigation, in order to gain the same yield without depletion of the soil N and without environmental risk.

As reported by Alva (2009), fertigation is expected to increase the nutrient uptake efficiency, thereby minimizing leaching losses compared to the application of fertiliser in dry granular form broadcast over a large soil area at less frequent intervals. However, among the practices tested in this study on a sandy-loam agricultural soil and under the environmental conditions with precipitation somewhat below and temperature above the 30 year average (Table 2), fertigation with three-split applications of the fertiliser did not result in the lowest N leaching potential. The highest yield, N uptake and lowest leaching potential, but also highest NO_3^- content at final harvest, were determined for treatment with broadcast fertilisation and farmer's practice of irrigation.

5. Conclusions

Farmer's practice, i.e. broadcast fertiliser application plus irrigation the day before and after transplanting, resulted in the highest yield and highest nitrogen uptake, suggesting that among the tested practices and under the given experimental conditions farmer's practice is the most appropriate for growing cabbage from the economic (yield) as well as the environmental point of view (lowest leaching potential). Broadcast fertilisation with drip irrigation covering 50% of the crop's water requirements was found, on the other hand, to result in the lowest yield and N uptake and hence in the highest potential for N losses and was found to be the least appropriate among the tested practices. However, in future more research should be undertaken under different environmental conditions, as well as in different soil types.

Acknowledgements

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Appendix 11: Article: Determination of isotopic composition of nitrate nitrogen in soil water, *Slovenski kemijski dnevi 2010*, 1–7, 2010.

DOLOČANJE IZOTOPSKE SESTAVE NITRATNEGA DUŠIKA V TALNI RAZTOPINI
DETERMINATION OF ISOTOPIC COMPOSITION OF NITRATE NITROGEN IN SOIL WATER

Martina Šturm, Sonja Lojen

Odsek za znanosti o okolju, Institut "Jožef Stefan", Jamova 39, SI-1000 LJUBLJANA, martina.sturm@ijs.si

Izvleček. Namen našega dela je bil izmed različnih metod priprave vzorca za določanje izotopske sestave nitratnega dušika (izotopsko razmerje $^{15}N/^{14}N$ v nitratu) v talni raztopini najti najbolj primerno metodo za rutinsko laboratorijsko uporabo. V prispevku so predstavljeni rezultati meritev izotopske sestave nitratnega dušika, ki smo ga iz pripravljene KNO_3 raztopine (naravna vsebnost ^{15}N) ter vzorcev talne raztopine (obogatene s sledilcem ^{15}N) izolirali z dvema metodama mikrodifuzije (metoda s kavljem in metoda s teflonsko pastjo) ter z modificirano metodo z anionskim izmenjevalcem. Vse tri metode so dale primerljive rezultate. Zaradi visoke cene Ag_2O, dolgotrajnega in zahtevnega postopka se je metoda z anionskim izmenjevalcem izkazala za manj primerno v primerjavi z metodama mikrodifuzije. Kot najbolj primerna metoda se je izkazala mikrodifuzijska metoda s teflonsko pastjo. Ker pri tej metodi pri večjih volumnih prihaja do nepopolne difuzije, smo uvedli korekcijski faktor, ki omogoča zanesljivo korekcijo $\delta^{15}N$ vrednosti v primeru, da je izkoristek difuzije manjši od 100 %, pri čemer prihaja do izotopske frakcionacije, ki daje lažno nizke $\delta^{15}N$ vrednosti.

Ključne besede: anionski izmenjevalec, mikrodifuzija, ^{15}N, nitrat, stabilni izotopi, talna raztopina

Uvod

Poznavanje izotopske sestave anorganskega dušika v tleh je pomembno za razumevanje procesov dušikovega cikla.[1] Natančno določanje izotopske sestave amonijskega in nitratnega dušika (N) je pomembno za prepozanavanje ali modeliranje stopnje transformacij N *in situ*,[1,2] virov rastlini dostopnega N[3] ter interakcij mikoriza–rastlina.[1,4]

Za določanje izotopske sestave nitratnega dušika (izotopsko razmerje $^{15}N/^{14}N$ v nitratu) je potrebno nitrat najprej izolirati iz raztopine, ga skoncentrirati in pretvoriti v obliko, primerno za analizo na masnem spektrometru (MS). S tem postopkom je potrebno pridobiti zadostno količino N, ki je potrebna za zanesljivo meritev na MS, ne da bi pri tem prišlo do kontaminacije in frakcionacije.[5] Za rutinsko laboratorijsko delo so zelo zaželene metode, ki so natančne in zanesljive ter hkrati enostavne in poceni. Hitrost in učinkovitost določanja izotopske sestave nitratnega dušika v vodnih raztopinah je omejena predvsem s pripravo vzorca.[6]

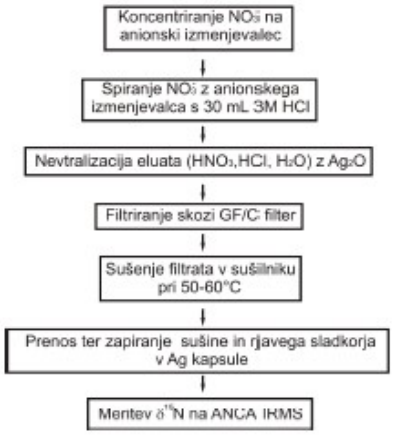
Namen našega dela je bil izmed treh različnih metod priprave vzorca najti najbolj primerno metodo za rutinsko laboratorijsko uporabo. V ta namen smo nitrat iz delovne ratzopine kalijevega nitrata izolirali z metodo z anionskim izmenjevalcem, ki so jo uvedli Silva in sodelavci[7] in modificirali Fukada in sodelavci[8], ter z metodama mikrodifuzije[6,9,10,11,12] – metodo s teflonsko pastjo (Teflon trap method) in metodo s kavljem (Hook method). Metodi mikrodifuzije smo primerjali tudi pri izolaciji nitratnega dušika iz realnih vzorcev talne raztopine, obogatenih s sledilcem ^{15}N ($K^{15}NO_3$; Shanghai Research Institute, Šanghaj, Kitajska). Analizirali smo 3 mL in 200 mL vzorce, ki so vsebovali 200–300 µg N. Izotopsko

sestavo nitratnega N smo določili z masnim spektrometrom Europa 20–20 z ANCA-SL preparativnim modulom za trdne in tekoče vzorce (ANCA IRMS).

Eksperimentalni del

Delovna raztopina in vzorci talne raztopine. Za pripravo delovne raztopine kalijevega nitrata smo uporabili ultra čisto destilirano vodo (Milli Q, Millipore Corporation, Bedford, MA, ZDA) in KNO_3 (Merck KGaA, Nemčija) z izotopsko sestavo oz. $\delta^{15}N$ vrednostjo 3,5±0,2‰. V polietilenske (HDPE) plastenke (difuzijske posodice) smo odpipetirali potrebno količino vzorca, tako, da je vsaka plastenka vsebovala 250 µg N v NO_3^- obliki (3 mL oz. 200 mL). Vzorci talne raztopine so bili odvzeti v okviru širšega poljskega poskusa z mladim zeljem, gnojenim z markiranim gnojilom, obogatenim s težkim dušikovim izotopom ^{15}N.[13] Vzorci talne raztopine so bili odvzeti na globini 40 cm s pomočjo keramičnih svečk (SDEC, Francija). Do izvedbe analiz so bili hranjeni pri –20 °C. Slepi vzorci so bili pripravljeni na enak način kot delovna raztopina, le da niso vsebovali merjenca. Pri metodi s teflonsko pastjo je bila uporabljena enaka količina KCl kot pri vzorcih. Pri vsakem poskusu je bil volumen vzorca, delovne raztopine in slepih vzorcev enak.

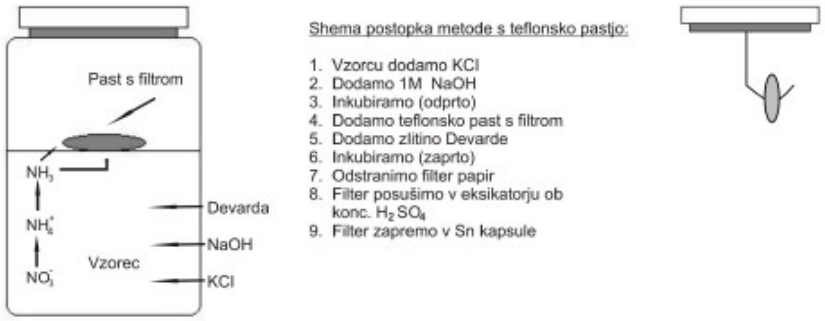
Metoda z anionskim izmenjevalcem. Nitrat smo iz raztopine skoncentrirali na anionski izmenjevalec (AG®1-X8 200-300 Mesh, Chloride form, BIO RAD, cat.#140-1441) in ga nato z izmenjevalca sprali s 30 mL 3 M HCl. Eluat smo nato v ledeni kopeli nevtralizirali z dodajanjem Ag_2O, pri čemer je nastal $AgNO_3$. Za nevtralizacijo posameznega vozrca smo porabili 10–13 g Ag_2O, ki smo ga v raztopino dodajali postopoma, da smo preprečili prekomerno segrevanje in izhlapevanje HNO_3 iz raztopine. Raztopino smo stalno mešali s stekleno palčko, da smo preprečili tvorbo skorje iz srebrovega klorida. Ko je reakcija potekla, se je raztopina vidno mlečno belo obarvala. Z mešanjem smo nadaljevali toliko časa, da je bela barva izginila in se je usedlina jasno ločila od raztopine. pH raztopine smo preverili s pH-papirčkom (pH = 5,5-6). Raztopino smo nato prefiltrirali (Whatman GF/C) in posušili v sušilniku pri 50–60 °C.[8] Potrebno količino nastale sušine smo na dan izvedbe meritev skupaj s trsnim sladkorjem (1:2,5; dodano za boljši sežig) zaprli v srebrove kapsule za analizo na ANCA IRMS (Slika 1).



Slika 1: Shematični prikaz metode z anionskim izmenjevalcem.

Metodi mikrodifuzije. Pripravo vzorcev za določanje izotopske sestave dušika v nitratu smo izvedli z dvema metodama mikrodifuzije, in sicer z metodo s teflonsko pastjo ter

metodo s kavljem. Metoda s kavljem ni primerna za volumne, večje od 3 mL, saj pri večjih volumnih prihaja do kopičenja vlage na filtru in s tem do spiranja amonija in kisline s filtra nazaj v raztopino. Zato smo vzorce talne raztopine po potrebi predkoncentrirali na 3 mL z izhlapevanjem v sušilniku pri 60 °C.[10] Vsem vzorcem (delovni raztopini, talni vodi in slepim vzorcem) smo z dodajanjem 1M NaOH (Acros Organics, Belgija, extra pure) dvignili pH >10. Vzorce talne raztopine smo pustili v odprtih difuzijskih posodicah stati čez noč (oz. 14 dni pri 200 mL vzorcih), da se je iz vzorcev odstranil amonij. Naslednji dan smo z dodatkom 200 mg zlitine Devarde (Merck KGaA, Nemčija) nitrat v vzorcih reducirali v amonij. Nastali amonij se je sprostil iz alkalnega medija in se skoncentriral na nakisan (10 µL 2,5 M $KHSO_4$; IAEA, Dunaj, Avstrija) filter papir (Schleicher & Schuell QF, narezan na manjše kroge s pomočjo navadnega luknjača za papir), ki smo ga bodisi namestili na kavelj iz nerjavečega jekla, pritrjen na pokrovček difuzijske posodice (metoda s kavljem), bodisi pustili, da prosto plava na vzorcu, tesno zaprt med dva PTFE teflonska trakova (metoda s teflonsko pastjo) (Slika 2). Pri metodi s teflonsko pastjo smo z dodatkom KCl (Sigma–Aldrich, Nemčija, p.a.) povečali ionsko moč raztopine in s tem preprečeli akumulacijo tekočine znotraj PTFE pasti. Vzorce smo zaprte inkubirali čez noč (3 mL) oz. 14 dni (200 mL) pri sobni T. Med inkubacijo smo vzorce dvakrat dnevno ročno pretresli. Po koncu inkubacije smo filtre odstranili, jih posušili v eksikatorju ob koncentrirani H_2SO_4 in jih na dan izvedbe meritev zaprli v kositrne kapsule za analizo na ANCA IRMS.



Slika 2: Shematski prikaz postopka mikrodifuzije nitrata z metodo s teflonsko pasjo (levo; prirejeno po Holmes in sod.[5]). Na desni strani slike je prikaz namestitve filtra pri metodi s kavljem.

Določanje izotopske sestave. Izotopsko sestavo dušika ($^{15}N/^{14}N$ razmerje) smo izmerili na masnem spektrometru Europa 20–20 (PDZ Europa Scientific) s preparativnim modulom za trdne in tekoče vzorce. Rezultati neobogatenih (»naravnih«) vzorcev so podani v obliki δ vrednosti in so izraženi v promilih (‰) glede na mednarodno dogovorjeni standard – zračni dušik, kot prikazuje Enačba 1:

$$\delta^{15}N_{vzorec}\,(‰) = \left(\frac{R_{vzorec}}{R_{standard}} - 1\right) \times 1000 \qquad (1)$$

kjer R predstavlja razmerje $^{15}N/^{14}N$, standard predstavlja zračni dušik z $^{15}N/^{14}N$ razmerjem 0.00368 in $\delta^{15}N$ vrednostjo 0 ‰. Rezultati ^{15}N obogatenih vzorcev so izraženi v atom % ^{15}N, kot prikazuje Enačba 2[14]:

$$\text{atom}\,\%\ ^{15}N = \left(\frac{R_{vzorec}}{R_{vzorec} + 1}\right) \times 100 \qquad (2).$$

Natančnost meritev (ponovljivost, obnovljivost) smo določili na podlagi dveletnih meritev certificiranih referenčnih materialov IAEA–N1 (+0,4‰), IAEA–N2 (+20,3‰), IAEA–

NO–3 (+4,7‰) in laboratorijskega referenčnega materiala europa–N (+2,5‰) za neobogatene vzorce ter IAEA–32 (0,432 atom %), IAEA–305B (0,488 atom %) in IAEA–311 (2,03–2,06 atom %) za ^{15}N obogatene vzorce. Ocenjena natančnost meritev je bila za neobogatene vzorce boljša od 0,2 ‰, medtem, ko je bila pri obogatenih vzorcih le–ta odvisna od stopnje obogatitve, vendar je bila vedno boljša od 0,006 atom % ^{15}N.

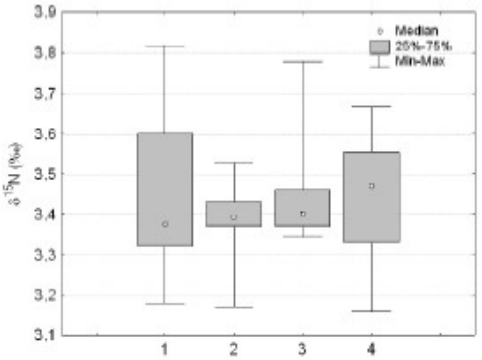
Rezultati so bili statistično obdelani s programom Statistica 6.0, enosmerna ANOVA, Post hoc LSD Fisher Test.

Rezultati in razprava

Med rezultati meritev $\delta^{15}N$ trdnega KNO_3, uporabljenega za pripravo delovne raztopine ter N, ki smo ga iz delovne raztopine (3 mL) izolirali z različnimi metodami, ni statističnih razlik ($p>0,01$) (Tabela 1 ter Slika 3).

Tabela 1: $\delta^{15}N$ (‰) izmerjen v trdnem KNO_3 in v nitratu, izoliranem iz delovne raztopine z različnimi metodami (3 mL vzorci).

Metoda	N	Srednja vrednost ± S.D.	minimum	maximum
KNO_3 (trden)	12	+3,4 ± 0,2	+3,2	+3,8
Mikrodifuzija (metoda s kavljem)	6	+3,5 ± 0,2	+3,3	+3,8
Mikrodifuzija (metoda s teflonsko pastjo)	6	+3,4 ± 0,1	+3,2	+3,5
Metoda z anionskim izmenjevalcem	12	+3,5 ± 0,2	+3,2	+3,7



Slika 3: $\delta^{15}N$ (‰) izmerjen v trdnem KNO_3 (1), v dušiku, izoliranem iz delovne raztopine z mikrodifuzijsko metodo s teflonsko pastjo (2), z difuzijsko metodo s kavljem (3) in z metodo z anionskim izmenjevalcem (4) (3 mL vzorci).

Rezultati kažejo, da imajo pri 3 mL vzorcih vse metode dober izkoristek in je vpliv reagentov, predvsem KCl in zlitine Devarde, ki lahko vsebujeta manjše primesi dušika, zanemarljiv, saj ni statistično značilnih razlik v $\delta^{15}N$ med trdnim (neobdelanim) KNO_3 in tistim, ki smo ga z različnimi metodami izolirali iz delovne KNO_3 raztopine. Stephan in Kavanagh[1] navajata, da je vpliv reagentov pri vzorcih, ki vsebujejo ≥30 µg $N–NO_3^-$, minimalen. Na masnem spektrometru, ki smo ga uporabili v okviru predstavljenega dela, pa je minimalna količina N za meritev izotopske sestave 200 µg N, kar pomeni, da je vpliv reagentov v našem primeru tudi pri večjih volumnih (200 mL), kjer je potrebno za dosego 1M KCl raztopine dodati več KCl, zanemarljiv.

Pri večjih volumnih lahko prihaja do nepopolne difuzije. Ker je lažji izotop ^{14}N bolj reaktiven v primerjavi s težjim izotopom ^{15}N, ^{14}N preferenčno difundira iz raztopine in se posledično tudi hitreje veže na filtrsko past – ob nepopolni difuziji zato prihaja do izotopske

frakcionacije, ki daje lažno nizke izmerjene δ^{15}N vrednosti. V skladu z ugotovitvami Holmesa in sodelavcev[5] tudi naši rezultati kažejo, da prihaja do frakcionacije in da je le–ta konsistentna med vzorci, ki so bili inkubirani pod istimi pogoji (Tabela 2), kar potrjuje, da je uvedba korekcijskega faktorja (Enačba 3), ki eliminira vpliv frakcionacije, nujno potrebna.

$$\delta^{15}N_{popravljena} \pm SD_{popravljena} = (I + X) \pm \sqrt{(SD^2_{standard} + SD^2_{vzorec})} \quad (3)$$

Tabela 2: Izračun korekcijskega faktorja na primeru standarda (200 mL vzorci).

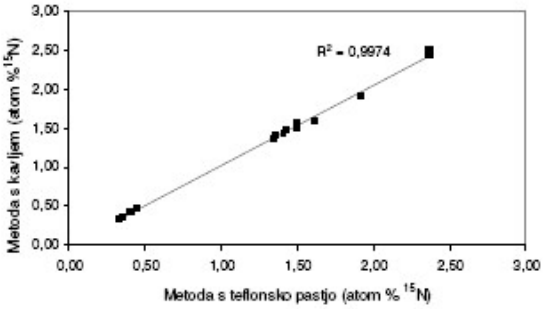
Ponovitev	δ^{15}N (‰)			
	Prava vrednost (P)	Izmerjena vrednost (I)	Frakcionacija (P – I)	Srednja vrednost ± SD (X ± SD)
1	+3,5	–5,5	9,0	9,2 ± 0,1
2	+3,5	–5,6	9,1	
3	+3,5	–5,7	9,2	
4	+3,5	–5,8	9,3	
5	+3,5	–5,8	9,3	

Primer uvedbe korekcijskega faktorja pri vzorcu z izmerjeno δ^{15}N vrednostjo –3,3 ± 0,4‰ (Enačba 4):

$$\delta^{15}N_{popravljena} \pm SD_{popravljena} = (-3,3 + 9,2) \pm \sqrt{(0,1^2 + 0,4^2_{vzorec})} = 5,9 \pm 0,4 \quad (4).$$

Stephan in Kavanagh[1] navajata, da napako v izmerjeni δ^{15}N vrednosti lahko enostavno korigiramo, kadar potrebna točnost in natančnost rezultata ni večja od 1,3‰. Po podatkih iz literature[5,10,15] je pri večjih volumnih z dvigom temperature inkubacije (65 °C) ter stresanjem vzorcev na stresalniku mogoče skrajšati čas difuzije ter izboljšati njen izkoristek.

Metodi mikrodifuzije smo primerjali tudi na vzorcih talne raztopine, obogatenih s sledilcem ^{15}N (0,34 – 2,51 atom % ^{15}N). Kot je razvidno iz Tabele 3 in Slike 4, sta uporabljeni metodi dali zelo skladne rezultate (R^2 = 0,9974). Ker je metoda s teflonsko pastjo primerna tudi za večje volumne (200 mL), predkoncentracija vzorca na 3 mL pri tej metodi ni potrebna. To pa pomeni krajši čas priprave vzorca in manjšo možnost kontaminacije vzorca med pripravo, zato je ta metoda bolj primerna za rutinsko laboratorijsko pripravo vzorcev za določanje izotopske sestave nitratnega dušika v vzorcih talne raztopine.



Slika 4: Primerjava rezultatov (atom % ^{15}N) v vzorcih talne raztopine po pripravi vzorcev z mikrodifuzijskima metodama (3 mL vzorci).

Tabela 3: Izmerjene atom % ^{15}N vrednosti v vzorcih talne raztopine po pripravi vzorcev z metodama mikrodifuzije (3 mL vzorci).

Vzorec	Metoda s teflonsko pastjo (atom % ^{15}N)	Metoda s kavljem (atom % ^{15}N)	Razlika (%)
1	0,337	0,345	2,4
2	0,351	0,354	8,5
3	0,398	0,414	4,0
4	0,417	0,428	2,6
5	0,447	0,458	2,5
6	1,344	1,361	1,3
7	1,360	1,415	4,0
8	1,409	1,424	1,1
9	1,429	1,469	2,8
10	1,496	1,573	5,1
11	1,497	1,521	1,6
12	1,500	1,488	0,8
13	1,610	1,582	1,7
14	1,919	1,919	0,0
15	2,366	2,513	6,2
16	2,370	2,435	2,7

Zaključek

Zaradi visoke cene Ag_2O, dolgotrajnega in zahtevnega postopka, se je metoda z anionskim izmenjevalcem izkazala za manj primerno v primerjavi z metodama mikrodifuzije. Kot najbolj primerna metoda se je izkazala mikrodifuzijska metoda s teflonsko pastjo. Ker pri tej metodi pri večjih volumnih prihaja do nepopolne difuzije, smo uvedli korekcijski faktor, ki omogoča zanesljivo korekcijo δ^{15}N vrednosti v primeru, da je izkoristek difuzije manjši od 100 %, pri čemer prihaja do izotopske frakcionacije, ki daje lažno nizke δ^{15}N vrednosti. Korekcija pri vzorcih, obogatenih s sledilcem ^{15}N, ni potrebna. Pri naravnih (neobogatenih) vzorcih pa na podlagi pridobljenih rezultatov ter podatkov iz literature svetujemo vključitev delovne raztopine z znano izotopsko sestavo (δ^{15}N) v vsako serijo vzorcev in korekcijo rezultatov glede na frakcionacijo, izračunano za uporabljeno delovno raztopino.

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Abstract. In order to find the most appropriate method for routine laboratory use, i.e. accurate, reproducible, as well as fast and non–expensive method for sample preparation for the determination of soil water nitrate nitrogen isotopic composition, different methods of nitrate nitrogen isolation were compared. Results of nitrate nitrogen isotopic composition after an anion exchange method and two microdiffusion methods, i.e. the Hook method and the Teflon trap method, were compared, analyzing a KNO_3 standard solution at a natural ^{15}N abundance level as well as soil water samples at ^{15}N enriched level. All three methods gave comparable and satisfactory results. However, due to high costs of Ag_2O, laborious and time consuming procedure, the anion exchange method was found to be less appropriate as compared to the microdiffusion methods. The Teflon trap method, on the other hand, was found to be the most appropriate among the tested methods. However, at larger sample volumes, incomplete diffusion was observed, which resulted in isotopic fractionation, causing too low $\delta^{15}N$ determinations. Hence a correction factor was proposed, which allows precise correction of measured $\delta^{15}N$ values for incomplete recoveries.

Appendix 12: Article: Isotopic composition in organic and conventionally grown vegetables available on the Slovenian market. *Proceedings of symposium, New challenges in field crop production 2010*, 232-238.

Izotopska sestava dušika v ekološko in konvencionalno pridelani zelenjavi na slovenskem trgu

Martina ŠTURM[87], Sonja LOJEN[2]

Izvleček
Namen opravljene raziskave je bil pridobiti podatke o izotopski sestavi dušika ($\delta^{15}N$) v ekološko ter konvencionalno pridelani zelenjavi v prodaji na slovenskem trgu ter ugotoviti ali obstajajo sistematične razlike v $\delta^{15}N$ vrednostih, ki bi lahko služile kot orodje za ugotovljanje nepravilnega označevanja ekoloških pridelkov. V ta namen je bilo analiziranih štirinajst različnih vrst ekološko in konvencionalno pridelane zelenjave. Poleg tega je bila določena tudi izotopska sestava dušika v sedmih sintetičnih in štirih organskih gnojilih. Rezultati analiziranih vzorcev zelenjave kažejo jasne razlike v srednjih $\delta^{15}N$ vrednostih med ekološko in konvencionalno pridelano zelenjavo (do 6,3 ‰). Kljub razlikam v srednjih $\delta^{15}N$ vrednostih pa kar pri osmih (cvetača, paradižnik, česen, čebula, rumena koleraba, peteršilj, paprika in korenček) od štirinajstih analiziranih vrst zelenjave zaradi prekrivanja podatkov na podlagi $\delta^{15}N$ vrednosti ni bilo mogoče ločiti med ekološko oziroma konvencionalno pridelavo. Pri šestih vrstah zelenjave (endivija, rukola, radič v tipih "Palla rosa" in "Pan di zucherro", por in krompir) pa se je $\delta^{15}N$ zapis v zelenjavi izkazal kot hitro in poceni orodje za kontrolo ekološke pridelave. Rezultati kažejo, da se $\delta^{15}N$ vrednosti lahko uporabljajo le kot dodatno, ne pa tudi kot edino orodje za nadzor ekološke pridelave zelenjave.
Ključne besede: ekološka pridelava, dušik, stabilni izotopi, ^{15}N

Isotopic composition of nitrogen in organically and conventionally grown vegetables available on the Slovenian market

Abstract
The aim of the study was to obtain data on the isotopic composition of nitrogen ($\delta^{15}N$) in organically and conventionally grown vegetables available on the Slovenian market to determine whether there are any systematic differences in $\delta^{15}N$ values, which could serve as a screening tool to differentiate between organically and conventionally grown produce. In order to do so, fourteen different varieties of organically and conventionally grown vegetables from Slovenian market were analyzed. In addition, the isotopic composition of nitrogen in seven synthetic and four organic fertilizers was also determined. Results show clear differences in mean $\delta^{15}N$ values between organic and conventionally grown vegetables (up to 6.3 ‰). However, due to overlapping results, despite the differences in mean $\delta^{15}N$ values, it was not possible to differentiate between organically and conventionally grown counterparts in eight (cauliflower, tomato, garlic, onion, kohlrabi, parsley, sweet pepper and carrot) out of fourteen vegetable varieties. Nevertheless, in six vegetable varieties (endive, rocket, chicory in type "Palla rosa" and "Pan di zucherro", leek, and potato) $\delta^{15}N$ values proved to be a fast and relatively cheap screening tool to differentiate between organically and conventionally grown produce. Results obtained suggest that $\delta^{15}N$ could be used as a marker of organic production but only as supporting, additional marker and not as the unequivocal marker of organic vegetable production.
Key words: organic production, nitrogen, stabile isotopes, ^{15}N

[87] Univ. dipl. inž. geol., Institut "Jožef Stefan", Odsek za znanosti o okolju, Jamova cesta 39, SI-1000 Ljubljana, e-pošta: martina.sturm@ijs.si
[2] Doc. dr., Institut "Jožef Stefan", Odsek za znanosti o okolju, Jamova cesta 39, SI-1000 Ljubljana, e-pošta: sonja.lojen@ij.si

1 Uvod

Ekološko kmetijstvo je v zadnjih letih doživelo precejšen porast. Leta 2000 je bilo v Sloveniji registriranih 115 kmetijskih gospodarstev z ekološkim kmetovanjem, leta 2009 pa že 1.853 (SURS, 2010). Ker ekološko pridelana zelenjava na trgu dosega višje cene v primerjavi s tisto, pridelano na konvencionalen način (Bateman in sod., 2007), obstaja nevarnost goljufij oziroma nepravilnega označevanja konvencionalno pridelane zelenjave z oznako "ekološki", zato se pojavlja potreba po metodah za preverjanje avtentičnosti ekološke pridelave. Uporaba stabilnih izotopov ogljika, vodika in kisika kot indikatorjev avtentičnosti hrane je znana in uveljavljena praksa (Krueger in Reesman, 1982; Rossmann, 2001; Jamin in Wietzerbin, 2003; Rogers, 2008). Nekoliko manj znana in raziskana pa je uporaba stabilnih izotopov dušika pri kontroli uporabe sintetičnih gnojil v ekološki pridelavi. Možnost uporabe izotopov dušika temelji na predpostavki, da imajo konvencionalno pridelane rastline nižjo izotopsko sestavo (nižje $\delta^{15}N$ vrednosti oz. $^{15}N/^{14}N$ razmerje, t.j. razmerje težjega proti lažjemu stabilnemu izotopu dušika) v primerjavi z istimi rastlinami, gnojenimi z organskim gnojilom, saj imajo zaradi različnih procesov izdelave gnojil sintetična gnojila nižje $\delta^{15}N$ vrednosti od organskih (Bateman in Kelly, 2007).

Sintetična gnojila se izdelujejo iz zračnega dušika z $\delta^{15}N$ vrednostjo 0 ‰. Ker med procesom izdelave ne prihaja do večje frakcionacije, je njihova $\delta^{15}N$ vrednost blizu 0 ‰. Živali se prehranjujejo z rastlinami, katerih $\delta^{15}N$ odraža $\delta^{15}N$ v tleh, na katerih so uspevale (izjema so N–fiksatorske rastline, ki imajo zaradi vezave zračnega dušika $\delta^{15}N$ vrednosti podobne zračnemu dušiku). Živali pri izločanju z urinom prednostno izločijo lažji izotop dušika (^{14}N), trdni živalski izločki zato vsebujejo več težjega izotopa (^{15}N), poleg tega pa so zaradi izhlapevanja amonija, denitrifikacije in bakterijskega delovanja podvrženi še dodatni obogatitvi s težjim dušikovim izotopom (Kendall, 1998; Rogers, 2008), saj se pri vseh procesih lažji izotop prednostno porablja, preostali trdni izločki pa posledično postanejo obogateni s težjim izotopom. Po podatkih iz literature (Kreitler, 1979; Kendall, 1998; Bateman in sod., 2007) lahko nitrat, nastal iz trdnih živalskih izločkov z $\delta^{15}N$ vrednostjo +5 ‰, zaradi omenjenih procesov doseže $\delta^{15}N$ vrednosti med +10 in +30 ‰.

Večina raziskav, pri karerih so proučevali možnost uporabe izotopske sestave dušika pri kontroli ekološke pridelave, je temeljila na nadzorovanih laboratorijskih poskusih (Choi in sod., 2002; Nakano in sod., 2003; Bateman in sod., 2005; Yun in sod., 2006; del Amor in Navarro, 2008; Šturm in sod., 2010), manj pa na komercialno dostopni ekološko in konvencionalno gojeni zelenjavi (Bateman in sod., 2007; Rogers, 2008; IEH Lab., 2010). Najboljšo oceno primernosti uporabe $\delta^{15}N$ kot orodja za ločevanje med ekološko in konvencionalno pridelano zelenjavo pa lahko dobimo prav z analizo avtentičnih komercialno dostopnih pridelkov, zajetih iz širokega geografskega območja, s širokim razponom okoljskih razmer in kmetijskih praks (Bateman and Kelly, 2007). Na ta način, za razliko od nadzorovanih laboratorijskih poskusov, upoštevamo tudi veliko drugih dejavnikov, ki lahko vplivajo na $\delta^{15}N$ vrednosti pridelka, kot npr. razlike v talnih tipih, razlike v količini zračno odloženega N in razlike v lokalnih kmetijskih praksah.

Namen opravljene raziskave je bil pridobiti podatke o izotopski sestavi dušika v ekološko ter konvencionalno pridelani zelenjavi v prodaji na slovenskem trgu ter ugotoviti ali obstajajo sistematične razlike v $\delta^{15}N$ vrednostih, ki bi lahko služile kot orodje za ugotovljanje nepravilnega označevanja ekoloških pridelkov.

2 Material in metode dela

Za potrebe raziskave smo izbrali štirinajst različnih vrst ekološko in konvencionalno pridelane zelenjave v ponudbi na slovenskem trgu ter jim določili izotopsko sestavo dušika. Pri izbiri ekološko pridelane zelenjave iz različnih geografskih območij so imeli prednost certificirani vzorci (oznaka »ekološki«). Vzorci konvencionalno pridelane zelenjave so bili kupljeni v maloprodaji pri različnih trgovskih ponudnikih. Za vsako posamezno vrsto zelenjave ter način pridelave smo analizirali 3–6 vzorcev. Dodatno smo analizirali tudi vzorce organskih gnojil (hlevski gnoj in kompost ter komercialni gnojili za ekološko pridelavo Biogrena in Valentin naravno organsko gnojilo) ter vzorce različnih sintetičnih (anorganskih) dušikovih gnojil (N:P:K, KAN, KNO_3, $Ca(NO_3)_2$ ter $MgNO_3$), ki se v Sloveniji pogosto uporabljajo.
Vzorce zelenjave smo oprali z ultra čisto destilirano vodo (Mili–Q), narezali na manjše dele ter jih posušili v sušilniku pri 60 °C do konstantne mase. Posušene vzorce smo homogenizirali med mletjem v droben prah v terilnici. Za analizo izotopske sestave smo v kositrne kapsule natehtali 8–11 mg posameznega uprašenega vzorca, kapsule zaprli in oblikovali v kroglico. Organska gnojila (hlevski gnoj, kompost) smo zračno posušili. Vzorce organskih in sintetičnih gnojil smo homogenizirali med mletjem v terilnici, potrebno količino posameznega gnojila smo natehtali in zaprli v kositrne kapsule.
Izotopsko sestavo dušika ($^{15}N/^{14}N$) smo izmerili na masnem spektrometru Europa 20–20 (PDZ Europa Scientific) s preparativnim modulom za trdne in tekoče vzorce (ANCA–SL). Rezultati so podani v obliki δ vrednosti in so izraženi v promilih (‰) kot relativne vrednosti glede na mednarodno dogovorjeni standard – zračni dušik, kot prikazuje spodnja enačba:

$$\delta^{15}N_{vzorec}\ (‰) = \left(\frac{R_{vzorec}}{R_{standard}} - 1\right) \times 1000$$

kjer R predstavlja razmerje $^{15}N/^{14}N$, standard predstavlja zračni dušik z $^{15}N/^{14}N$ razmerjem 0,00368 in $\delta^{15}N$ vrednostjo 0 ‰. Vsi vzorci so bili analizirani v dveh ponovitvah. Natančnost meritev (ponovljivost, obnovljivost) smo določili na podlagi meritev certificiranih referenčnih materialov USGS34 (–1,8‰), IAEA–N2 (+20,3‰) in laboratorijskih referenčnih materialov europa–N (+2,5‰) ter Lucerna (–0,6‰), in sicer na ±0,2 ‰. Točnost meritev $\delta^{15}N$ je bila potrjena z uspešnim sodelovanjem v mednarodnih medlaboratorijskih primerjavah (WEPAL Interlaboratory Plant–Analytical Exchange Programme, IPE2009.2, IPE2010.2).

3 Rezultati z diskusijo

3.1 IZOTOPSKA SESTAVA DUŠIKA V GNOJILIH

Organska gnojila, ki so dovoljena v ekološki pridelavi, imajo zaradi zelo različnega izvora (kompost, hlevski gnoj, rogovi, parklji, kostna moka, ostanki rib, morska trava, kri, tropine) večji razpon $\delta^{15}N$ vrednosti od sintetičnih (Bateman in sod., 2007). Povprečna izotopska sestava ter razpon sintetičnih in organskih gnojil, analiziranih v okviru prikazane raziskave, sta podana v preglednici 1, skupaj s podatki iz literature. Organska gnojila, analizirana v okviru naše raziskave, imajo višje $\delta^{15}N$ vrednosti v primerjavi z analiziranimi sintetičnimi gnojili. Pridobljeni rezultati so primerljivi s podatki iz literature (preglednica 1).
$\delta^{15}N$ vrednosti celotnega dušika v tleh se gibljejo med +2 in +5 ‰ (Kendall, 1998), z mediano +5 ‰ (Amundson in Baisden, 2000; Rogers, 2008). $\delta^{15}N$ vrednost talnega dušika je odvisna od rastlin, ki na njih uspevajo, od okoljskih razmer (npr. klima) (Amundson in Baisden, 2000; Rogers, 2008) ter od vrste uporabljenih gnojil. Uporaba sintetičnih gnojil zaradi mešanja med dušikom iz gnojila in dušikom iz tal povzroči znižanje $\delta^{15}N$ celotnega dušika v tleh, medtem

ko uporaba organskih gnojil povzroči dvig $\delta^{15}N$ vrednosti celotnega dušika v tleh (Rogers, 2008), kar se odraža tudi v rastlinah, ki na teh tleh uspevajo.

3.2 IZOTOPSKA SESTAVA DUŠIKA V ZELENJAVI

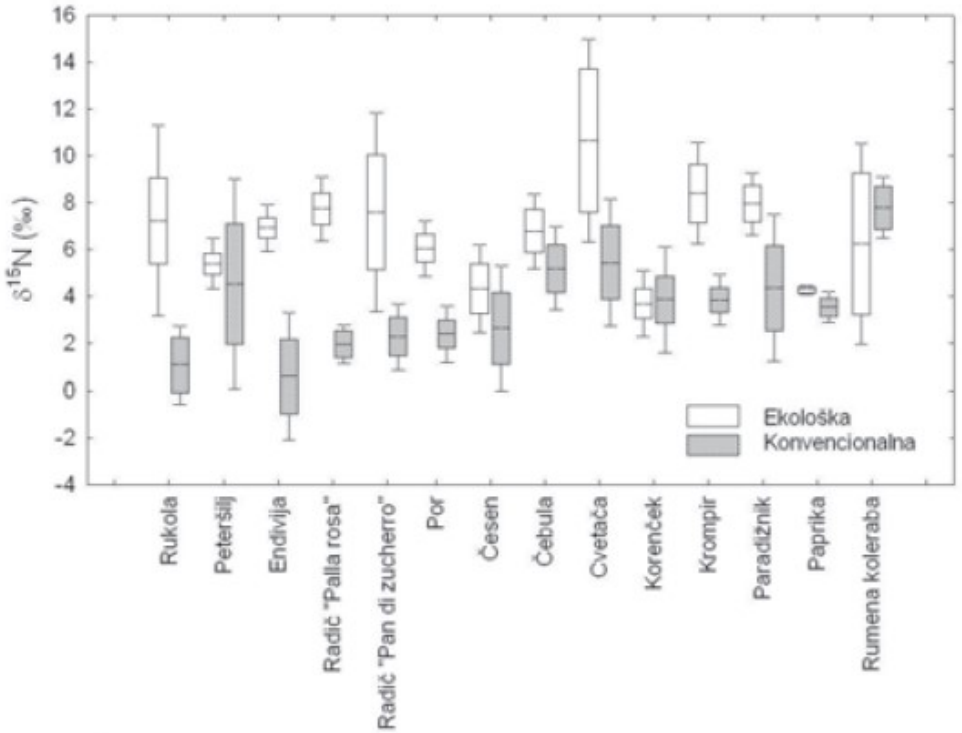
Izotopska sestava dušika v analiziranih vzorcih zelenjave je prikazana na sliki 1. Ekološko pridelana zelenjava je imela med 0,8 in 6,3 ‰ višje srednje $\delta^{15}N$ vrednosti v primerjavi s konvencionalno pridelano zelenjavo iste vrste. Izjema je korenček, kjer so se $\delta^{15}N$ vrednosti ekološko in konvencionalno pridelanih vzorcev skoraj popolnoma prekrivale, srednja vrednost ekološko pridelanega korenčka (razpon $\delta^{15}N$ vrednosti med 2,1 in 5,8 ‰) je bila celo za 0,2 ‰ nižja od srednje vrednosti konvencionalno pridelanega korenčka (razpon med 3,0 in 7,7 ‰). Ti rezultati so v skladu z ugotovitvami Bateman in sod. (2007), ki so določili razpon $\delta^{15}N$ vrednosti pri ekološko pridelanem korenčku med 0,7 in 11,2‰ in med 1,0 in 9,1‰ pri konvencionalno pridelanem korenčku. Kot verjetna vzroka za tako prekrivanje $\delta^{15}N$ vrednosti omenjeni avtorji navajajo nizko potrebo korenčka po dušiku ter običajno prakso, da se v ekološki pridelavi sejanje korenčka ne izvaja takoj po gnojenju s hlevskim gnojem, saj to povečuje verjetnost nepravilnega razvoja koreninskega sistema (Bateman in sod., 2007).

Preglednica 5: Izotopska sestava dušika ($\delta^{15}N$ v ‰) v različnih vrstah gnojil

Vrsta gnojila	Srednja vrednost	Min.	Maks.	Število vzorcev	Reference
Sintetično	+3,0	–0,9	+5,8	7	Šturm in Lojen, 2010
	–0,2	–5,9	+6,6	29	Bateman in Kelly, 2007
	+0,4	–5,9	+2,8	44	IEH Lab.[1], 2010
	–1,6	–1,2	–1,7	12	Rogers, 2008
	0,2	–1,7	+3,9	22	Vitoria in sod., 2004
	/	–4,0	+4,0	/	Kendall, 1998
Organsko	9,5	6,2	+14,8	4	Šturm in Lojen, 2010
	/	+2,0	+30,0	/	Kendall, 1998
	+8,1	+3,5	+16,2	11	Bateman in Kelly, 2007
	+8,3	+4,4	+26,7	9	IEH Lab., 2010
	+6,3	+2.7	+11,3	12	Rogers, 2008

Na podlagi izmerjenih $\delta^{15}N$ vrednosti je bilo mogoče ločiti med ekološko in konvencionalno pridelano envidijo (razlika v srednjih $\delta^{15}N$ vrednostih med ekološko in konvencionalno pridelanimi vzorci: $\Delta\ \delta^{15}N_{ekol.-konv.} = 6,3$ ‰), rukolo ($\Delta\ \delta^{15}N_{ekol.-konv.} = 6,1$ ‰), radičem v tipu "Palla rosa" ($\Delta\ \delta^{15}N_{ekol.-konv.} = 5,7$ ‰), porom ($\Delta\ \delta^{15}N_{ekol.-konv.} = 5,4$ ‰), krompirjem ($\Delta\ \delta^{15}N_{ekol.-konv.} = 4,6$ ‰) ter radičem v tipu "Pan di zucherro" ($\Delta\ \delta^{15}N_{ekol.-konv.} = 3,0$ ‰). Pri osmih od štirinajstih analiziranih vrst zelenjave, t.j. pri cvetači ($\Delta\ \delta^{15}N_{ekol.-konv.} = 5,2$ ‰), paradižniku ($\Delta\ \delta^{15}N_{ekol.-konv.} = 3,6$ ‰), česnu ($\Delta\ \delta^{15}N_{ekol.-konv.} = 1,7$ ‰), čebuli ($\Delta\ \delta^{15}N_{ekol.-konv.} = 1,6$ ‰), rumeni kolerabi ($\Delta\ \delta^{15}N_{ekol.-konv.} = 1,6$ ‰), peteršilju ($\Delta\ \delta^{15}N_{ekol.-konv.} = 0,9$ ‰), papriki ($\Delta\ \delta^{15}N_{ekol.-konv.} = 0,8$ ‰) ter korenčku ($\Delta\ \delta^{15}N_{ekol.-konv.} = 0,2$), pa kljub razlikam v srednjih $\delta^{15}N_{ekol.-konv.}$ vrednostih ni bilo mogoče zanesljivo ločiti med ekološko in konvencionalno pridelanimi vzorci, saj se njihove $\delta^{15}N$ vrednosti delno prekrivajo (slika 1). O podobnem prekrivanju podatkov pri določenih vrstah zelenjave poroča tudi študija, ki so jo opravili v IEH Laboratories and Consulting Groups (2010), ki so analizirali ekološko in konvencionalno pridelano zelenjavo, dostopno na trgu v Kanadi in Britanski Kolumbiji. Rogers (2008), ki je proučeval razlike v $\delta^{15}N$ vrednostih med ekološko in konvencionalno pridelano zelenjavo na trgu v Novi Zelandiji, pa na drugi strani poroča o jasnih razlikah v srednjih $\delta^{15}N$ vrednostih

med vsemi analiziranimi ekološkimi in konvencionalnimi pridelki (krompir, čebula, bučke, paradižnik, koruza, jajčevec, idr.), z izjemo graha, ki spada med N–fiksatorske rastline. Pomankljivost njihove raziskave pa je v tem, da so podane samo srednje $\delta^{15}N$ vrednosti, ne pa tudi celoten razpon vrednosti, zato neposredna primerjava podatkov ni ustrezna.
Prekrivanje $\delta^{15}N$ vrednosti pri ekološko in konvencionalno gojenih pridelkih pa ne pomeni nujno napačnega označevanja pridelkov. Nizke $\delta^{15}N$ vrednosti pri pridelkih, ki naj bi bili pridelani ekološko, niso nujno odraz uporabe sintetičnih N gnojil, ampak so lahko tudi posledica vključevanja N–fiksatorskih rastlin (npr. fižola) v kolobar. Za N–fiksatorske rastline je značilno, da preko mikorize v tla vežejo zračni dušik ($\delta^{15}N_{zrak}$ = 0 ‰), kar povzroči znižanje $\delta^{15}N$ celotnega dušika v tleh in posledično v rastlinah, ki bodo na teh tleh uspevale. Podoben vpliv lahko pričakujemo tudi zaradi gnojenja z rastlinskimi ostanki N–fiksatorskih rastlin, vendar ti vplivi še niso dobro raziskani. Na drugi strani pa lahko konvencionalni pridelovalec poleg sintetičnih N gnojil uporablja katero koli organsko gnojilo. V tem primeru lahko tudi pri konvencionalno gojeni zelenjavi določimo $\delta^{15}N$ vrednosti, značilne za ekološke pridelke (Bateman in sod., 2005).



Legenda: Črta – srednja vrednost; (□) srednja vrednost ± standardna napaka; (I) srednja vrednost ± standardni odklon.

Slika 1: Izotopska sestava dušika ($\delta^{15}N$ v ‰) v konvencionalno in ekološko pridelani zelenjavi v ponudbi na slovenskem trgu.

4 Sklepi

Z uporabo izotopske masne spektrometrije stabilnih izotopov smo zaradi razlik v $\delta^{15}N$ vrednostih med sintetičnimi in organskimi gnojili lahko pri šestih (endivija, rukola, radič v tipih "Palla rosa" in "Pan di zucherro", por in krompir) od štirinajstih analiziranih vrst zelenjave ločili med ekološko in konvencionalno pridelano zelenjavo, pri osmih vrstah zelenjave (cvetača, paradižnik, česen, čebula, rumena koleraba, peteršilj, paprika in korenček) pa so se $\delta^{15}N$ vrednosti ekološko in konvencionalno pridelanih vzorcev med seboj prekrivale. Ekološko pridelana zelenjava je, z izjemo korenčka, imela višje srednje $\delta^{15}N$ vrednosti v primerjavi s konvencionalno pridelano zelenjavo iste vrste. Na omejenem številu analiziranih vzorcev se je $\delta^{15}N$ zapis tako pri določenih vrstah rastlin izkazal kot hiter in relativno poceni indikator gnojenja s sintetičnim dušikovim gnojilom. Pri tem pa je potrebno poudariti, da z uporabljeno metodo lahko nadzorujemo samo vrsto uporabljenega gnojila, ne pa tudi izpolnjevanja ostalih pogojev, ki jih zahteva ekološka pridelava (prepoved uporabe pesticidov, gensko spremenjenih organizmov, itd.). Pri interpretaciji rezultatov je treba upoštevati še nekatere dodatne omejitve uporabe $\delta^{15}N$ zapisa v zelenjavi pri kontroli ekološke pridelave, kot na primer vpliv vključevanja N–fiksatorskih rastlin v kolobar ter gnojenje z rastlinskimi ostanki N–fiksatorskih rastlin. Poleg tega so predhodne študije pokazale, da predstavljena metoda ni primerna za ločevanje ekološko in konvencionalno gojenih N–fiksatorskih rastlin (Rogers, 2008) ter je premalo občutljiva za zanesljivo ugotavljanje kombinirane uporabe organskih in sintetičnih gnojil (Šturm in sod., 2010). Na podlagi naštetega lahko zaključimo, da se $\delta^{15}N$ zapis lahko uporablja le kot dodatno, ne pa kot edino orodje za kontrolo ekološke pridelave zelenjave.

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OUTLOOK

Briefly, the strategies regarding the environment management and protection in Romania should include 3 project categories, namely:

1. Projects regarding the rational use ofnon-renewable resources necessary in industry.

2. Projects regarding the rational use of renewable resources aimed at developing agro-ecosystems and at their rational use, in the sense of creating a sustainable development starting from the development of agriculture and the rural space.

3. Projects regarding the rational use of thetrained workforce.

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THE SUITABILITY OF NITROGEN ISOTOPIC FINGERPRINT IN LETTUCE AS AN INDICATOR OF FERTILIZATION REGIME

Martina Sturm[1], Nina Kacjan-Marsic[2], Sonja Lojen[1]

[1]Jozef Stefan Institute, Department of Environmental Sciences, Slovenia. Martina.Sturm@ijs.si, Sonja.Lojen@ijs.si

[2]University of Ljubljana, Biotechnical Faculty,Agronomy Department, Ljubljana, Slovenia, Nina.Kacjan.Marsic@bf.uni-lj.si

ABSTRACT

The use of nitrogen isotopic fingerprint ($\delta^{15}N$) in lettuce as a potential marker for identifying organic produce was tested on pot grown lettuce (*Lactuca sativa* L.), fertilized with synthetic and/or organic nitrogen fertilizer (single or split application). The study was based on the hypothesis that conventionally grown crops have significantly lower $\delta^{15}N$ values compared to those grown organically, since synthetic fertilizers have lower $\delta^{15}N$ values compared to organic fertilizers due to different fertilizer production processes. The $\delta^{15}N$ values of plants treated with different fertilizer differed significantly when fertilizer was applied in a single application. However, additional fertilization did not cause significant alteration of plant $\delta^{15}N$. Obtained results indicate that $\delta^{15}N$ of lettuce tissues could be used as a marker to reveal the history of nitrogen fertilization, but only in thecase of a single fertilizer application.

Keywords: nitrogen, stable isotopes, fertilization, organic produce, *Lactuca sativa* L.

INTRODUCTION

Organic products attain high prices on themarket hence there are concerns among users about mislabelling conventionally grown crops as "organic". The possible use of nitrogen isotopes to differentiate between crops grown with or without inputs of synthetic nitrogen is based on the hypothesis that the application of synthetic nitrogen (N) fertilizers with $\delta^{15}N$ values close to 0‰ will result in the $\delta^{15}N$ of plants grown in conventional regimes being lower than those in organic regimes (+10 to +20‰) due to different fertilizer production processes [1]. The aim of presented study was to test weather N fertilizer type and timing of fertilizer application leave specific $\delta^{15}N$ fingerprint in lettuce tissues (*Lactuca sativa* L.) which could be used as a potential marker to reveal the use of prohibited use of synthetic N fertilizers in organic farming. The effect of split N fertilization with combined usage of synthetic and organic fertilization, which might enable farmers to cover up the use of synthetic fertilizers, on plant $\delta^{15}N$ was also studied.

MATERIALS AND METHODS

A greenhouse pot experiment with lettuce (*Lactuca sativa* L.) was performed at the Biotechnical Faculty of Ljubljana, Slovenia. Natural commercial organic fertilizer with $\delta^{15}N$ of +14.8 ‰ was used as organic input and $Ca(NO_3)_2$ with $\delta^{15}N$ of +5.7 ‰ was used as synthetic fertilizer. Lettuce was sown into plug trays, containing Klasman tray substrate, individually transplanted into pots (7.5 kg of sandy loam soil) 35 days later and grown for 50 days. Seven treatments were applied in a completely randomized factorial design: a single basal organic fertilization of 40 mg N kg^{-1} soil (Organic), a single basal synthetic fertilization of 40 mg N kg^{-1} soil (Synthetic), a basal synthetic fertilization of 20 mg N kg^{-1} soil followed by an additional organic fertilization of 20 mg N kg^{-1} soil (Synth.+Org.), a

basal organic fertilization of 20 mg N kg^{-1} soil followed by an additional synthetic fertilization of 20 mg N kg^{-1} soil (Org.+Synth.), a basal synthetic fertilization of 20 mg N kg^{-1} soil followed by an additional synthetic fertilization of 20 mg N kg^{-1} soil (Synth.+Synth.), a basal organic fertilization of 20 mg N kg^{-1} soil followed by an additional organic fertilization of 20 mg N kg^{-1} soil (Org.+Org.), and unfertilized control. Additional application of N fertilizers was performed after sampling at 30 days after transplanting (DAT). Aboveground lettuce was destructively sampled at 20, 30 and 50 DAT. Samples were dried at 60°C, ground to fine powder, homogenized and weighed into tin cups for $\delta^{15}N$ determination using a PDZ Europa ANCA-SL elemental analyzer linked to a 20:20 continuous flow IRMS. The accuracy was checked with certified reference materials: USGS 34, IAEA-N-22 and in-house plant reference material. All samples were analysed in duplicate and $\delta^{15}N$ values were accepted when sample standard deviation was ≤0.2 ‰. Results are reported in δ-notation in units of permil (‰) with respect to atmospheric nitrogen (air) according to the Equation 1:

$$\ln \mathrm{Yield}_R = (\alpha_0 + \alpha_1 t) + \sum_j \beta_j \ln X_{jR} + w \ln C \qquad (1)$$

where R denotes $^{15}N/^{14}N$ and the standard denotes atmospheric nitrogen with a $\delta^{15}N$ value of 0 ‰. Data were verified statistically with the Factorial ANOVA using the Statistica 6.0 package. Significant differences are given at the 95 % level.

RESULTS AND DISCUSSION

The $\delta^{15}N$ of plants receiving organic fertilizer (single or split application) were significantly higher compared to the treatments with different N sources (i.e. synthetic fertilizer and soil), reflecting the higher $\delta^{15}N$ values of organic fertilizer-N (14.8‰) compared to that of synthetic fertilizer-N (5.7‰) and total soil-N (6.4‰) (Fig. 1). At final harvest, $\delta^{15}N$ of plants receiving single application of organic fertilizer was 9.6‰, and $\delta^{15}N$ of plants receiving split applications were 8.0‰ and 7.2‰ for Org.+Org. and Org.+Synth. Treatments, respectively. $\delta^{15}N$ of plants receiving single application of synthetic fertilizer was 5.3‰, whereas $\delta^{15}N$ of those receiving split applications were 5.2‰ and 6.0‰ for Synth.+Synth. and Synth.+Org. treatments, respectively. However, lettuce fertilized with synthetic fertilizer (single or split application) were significantly depleted with ^{15}N compared to unfertilized control plants (with $\delta^{15}N$=7.2‰), indicating that nitrogen derived from the synthetic fertilizer was so abundant in the soil that plants predominantly assimilated N from synthetic fertilizer over soil-N [2].

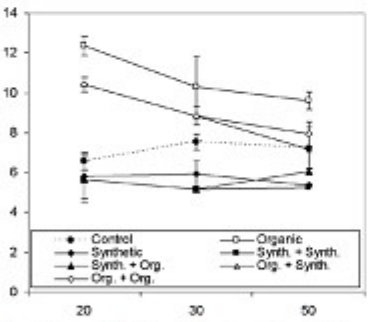

Figure 1: Lettuce $\delta^{15}N$ under different treatments at 20, 30 and 50 days after transplanting (DAT). Data are means ± SD for n = 3 plants per treatment.

Significantly higher $\delta^{15}N$ values were found in lettuce receiving one time application of organic fertilizer compared to those receiving split application, reflecting the proportionally greater contribution of organic fertilizer-N to total plant-N in the single application [2]. In contrast, no significant difference in $\delta^{15}N$ was found between lettuces receiving synthetic fertilizer as a single or split application. Additional fertilization did not cause significant alteration of plant $\delta^{15}N$, neither when isotopically similar (Org.+Org., Synth.+Synth.) nor when isotopically different (Org.+Synth., Synth.+Org.) additional N sources were applied. Decreasing of plant $\delta^{15}N$ with time was found in organically fertilized plants (single and split application with basal organic fertilization), which indicates increased contribution of soil-N to plant-N with time [2] [3] [4]. Decreasing of $\delta^{15}N$ with time in the treatment with basal organic and additional synthetic fertilization additionally indicates also the contribution of synthetic fertilizer-N. The $\delta^{15}N$ of plants treated with synthetic fertilizer on the other hand was relatively constant and indicated the $\delta^{15}N$ of synthetic fertilizer-N during the whole plant growth. The addition of organic fertilizer to basal synthetic fertilization did elevate the mean $\delta^{15}N$ value of lettuce tissues for about 0.7‰ as compared to synthetic fertilization but the difference between the treatments is not significant

CONCLUSION

Obtained results indicate that $\delta^{15}N$ of aboveground plant lettuce tissues could be used as a marker to reveal the use of synthetic N fertilizer when it is applied in a single application, however in the split fertilizer application, the addition of synthetic fertilizer to the basal organic fertilization and vice versa could not be confirmed by this method.

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