Univerzita Karlova v Praze

Přírodovědecká fakulta

Studijní program: Geologie

Studijní obor: Praktická geobiologie

Petra Zahajská

Carbon isotopes as a tool for study of palaeoclimate

Izotopy uhlíku jako nástroj ke studiu paleoklimatu

Bakalářská práce

Školitel: RNDr. Jiří Kvaček, Csc.

Praha 2014

Charles University in Prague

Faculty of Science

Study program: Geology

Study course: Practical geobiology

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Podpis

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Abstract

Poměr izotopů uhlíku ^{12}C a ^{13}C fixovaných v tělech rostlin kolísá podle dostupnosti vody v prostředí, v kterém rostou. Přesným měřením poměrů izotopů lze stanovit na jakém stanovišti konkrétní rostlina rostla, zda ve vlhku nebo v suchu. Tato metoda používaná v současnosti hlavně u recentních rostlin může významně přispět ke studiu a rekonstrukci paleoprostředí.

Prvním krokem k výzkumu bude důkladná rešerš literatury. Dalším krokem bude studium a analýza fosilního rostlinného materiálu z lokality Valča, která je opěrnou lokalitou pro studium holocénu střední Evropy. Závěrečným úkolem bude zhodnocení aplikovatelnosti metody pro využití analýz izotopů uhlíku fosilních rostlin pro rekonstrukci paleoprostředí.

Abstract

A ratio of carbon isotopes ^{12}C and ^{13}C fixed in plants can vary according to water availability in environment where they grow. Applying high resolution measurements of the isotopes it is possible to define environment in which plants grow, either in moisture or either in dry conditions. This method is used mainly in recent plants, but it can improve importantly our studies of fossil paleoenvironments.

The first task of the study will be careful research of literature. The next step of analysis will be the fossil material from Valča. This locality is a principal for study of the Central European Holocene. The final task will be evaluation and applicability of the method fossil plants carbon isotopes for reconstructions of paleoenvironments.

1 Introduction

The photosynthesis is regarded as the most essential life process of plants on the Earth. The processes of photosynthesis are important because of their ability to incorporate inorganic carbon into plants or simply make an organic carbon from inorganic. Generally, the photosynthesis is the process that stores carbon in biomass and in contrast the respiration is considered as a process of releasing carbon from biomass. This thesis might show the methods using ratio of stable carbon isotopes for modeling paleoclimate. At first is very important to understand recent processes of photosynthesis, respiration, their relation and find all possible factors which could influence stable carbon isotope ratio.

2 Photosynthesis, respiration, carbon cycle

2.1 Photosynthesis as a fixation of inorganic carbon and incorporation in organic compounds

Photosynthesis is a process where is about 1.6×10^{14} kg of carbon are fixated annually into organic compounds due to photosynthetic organisms - the net primary productivity. The carbon source for this process is the $0,04\%$ CO_2 from the air and CO_2 or HCO_3^- dissolved in lakes and oceans. The photosynthesis can be divided into three stages (Fig. 1):

1. Photochemical steps - primary events, absorbing photons.
2. Electron transfer stage - ATP and NADPH formation.
3. Biochemical reactions - incorporating CO_2 into carbohydrates (Calvin cycle - see below).

The energy bilance of photosynthesis enables that from 8 inputed photons is absorbed one CO_2 and evolved one O_2 (Nobel, 2009).

The estimate of carbon saved in oceanic floor is approximately $0,1Gt$ per year. The total biomass represents $60Gt$ of carbon per year. The $40 - 50\%$ of dry weight of plants creates only carbon mainly of atmospheric origin. The photosynthesis reduction cycle

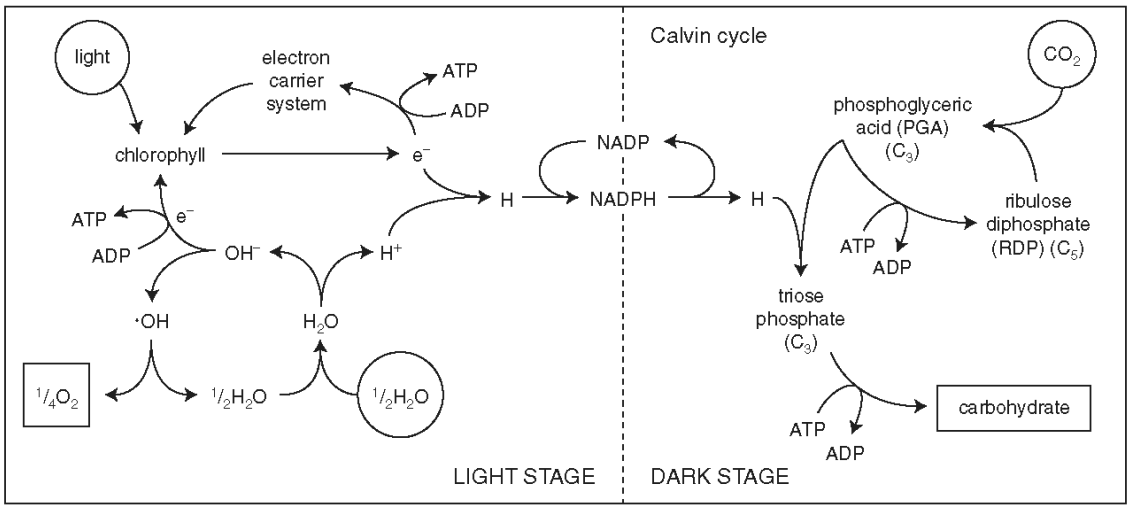

Figure 1: Scheme of photosynthesis, (Killops et al., 2005a).

(PRC) called also Calvin cycle builds on fixation of carbon accompanied by energy from sunlight to synthesize organic carbohydrates compounds. During the photosynthesis plant absorbs CO_2 (in secondary photosynthesis phase) and use ATP and NADPH emerging in first photosynthesis phase (PSII) - light stage (Fig. 1). Absorbed CO_2 is reduced in PRC (in chloroplast stroma - dark stage) to organic compounds. PRC is processed in 3 basic phases of Calvin cycle (also in Fig. 1) (Marek, 2011) :

1. carboxylating phase - covalent binding CO_2 to carbon skeleton of acceptor RuBP (ribulose-1,5-biphosphate),

2. reducing phase - reduction 3-phosphoglycerate to glyceraldehyde-3-phosphate with using ATP and NADPH,

3. regenerating phase - formating new RuBP.

2.1.1 C_3, C_4 and CAM metabolism

In general, we have three basic metabolic pathways in plants. The most widespread metabolic pathway is C_3. The CO_2 is bounded by RUBISCO in the mesophyl cell and chloroplasts. As C_3 is called because the product of Calvin cycle has 3 carbons (Nobel, 2009). Into the group of C_3 plants are included gymnosperms, pteridophytes and some angiosperms.

The other metabolic pathway is C_4. This type of metabolism have advantage over C_3 plants particularly in hot and dry environments with high light intensity (Bocherens et al., 1994). CO_2 is bounded by PEP carboxylase in mesophyll cell and cytosol. The product is 4 carbons acid and it is bounded into the second phase of photosynthesis in chloroplast (Nobel, 2009). C_4 plants are graminoids or in general forms a big part of monocots plants.

Crassulacean acid metabolism (CAM) is the last type of metabolism and it is typical for arid environment, for example Crassulaceae. This metabolism has its night and day phases and plants fix CO_2 in the dark (Bocherens et al., 1994). CO_2 is fixed by PEP carboxylase in mesophyll cell and cytosol during the night and the product is C_4 acid. It is bounded by RUBISCO in mesophyll cell and chloroplast during the day (Nobel, 2009). For comparision of all three metabolic pathways see Fig. 2.

In the text below the C_3 metabolism is only considered in case, it is not written something different.

2.1.2 Carboxylating phase

Initial compound of this phase is ribulose-1,5-biphosphate (RuBP) and main catalyzator which enable binging of CO_2 to RuBP is RUBISCO. The reactions are located in a stomatal space of the chloroplast (Lodish et al., 2004a). In processes of assimilation RUBISCO has affinity to CO_2, but in processes of photorespiration it has affinity to O_2 (Marek, 2011). This phase is the first entry, where the isotopic discrimination is known, which is described in detail in chapter 4.

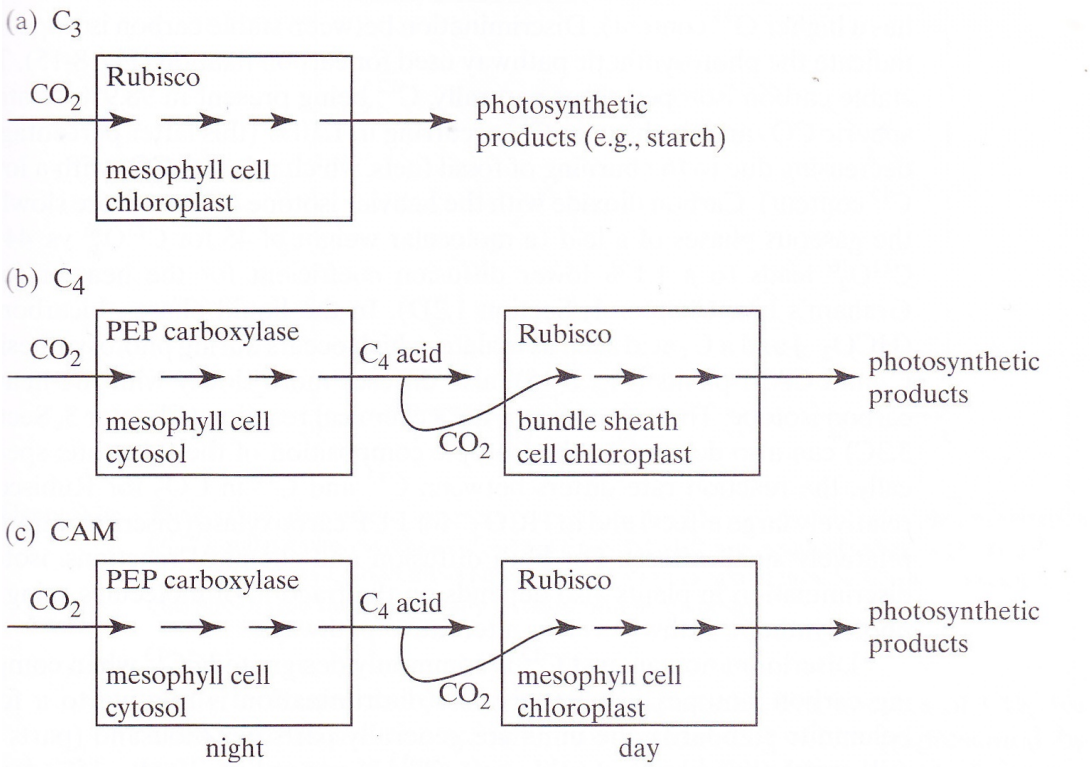

Figure 2: Three photosynthetic pathways: (a) C_3, (b) C_4 and (c) Crassulacean acid metabolism (CAM) (Nobel, 2009).

2.1.3 Reducing phase

From the compound RuBP+RUBISCO+CO_2 arises non-stable compound with 6 carbons as an intermediate. The first product of carboxylation is 3-phosphoglycerate with 3 carbon molecules (C_3). Plants with this type of photosynthetic fixation of CO_2 are termed C_3-plants. In next phase the 3-phosphateglycerate is reduced to glyceraldehyde-3-phosphate (G-3-P). Six molecules of G-3-P arised there. One molecule G-3-P is leaving the cycle and others compounds as saccharides, proteins etc. are synthesized from G-3-P. Five remaining G-3-P stay in the cycle and they are used in the next phase (Marek, 2011).

2.1.4 Regenerating phase

Glyceraldehyde-3-phosphate is also substrate for last phase - regeneration. It is based on creating compounds ribulose-5-phosphate from 5 remaining molecules G-3-P. At the end is necessary the last process - phosphorylation, where RuBP arise from ribulose-5-phosphate. And then the Calvin cycle can continue with the first phase (Marek, 2011).

2.2 Limiting factors for net photosynthesis

2.2.1 Influences of CO_2 concentration

The amount of dissolved CO_2 is one of the limiting factors for the photosynthetic process. The relation between rate of assimilation (A) an CO_2 concentration is described by CO_2-curve of assimilation rate. Important parameters are total conductivity of boundary layer and stomata for diffusion CO_2, rate of transpiration and concentration of surrounding CO_2. The curve is applicable for stomatal counting and mesophyl limitation or it can be used for counting the maximal rate of carboxylation and maximal rate of transportation electrons in leaf. The shape of curve is very similar to the light intensity curve (see Fig. 3) and the limiting factors form the first linear phase of curve (the concentration of CO_2 is small) an enzymatic activity of RUBISCO. In the last linear phase (the concentration of CO_2 is high) the concentration of RuBP is the limiting factor, which bounds CO_2.

2.2.2 Influences of light intensity

Light intensity is one of the very important limiting factors in photosynthesis, because light influences the assimilation of CO_2. For measuring effects of light intensity variability it is necessary to have a constant concentration of CO_2. The rate of assimilation is A and for expression depending A to light intensity (FAR), it is used a light curve of assimilation rate (Fig. 3). The curve prescription is the quadratic equation (1) where A is actual assimilation, A_{max} is a light saturated rate of assimilation CO_2, φ is a number modeling the shape of curve (0 - 1) and α is quantum yield of assimilation, which points the change of rate of assimilation CO_2 when the intensity of FAR is changed about 1 $\mu mol(fotons)m^{-2}s^{-1}$.

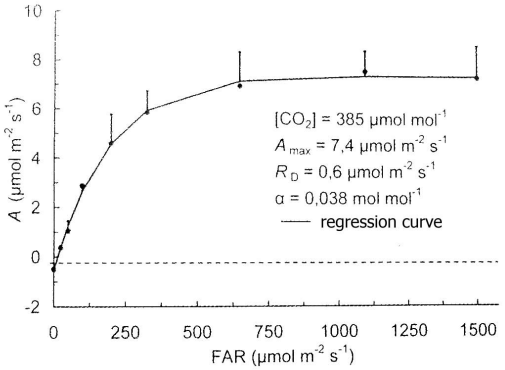

Figure 3: Light curve of assimilation rate, A is rate of assimilation, FAR is light intensity, (Marek, 2011).

$$\varphi A^2 - (\alpha I + A_{max})A + \alpha I A_{max} = 0 \tag{1}$$

From this relation it is clear that in the rapidly increasing part of curve the light intensity is limiting factor. Also in this phase the amount of RuBP which binds CO_2 is the limiting factor. With increasing value of light intensity the limiting factor are not fotons but pigments in leaves. And in the linear part of curve the amount of enzyme RUBISCO is the limiting factor.

For majority of C_3 plants assimilation is saturated with light intensity $500-1000$ μmol fotons $m^{-2}s^{-1}$, what is $25-50$ % sun light intensity. C_4 plants need for saturation different light intensity, but in general the C_4 plants have A_{max} value higher than C_3 plants (Marek, 2011).

2.2.3 Influences of temperature

The temperature mainly influences the metabolic processes due to an influencing the kinetic of chemical reactions and efficiency of enzymes. For C_3 plants their photosynthetic process have assumption that the rate of assimilation CO_2 is limited by activity of RUBISCO or by rate of regeneration RuBP. In the regeneration of RuBP an electron transport and output of ATP is included. The temperature causes that plants from different temperature conditions have a different temperature optimum of photosynthesis. The variability of assimilation of CO_2 depending on temperature is caused by
1) changes in intracellular concentration of pCO_2,
2) changes in maximal rate of carboxylation and
3) changes in maximal rate of electron transport.
There are mentioned 3 possible causes of influence, but I am not going to explain these in detail, because it is not the main topic of this thesis. For more information see Marek (2011).

2.3 Respiration as a carbon releasing process from biomass

All plants have to respirate, what means that they consume O_2 and evolve CO_2. In our case the relation between the respiration and the net photosynthesis is important . The respiration is the reverse process and the efficiency of both processes are shown in Fig. 4. Photosynthesis and respiration are naturally in balance (Killops et al., 2005b).
Respiration can be described by the equation 2

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O \quad (2)$$

The oxygen-dependent degradation of glucose is the major pathway for a generating ATP in all nonphotosynthetic plant cells (Lodish et al., 2004b). It is an initial reaction for glucose metabolism which takes place in cytosol. In aerobic cells the product (puryvate) is transported into the mitochondria, where it is oxidized by O_2 to CO_2 (Lodish et al., 2004a).

2.4 Global carbon cycle

The carbon cycle is usually divided into two subcycles. One - larger, is considered as geochemical subcycle and involves sedimentary rock. The second one - smaller, is shorter and refers to watter masses, primary oceans, soil and biota biomass. It is termed biochemical subcycle and involves biological recycling in short time period as hundred years. Both subcycles are linked together by small two-way flux. Thanks to the fact that, the carbon from biochemical subcycle is incorporated into sedimentary rock in carbonate and kerogen form. Carbon cycle is in a steady state if there is no anthropological influence. There is an equal flux in both direction - in the incorporating carbon into the sedimentary rock as well as in the opposite direction, where is erosion and weathering of sedimentary rock. Because of this processes sedimentary rock, concrete carbonates are the largest reservoir of carbon. At the end of this part, it is necessary to say, that the quantity of free oxygen in the atmosphere is dependent on the amount of reduced carbon compounds saved in sedimentary rocks. The biochemical and geochemical subcycles are related in this way (Fig. 4) (Killops et al., 2005b).

2.4.1 Biochemical subcycle

The most prevalent form of carbon in the atmosphere is CO_2, which is consumed by plants for photosynthesis and released in respiration. This cycle is influenced primarily by intensity and efficiency of photosynthesis, which is referred as gross primary production. Values of gross primary production correspond to the captured solar energy by plant and they are measured by the amount of fixed carbon dioxide. Some of the gross primary production is consumed by respiration which is understood as a releasing carbon

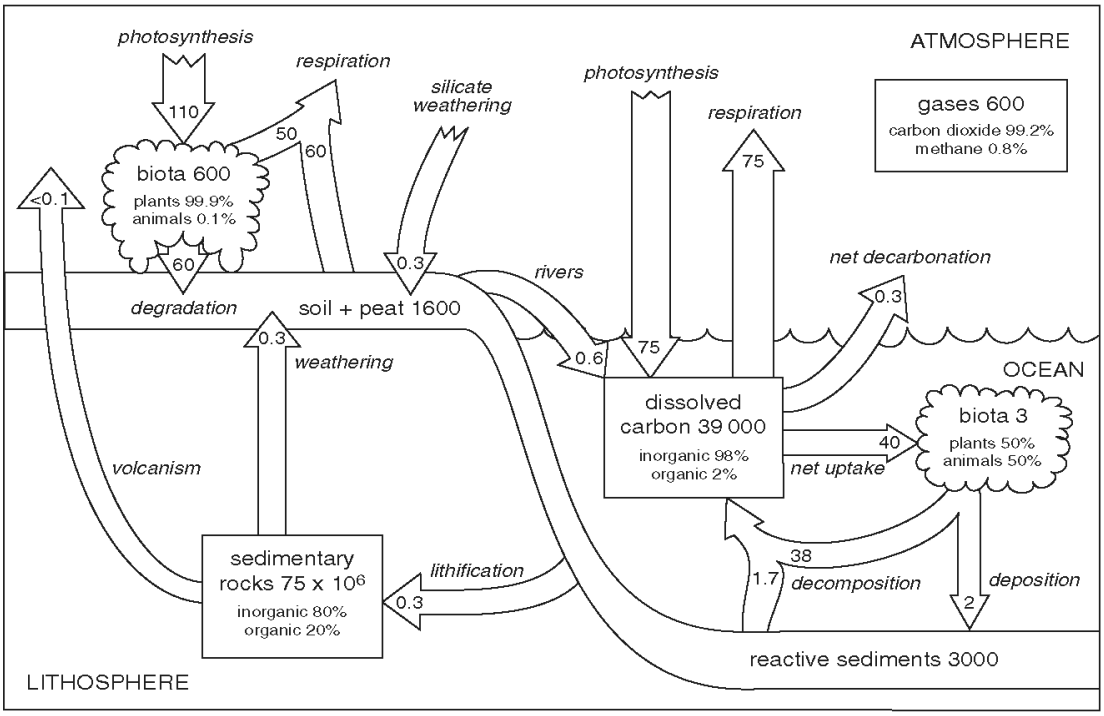

Figure 4: Carbon cycle, (Killops et al., 2005b).

dioxide back to the atmosphere, conversion organic carbon to inorganic. The rest of the gross primary production which is not consumed is termed the net primary production. Annual rest of primary production is similar to land plants as well as to marine plants, although the biomass of land plants is much more bigger than that formed by marine plants. Another big difference between marine and land plants is in residence time of C in reservoirs. residence time of C for terrestrial plants is 5,5 years and 26 years in soil organic matter. On the other hand, the residence time of C for marine phytoplankton biomass is 2 years, but in contrast to it is 338 years for dissolved carbon in oceans (Killops et al., 2005b).

Terrestrial component of the biochemical subcycle Land primary production varies between biomass and a relatively large storage of biomass are woody materials, because of their relatively long life. The residence time of C in soil is 25 years, but organic matter is recycled. Peat formations enable better places for carbon preservation in moorland bogs and low-lying swamps. In these places the size of carbon rezervoir is not

known, but it is suggested that peat formations played a great importance in the past. The storage of carbon as freshwater environments have the relatively small net primary production. There are carbon-rich deposits formed under certain conditions in particular lakes, but the deposits are of small volume. The dissolved and particulate organic carbon is represented by a river and in general by fluvial particles. In a fluvial environment there are also inorganic carbon particles from rock weathering. This particles are mainly deposited in estuaries and deltas (Killops et al., 2005b).

Marine component of the biochemical subcycle In a marine environment phytoplankton is the biggest primary producer. However, the phytoplankton is short-lived compared with land plants and do not need to produce so difficult structure as it is e.g. in case of wood. These facts could be advantages, but phytoplankton is reduced by herbivorous zooplancton so that the phytoplankton biomass is low and the ratio of animal to the plant biomass in the oceans is greater than on the land. The dissolved carbon in water represents a large reservoir of carbon in oceans. The dissolved inorganic carbon (DIC) occurs in form CO_2 and is very important for aquatic plants, which are utilized for photosynthesis. Because of this usage of carbon dioxide, it is necessary to have a dynamic equilibrium whereby molecules of CO_2 are exchanging between the atmosphere (CO_2) and oceans (DIC). Therefore, if the concentration of CO_2 in atmosphere, in constant temperature, increases, the concentration of DIC rise as well, thanks the dynamic equilibrium. Additionally, the concentration of DIC is unstable in water column: Deeper waters are DIC enriched, the residence time is around 1000 years and there is also organic carbon from remains of organisms - particulate organic carbon (POC) (Killops et al., 2005b). The marine part of carbon cycle is complicated, but very important in global scale. In this thesis it is not necessary to describe it in detail, but is necessary to keep in mind reactions and processes of the marine biochemical subcycle for better understanding of the whole carbon cycle.

2.4.2 Geochemical subcycle

The geochemical subcycle is a part of the carbon cycle, which has a long-time duration. Only 5% of the marine primary production enters sediments and the greater part

is recycled in the biochemical cycle. The residence time of C in the reactive sediments surface is 1,5 kyr, but after lithification residence time increases to 250 Myr. This time is dependent on a tectonic cycling of lithospheric plates, volcanism and also a weathering of uplifted sedimentary rocks. Therefore, the geochemical subcycle plays a minor role, but involves a large reservoir of carbon that is important over geological time scale. This cycle can be considered as a large sink for atmospheric CO_2, which involves the subaerial weathering of carbonates and silicates in the sedimentary rock. The product of this chemical weathering by the carbonic acid is CO_2, which may be precipitated by calcareous organisms as is calcium carbonate. The carbon weathering and the subsequent precipitation do not result in the net change in atmospheric CO_2 levels.

On the other hand, there exists also the silicate weathering, which after a precipitation of calcium carbonate releases back to the atmosphere with only half amount of originally drawn down CO_2 during the weathering process.

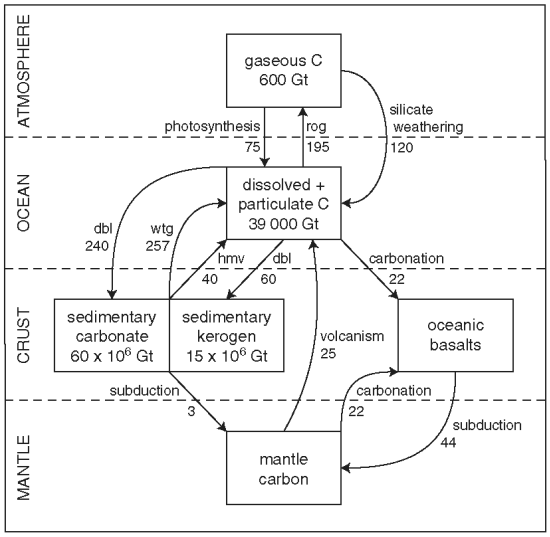

Figure 5: Summary of geochemical carbon subcycle, (Killops et al., 2005b).

The weathering of kerogen in sedimentary rocks is a process equivalent to the aerobic respiration, thus the opposite process of photosynthesis. The silicate, coal and kerogen weathering is limited by the uplifting, because weathering itself is the very fast process in a geological time scale. Additionally, the kerogen weathering consumes oxygen, so it can be considered to have an impact on atmospheric O_2 levels.

It is necessary to mention the level of atmospheric CO_2 is influenced by volcanism, metamorphism and diagenesis. In Fig.5 processes of geochemical carbon subcycle are summarized, there are formed closed loops which are important for overall balance (Killops et al., 2005b).

3 Carbon isotopes and fractionation

Carbon is a biogenic element and as a light element have two natural isotopes - differing in the number of an elemnet neutrons in nuclei. This difference causes that their atomic mass (the sum of neutrons and protons) has two variants. In nature the carbon is a mixture of ^{13}C and ^{12}C izotopes, where ^{13}C is in 1,106% of carbon mixture and the rest - 98,894% is the lighter isotope - ^{12}C (Killops et al., 2005c). The isotope ^{13}C is heavier than the isotope ^{12}C and is known that in some processes this isotope is preferred more than the other one (Killops et al., 2005a; Farquhar et al., 1989). Generally, the heavier isotopes of carbon are contained in inorganic compounds as carbonates and kerogen, and contrarily the lighter are more common in biogenic compounds. This is termed as **isotopic fractionation** (Killops et al., 2005c). The phenomenon during the primary carbon fixation reaction of photosynthesis prefers the lighter isotopes, because they are assimilated slightly faster (thanks their weight). This is termed the **kinetic isotope effect** (in details in chapter 4) (Killops et al., 2005a).

The isotopic fractionation may occur at diffusion of CO_2 into leaf, diffusion from the leaf atmosphere into the chloroplast, carboxylase catalyzed fixation and subsequent metabolic changes. Environmental parameters may affect any of these to some degree. As the most strongly influenced by external parameters can be the carboxylase reaction and subsequent metabolic fractionation (Smith et al., 1976).

Very important in isotopic distribution is that it can reveal information about physical, chemical and metabolic processes, which is possible to find in carbon transformations. In plant tissue the isotope ^{12}C is common and the ratio $\frac{^{13}C}{^{12}C}$ in atmospheric CO_2 is therefore lower (Farquhar et al., 1989).

The carbon isotopic fractionation in living terrestrial and freshwater plants is dependent on three basic factors:

1. $\frac{^{13}C}{^{12}C}$ value in the inorganic-carbon source, which the plants use for photosynthesis (see chapter 2.4)

2. photosynthetic patway (see chapter 2)

3. environmental parameters (see chapter 9).

4 Isotopic discrimination

Isotopic discrimination is a phenomenon when for some reason one isotope is preferred over the other. In our case, this phenomenon happens during photosynthesis and during many other biosynthetic processes where carbon is involved. It can be expressed by ratio of isotopes - $\frac{^{13}C}{^{12}C}$- and the variation on this ratio is influenced by "isotope effects".These effects happen during the formation and the destruction bonds with carbon atoms which are involved in biosynthetic processes. Every reaction with carbon in plant body is accompanied by carbon discrimination effect, because the processes are affected by atomic mass. As an example one reaction can be gaseous diffusion where the heavier isotope is bigger and slower due to the lighter isotope is preferred.

Isotope effects (also fractionation factors) are classified as **kinetic** or **thermodynamic** and the difference is according to the type of non-equilibrium and equilibrium situations. An example of a kinetic type it is a difference between the kinetic constants for reaction of $^{12}CO_2$ and $^{13}CO_2$ with RUBISCO (Farquhar et al., 1989). In this reaction the heavier carbon isotope is discriminated because the atoms are bigger and the assimilation is slower than in the reaction with the lighter carbon isotope. The balance of the two kinetic effect at a chemical equilibrium is an example of thermodynamic effect and generally these effects are smaller then individual kinetic effect. Factually it is a case of an unequal distribution of isotope species among phases in a system - CO_2 in solution (water) against CO_2 in air. These effects are dependent on temperature.

Fractionation factors are defined along Farquhar et al. (1989) as a ratio of carbon isotope ratios in reactant and product:

$$\alpha = \frac{R_r}{R_p}, \tag{3}$$

also it can be thought of as ratio of the rate constants for ^{12}C and ^{13}C containing substrates -> k^{12} and k^{13}. If it is understood like the rate constants, the equation is:

$$\alpha_{kinetic} = \frac{k^{12}}{k^{13}}. \tag{4}$$

And the expression for simple equilibrium isotope effect would be the same. But we substitute the rate constants for the equilibrium constants for ^{12}C and ^{13}C containing compounds.

The isotope effects can be described in an every reaction, but the overall isotope effect will only reflect the effects of partially reverse reactions or of the reactions where are alternative possible fates for atoms, until the irreversible step is reached. This is in detail described in O'Leary (1988).

Farquhar and Richards (1984) described the whole plant processes as a chemical processes. They measured ratios of carbon isotope in reactant (R_r) and product(R_p). As a reactant they selected isotopic abundance in the air - R_a and as product the isotopic abundance in the plant - R_p. For better numerical expression they used Δ - deviation of α - as a rate of the carbon isotope discrimination by the plant:

$$\Delta = \alpha - 1 \tag{5}$$

$$\Delta = \frac{R_a}{R_p} - 1 \tag{6}$$

But these equations count with absolute isotopic composition of a sample and it is not easy to measure directly therefore the mass spectrometer measures the deviation of the isotopic composition of material related to standard - R_s:

$$\delta_p = \frac{R_p - R_s}{R_s} \tag{7}$$

$$\delta_p = \frac{R_p}{R_s} - 1 \tag{8}$$

after substituing

$$\delta_p = \frac{R_{sample}}{R_{standard}} - 1 \tag{9}$$

it correspond with frequently used expression of $\delta^{13}C$ in promilles

$$\delta^{13}C = \frac{^{13}R_{sample}}{^{13}R_{standard} - 1} \times 1000 \tag{10}$$

$$[\delta^{13}C] = ‰ \tag{11}$$

As a reference material for determination carbon isotopic ratios have been set a carbon from carbon dioxide generated from a fossil belemnite from Pee Dee Formation - PDB, which $R = 0,01124$ (Farquhar et al., 1989).

The isotope discrimination was experimental measured by Evans et al. (1986) and it was found that preferential intake of $^{12}CO_2$ against $^{13}CO_2$ was incorporated into the

leaf. Using the equation (11) the results of $\delta^{13}C$ values of C_3 plants are in range between $-23‰$ and $-34‰$ (average $-27‰$). For C_4 plants is range between $-8‰$ and $-16‰$ (average $-13‰$) (Gröcke, 1998). Values of the atmospheric $\delta^{13}C$ reservoir, which is used by terrestrial plant is $-7‰$ (Killops et al., 2005b). That points at an existence of isotopic discrimination, plant has less of heavier isotopes in ratio to amount of light isotopes than surrounding atmosphere. They are depleted of the heavier isotope relative to ratio in atmosphere.

There are many experiments which describe isotope discrimination. Additionally, it is known that there are differences in isotope discrimination between different metabolic pathways which are used by C_3, C_4 and CAM plants. For example: The ethanol and CO_2 derivated by C_3 plants have less ^{13}C than that from C_4. Therefore we can observe the difference between carbon isotope ratio of C_4 and C_3 plants (Hobbie and Werner, 2004).

5 What can we use carbon isotopes for?

In general, carbon is exchanged between the atmosphere, the terrestrial biosphere and the oceans slower than between sediments and sedimentary rocks. Carbon isotopic composition in particular air-CO_2 provides a tool towards the quantifying the contribution of different components to ecosystem exchange. Thanks to it is possible to find sources and sinks of CO_2 in the ecosystem. The important information is that the plant photosynthesis discriminates ^{13}C - carbon absorbed and used by plants and tends to have less ^{13}C than surrounding CO_2. Due to this discrimination it is possible to interpret changes in $\delta^{13}C$ of atmospheric CO_2, what can be applied in three ways (Ghosh and Brand, 2003):

1. For partition net CO_2 fluxes between land and ocean and finding the carbon missing sink and processes of creating it

2. To interpret changes in $\delta^{13}C$ of atmospheric CO_2 in terms of environmental changes: The level of discrimination by C_3 plants is mainly influenced by environmental factors as availability of water, nutrients and also light. Depending on this factors the C_3 plants save different ratios of $\delta^{13}C/\delta^{12}C$ and this is possible to apply the process reverse - analyze the ratio and interpret the climate or environmental conditions which are typical for the ratio values. Preparing, finding and analyzing interpretations of different ratios are the

main goal of this thesis.

3. For understanding of carbon isotope disequilibrium caused by ^{13}C- depleted fossil fuels.

All three methods are very useful and important for understanding environment and processes in it. Also there is another usage of carbon isotope as reconstruction of vertebrate paleodiets or recording the changes in plant community composition - global shift from C_3 to C_4 grasslands (Arens et al., 2000; Koch, 1998). Additionally, it can be used for finding changes of physiology, ecology and taphonomy of individual plants. There are many other applications of carbon isotopic composition with paleosols and using them for the chemistratigraphic correlation between marine and terrestrial rocks (Arens et al., 2000).

6 Storage of C in plant body and saving mechanisms

For using the application mentioned above, it is necessary to understand clearly, how land plants reflect the isotopic composition of atmospheric CO_2. It is known, that C_4 plants record the isotopic composition of atmospheric CO_2 . The problem is their late coming. They are common from the latest Miocene, so it is limiting. C_3 plants have been here since the Devonian, but they display more variable carbon isotopic discriminations (Arens et al., 2000). Thanks Farquhar et al. (1989), we have a well-established conceptual model, which describes an isotopic fractionation during carbon assimilation in C_3 vascular plants. The carbon isotope ratio of C_3 plant tissues is proportional to the ratio of the partial pressure of CO_2 (pCO_2) inside the leaf (c_i) to the pCO_2 in the atmosphere (c_a) according to:

$$\delta^{13}C_p = \delta^{13}C_a - a - (b - a)\frac{C_p}{C_a} \tag{12}$$

where $\delta^{13}C_p$ is the carbon isotope ratio of plant tissue, $\delta^{13}C_a$ is the carbon isotope ratio of the atmosphere, a is the isotopic discrimination due to the diffusion of $^{13}CO_2$ versus $^{12}CO_2$ through air (4.4 ‰), b is the isotopic discrimination imparted during carboxylation by ribulose-1,5-bisphospathe carboxylase-oxygenase (RUBISCO - the primary carbon-fixation enzyme in C_3 plants) ($\sim$ 27‰). C_p/C_a is the ratio of intracellular to atmospheric pCO_2 expressed in parts per million volume (ppmv) (Powers et al., 2008).

The simplest form of equation (12) used for understanding of C_3 photosynthesis discrimination in leaves is equation (13).

$$\Delta = a + (b - a)\frac{c_i}{c_p} \tag{13}$$

a is the fractionation occurring due to diffusion in air (4.4‰) and b is the net fractionation caused by carboxylation (approximately 27‰). $\frac{c_i}{c_p}$ is ratio of CO_2 concentrations in the leaf intercellular spaces to that in atmosphere (Ehleringer et al., 1992; Farquhar et al., 1989).

The equation (12) or (13) describes three important factors influencing the carbon isotopic composition of C_3 vascular land plants:

1. Ecological factors influence the degree to which stomata remain open and CO_2 is absorbed (it is expressed $\frac{C_p}{C_a}$, thus it directly influence $\delta^{13}C_p$. This ecological factor can be water and nutrient stress, light limitation, thermal load. They are described in chapter 9.

2. Physical and biochemical fractionation during CO_2 absorption (a) and during carboxylation (b), in detail see chapter 4.

3. Atmospheric CO_2 in the substrate for the fixation, that means the carbon isotopic composition of atmosphere ($\delta^{13}C_a$) directly influences the composition of the resulting plant tissue.

All three factors are similar to factors for the isotopic fractionation of carbon in living terrestrial and freshwater plants (see chapter 3), that means the relation between pCO_2 atmospheric and isotopic fractionation. This fact is known thanks Farquhar et al. (1989), who described, that partial pressure of CO_2 in the leaf related to the fact that in the atmosphere surrounding the leaf, is the primary cause of isotope fractionation in C_3 plants (Gröcke, 1998).

6.1 Wax biosynthesis

The aerial surfaces of plants are covered by a wax layer -plant cuticle, that is primarily a waterproof barrier and also provides the protection against environmental stresses (Beittenmiller, 1996). The cuticle plays an important role in controling non stomatal water loss

and protect a plant surface against pathogens and insect herbivores (Kunst and Samuels, 2009). Lipid components of the cuticle covering the outer surface on plant tissue are collective understood as plant wax. Cuticular waxes are the hydrophobic compounds that are removed by a brief immersion in an inorganic solvent such as chloroform of hexane (Beittenmiller, 1996).

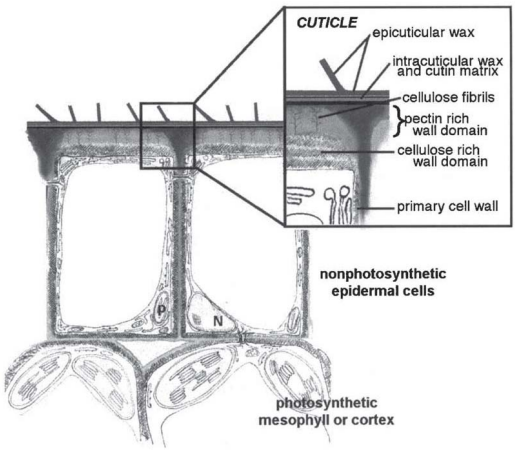

Figure 6: Cross-section by plant epidermis and cuticle, (Kunst and Samuels, 2003).

Terminology of plant surface is shown in the Fig. 6 (Kunst and Samuels, 2003). The components can be distinguished into two major types of lipids: cutin and cuticular waxes. Cutin is the core structural polymer composed of $\omega-$ and mid-chain hydroxy and epoxy C_{16} and C_{18} fatty acids and glycerol (Kunst and Samuels, 2009). The waxes are composed of very long chain fatty acid (VLCFA), triterpenoids or minor secondary metabolites - sterols or flavinoids (Kunst and Samuels, 2003). The physical and chemical properties of cuticular wax restrict non-stomatal water loss, protect plant against UV radiation and reduce water retention on plant's surface, that means minimizing dust, pollen and air pollutants deposition. It is possible, that plant waxes are important in defense against bacterial and fungal pathogens (Kunst and Samuels, 2003).

In the Fig. 6, it is shown, that the cuticle is compounded of cutin, intracellular wax and epicuticular wax. Cutin is insoluble, covalently cross-linked polymer, intracellular wax is amorphous mixture of lipids inserted in the cutin linking the cuticle with the cell wall matrix. Epicuticular wax is represented by surface lipids, which are forming the crystalloids or smooth film exterior to the cuticle (Kunst and Samuels, 2003). It reflects a characteristic cell pattern and structures (stomata, papillae, hairs) and it is a source of cellular information in case of fossil plants. The primary chemical composition and the preservation cause the cuticle resistance (Kerp and Krings, 1999).

6.1.1 Biosynthesis of VLCFA

Elongation of the fatty acyl chains In epidermal cells aliphatic components of cuticular wax are synthesized from VLCFA. VLCFA wax precursors are formated in complex process composed of two stages:

1. **De novo fatty acid synthesis**of C_{16} and C_{18} acyl chains (precursors) located in plastids stroma, which helps to enzyme forming the fatty acid synthetaze complex (FAS). During the synthesis there are the elongating acyl chains connected to acyl carrier protein (ACP). The ACP is essential protein cofactor, which is considered as a component of FAS. Fatty acid synthesis can be divided into four reactions: 1) condensation 2) reduction 3) dehydration and 4) reduction (Kunst and Samuels, 2003). For each two-carbon addition it is necessary to pass this sequential round of four reactions. New formed fatty acids are utilized for glycerolipids, waxes or cutin and suberin biosynthesis (Beittenmiller, 1996).

2. **Fatty acid elongation** - it is located in epidermal tissues. It is extension process of the C_{16} and C_{18} fatty acids to VLCFA chains, which are used for production of aliphatic wax components. Extra-plastidial membrane-associated multienzyme complex = fatty acid elongases (FAE) is catalyzed the extension. As in de novo FAS, there are sequence of four reactions and the result is extension of 2 carbons. Aliphatic wax constituents have typically 20-34 carbons, therefore the reaction cycle have to go through about 10 times and more (Kunst and Samuels, 2003).

6.1.2 Aliphatic wax constituents biosynthesis

From VLCFA produced by elongation in epidermal tissue there are synthesized other wax components. There are usually two principal wax biosynthetic pathways (Kunst and Samuels, 2003):

1. Acyl reduciton pathway - the products are primary alcohols and wax esters.

2. Decarbonylation pathway - the products are aldehydes, alkanes, secondary alcohols and ketones.

In the Fig. 7, there are shown three primary pathways of wax biosynthesis, there is one more in contrast with two pathways defined by Kunst and Samuels (2003). Beittenmiller

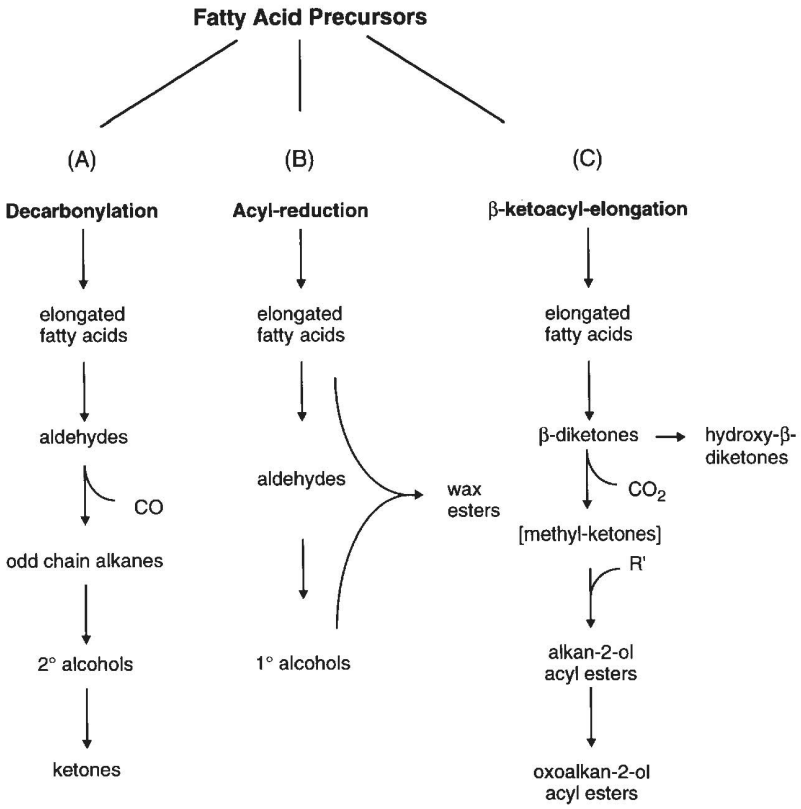

Figure 7: The primary wax biosynthesis pathways, (Beittenmiller, 1996).

(1996) earmarks the β-ketoacyl-elongation pathways. For our topic, it is not very important to know in detail the pathways, but it is important to know, how complicate are the reactions and consider, where the carbon discrimination can occur.

6.1.3 Cuticular wax export

Typical epidermal cell, which produce wax contains a large central vacuole surrounded by a very thin layer of cortical cytoplasm. In cytoplasm are leucoplasts, ER, Golgi, mitochondria and cytoskeletal elements. The transportation of synthesized wax starts in plastid where occurs FAS. Esterification to ACP is placed in stroma. The ACP can have two fates: 1) in procaryotic pathway - is transferred directly to glycerol-3-phosphate in plastid or 2) eucaryotic pathway - ACP is released by thioesterase and exported from plastid to the ER for further elongation (Kunst and Samuels, 2003). The transport of waxes into the cell wall is described by Kunst, but it is still a matter of speculation, see Kunst and Samuels (2009).

7 Methods

For measuring the carbon isotope composition there are several methods and their use is dependent on the matter of measurement. If the result of measurement is total organic carbon, than it is used CHN analyzer. For measurement of the volume or so called "bulk" of $\delta^{13}C$ values in our sample it is used EA-IRMS (see chapter 7.2.1). In case when it is necessary to measure compound specific $\delta^{13}C$ values, GC-IRMS is usually use (see chapter 7.2.2). This text is a preparation for further sample analysis (samples from Valča), therefore there are mentioned all possible ways of measurement $\delta^{13}C$.

For observation of structures on leaves surface scanning electron microscope (SEM) was used. The preparation of samples, methods and more details about samples from Valča are described in the chapter 7.3.2.

7.1 Total organic carbon analysis - TOC

This analysis is used, when the goal is to know the total volume of organic carbon in a sample. Problem is, that in every sample there will be some amount of inorganic carbon. Therefore there are methods how count with this fact. There are numerous methods of measurement of TOC, but the basic idea is to do some reactions and change organic carbon to inorganic and then measure it. All methods use acidification of the sample (for inorganic carbon) and than can proceed oxidation (for organic carbon), detection and quantification. TOC analyzers measure the CO_2, which is formed during the reactions - acidification and oxidation (Pedentchouk, 2014).

7.2 Isotope ratio mass spectrometry

Isotope ratio mass spectrometry is a method of measurements the stable isotope ratios. Mass spectrometers are based on combination of lens, magnetic components, flow of ions and collectors. Lens serve for focusing ion beam, magnetic components divide the ion beam by weight. Charge and divided beams are captured by collectors (see Fig. 8). The sample, which is introduced in mass spectrometer, must be converted to gas (Ghosh and Brand, 2003). There are two possibilities: combustion of sample or chromatographic separation of components followed by conversion to gas. The first one is used in case of

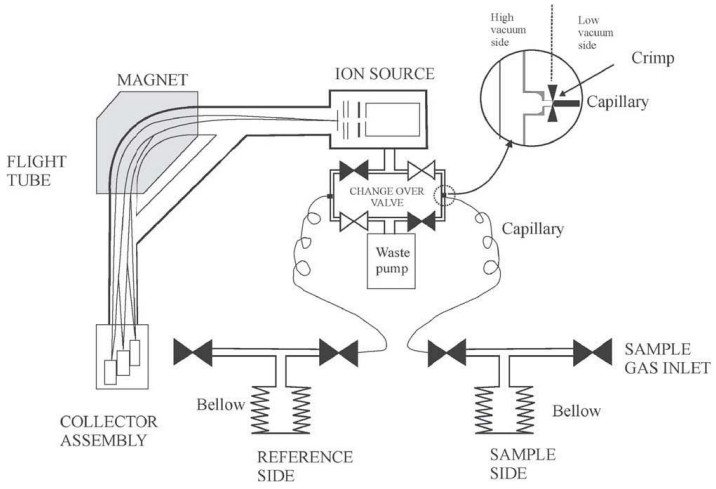

Figure 8: Model of IRMS with essential components (Ghosh and Brand, 2003).

EA-IRMS and the other one is typical for GC-IRMS (for measurement isotope ratio of specific compound).

7.2.1 EA-IRMS

Elementar analyzer isotope ratio mass spectrometry is a method used for measurement bulk data. It has been used for first information about samples and also for analysing δ^{13} C values in modern or fossil leaves. This method is appropriate for detection of nonspecific mass of δ^{13} C values. The analysis is divided into four steps (Carter et al., 2011):

- Combustion of the sample material using elementar analyzer (EA)- input can be wide rang of materials: solid substances, non-volatile liquids or liquid with limited viscosity. Combustion is followed by reduction and by a water-separation device. There is a packed GC column for separation of evolved gases - CO_2 or N_2. The combustion is take place in O_2 atmosphere (in quartz reactor) and products are CO_2, NO_x and H_2O. Temperature of reaction is typically between 900-1500 ℃. Therefore it is recommended the quartz inserts for collecting ash and residue from samples and thin capsules. At the end the CO_2 is separated by isothermal packed column GC.

- Introducing of evolved gases into the ion source of the MS using the interface - The interface enable the connection on-line EA with IRMS. It is very important because it regulates the gas volume entering the ion source and dilutes the sample gas with helium.

- Gas molecules ionization followed by separation and detection of the ions in MS -

The ion source of MS ionizes gas molecules thought interaction with the electron beam. Then the ions leave the source and are focused and accelerated thought high voltage. Ions pass thought the magnetic field and than reach the Faraday cup detectors. The strength of magnetic field determine the trajectory of ions and also which ions will enter the Faraday cups. For measurement carbon ratio are necessary three collectors - two are specifically spaced. The cups are connected with its own amplifier, which have different gain.

- Evaluation and interpreting of data - The signals from each amplifier are recorded every tenth of a second, digitalized and recorded by the IRMS data system. The result is a chromatogram where the peak area is proportional to the number of detected ions.

Preparation of samples Plant leaf tissues are milled to fine powder in cryogenic mill under liquid nitrogen. Samples are weighed into thin capsules, crimped and than analyzed by EA-IRMS. Before measurement, the in-house casein and collagen standards are analyzed to calibrate the CO_2 reference gas and monitor for any drift. This is repeated before each sequence of measurement. It is important to prepare all samples including standards by the same methods for ensuring the same conditions (Pedentchouk, 2014).

7.2.2 GC-IRMS

Gas chromatography isotope ratio mass spectrometry (GC-IRMS) is a method used for analyzing δ^{13} C values of specific compounds, which are separated in gas chromatography phase. It is appropriate for measurement carbon isotopic ratio in n-alkanes - lipids or waxes produced by plants. It enables the molecular specificity and isotopic signature of compounds and it is powerful tool for tracing the origin and fate of organic matter in recent, but also in fossil ecosystems (Evershed, 2007). The method is functional for fossil samples, because plant waxes are resistant to decay and thanks to them we can analyze the carbon isotopic ratio and interpret results (see chapter 10). In text below you can find the method suitable for analyzing δ^{13} C values of n-alkanes and plant waxes. For schematic figure of GC-IRMS see Fig. 9 or for more details about GC-IRMS see Sessions (2006); Evershed (2007).

Lipid extractions From the chapter 6.1 we know, that n-alkanes have to be extracted by sonication with HPLC grad hexane to obtain the total lipid fraction. The extract is

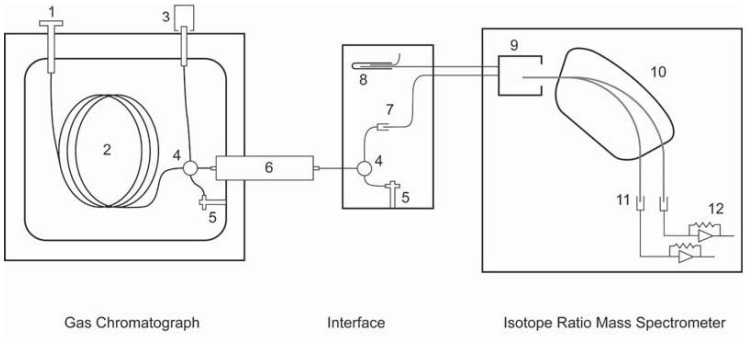

Figure 9: Typical GC-IRMS system, 1 - injector, 2 - analytical column, 3 - FID, 4 - unions, 5 - backflush valves, 6 - pyrolysis reactor, 7 - open split, 8 - reference gas injector, 9 - electron impact ionization source, 10 - magnetic sector mass analyzer, 11 - Faraday detectors, 12 - analog electrometers (Sessions, 2006).

concentrated to 1 mL under nitrogen gas using turbovap prior to chromatographic separation. During column chromatography, the hydrocarbon fraction is eluted with 4 mL of HPLC grade hexane using activated silicagel. Then it is injected into gas chromatograph with a flame ionization detector and capillary column for analyzing the molecular distribution and concentration of n-alkanes. The temperature is increased from 50 ℃ to 150℃ with steps of 20 ℃ min^{-1}. After reaching the 150 ℃, the step changed to 8 ℃ min^{-1} and the final temperature was 320 ℃. The specific n-alkanes are identified by comparison with their elution times with $n - C_{16}$ to $n - C_{30}$ alkane standard (Pedentchouk, 2014).

Carbon isotopes measurement and preparation of samples The carbon isotopes of n-alkanes are measured by IRMS interfaces with GC. The helium is used as a carrier and a reference gas is introduced into carrier gas (Ghosh and Brand, 2003). Mixtures of compounds are separated by high resolution capillary GC and than individually combusted on-line over a catalyst generating CO_2 (Evershed, 2007). For $\delta^{13}C$ analysis the sample must be combusted at 1020℃(it is combusted in steps, from 50 ℃ rising at a rate of 30 ℃ min^{-1} until reaching 220 ℃, than the final temperature 320 ℃ is reached by steps of 6 ℃ min^{-1}. The $\delta^{13}C$ values are expressed relative to PDB (see chapter 4). During the measurement the six reference gas pulses are passed trough the mass spectrometer (MS) (Pedentchouk, 2014).

7.3 Samples from Valča

The Valča locality is situated between Malá and Velká Fatra Mountains in Valčianska dolina - 49°00'03,4"N; 18°47'29,6"E. This locality is very important for study of the Central European Holocene. The samples are needles of *Picea abies* and its age is dated using ^{14}C isotopes. From this locality we have 30 samples from different sediment layers (see Attachments). The youngest layers which were dated are 4730 BP ± 25 years and the lowest/oldest layers are 8170 BP ± 40 years (Pokorný, 2013). For detailed stratigraphy see Attachments. Samples were collected from carbonate sediments (tufa) and stored in glass tubes.

7.3.1 Preparing samples for SEM

The samples from Valča are composed from needles of *Picea abies* and for SEM, four needles was chosen from sample 16 (the layer number 16, see in Attachment 12). Because of adhering sediments on needles, it was necessary to clean them in HF. The samples were leached in HF during 30 minutes and after that they were washed out ten times in water. Then they were dried in alcohol and prepared on stubs for observing with SEM HitachiS-3700. Sample were not gilded, the observation were performed in low vacuum mode where the gold couating is not necessary.

7.3.2 SEM

Scanning electron microscope (SEM Hitachi S-3700N) was used for detailed observation of the material. The samples for SEM were air dried. Samples are placed on stubs, glued using nail polish. After drying, the samples were observed in low vacuum. Our samples were observed by SEM Hitachi S-3700N in National museum in Prague. The goal of the observing was to find structures on the needles surface for possible detecting of stomata. Also crystals created by epicuticular waxes were observed (see chapter 6.1).

8 Samples from Valča - description

In pictures from SEM - Fig. 10 periclinal site of cuticle with distinguishable stomata were observed. Stomata forming oval to elongate structures in our samples are oriented

longitudially to the leaf margin in continuous lines. Not everywhere stomata were clearly seen because of thick cuticle and their immersion.

9 Changes of δ^{13}C values in dependence on environment

The isotopic composition of plant tissue provides an estimate of environmental or physilologal effects on rate of CO_2 assimilation in photosynthesis and stomatal conductance. Therefore δ^{13}C of tissues reflects the δ^{13}C of atmospheric CO_2 during fixation and fractionation of CO_2 by plant deposit processes (Warren et al., 2001). Another factor is latitude (Gröcke, 1998) and altitude (Warren et al., 2001). Both factors indirectly influence the δ^{13}C. Within single environmental variables include temperature, irradiance and humidity which have direct influence on discrimination. In contrast, δ^{13}C values are weakly related to precipitation and evaporation gradients (Warren et al., 2001). The δ^{13}C value depend, in general on three factors:

1. δ^{13}C value of atmosphere,
2. the atmospheric CO_2 concentration,
3. on the $\frac{p_i}{p_a}$ ratio.

For data interpretation, all external influences which can change one of these factors must be considered.

9.1 Influence of humidity and water-use efficiency

The influence of moisture and water is generally very important for δ^{13}C values. There are direct relationships between soil moisture, rate of photosynthesis, transpiration and leaf conductance (Farquhar et al., 1989). All factors are linked and we have to understand is as one functional unit. Also the amount of cuticular wax is response on environmental conditions. When the relative humidity is high, the wax production is low (Beittenmiller, 1996). Hence, when the soil moisture decrease, the rate of photosynthesis decreased too as well as rate of transpiration and leaf conductance. As a result the δ^{13}C increased and

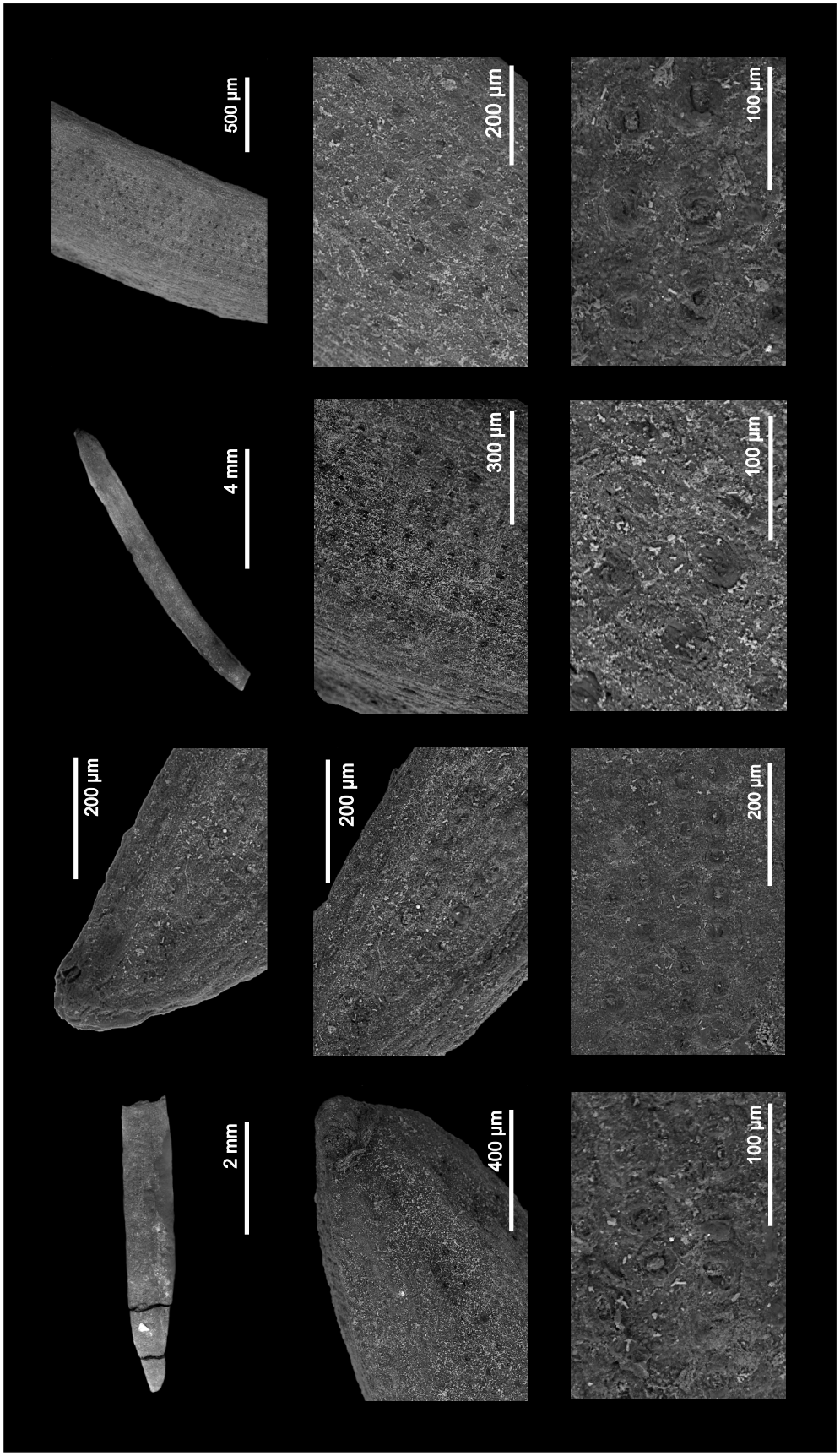

Figure 10: Table 1: Stomata on needles from Valča.

in plant tissue is represented more ^{13}C isotope than would be in beneficial conditions. During the water-stress the carbohydrates are fractionated in leaves and the carbon from this source is recyclated. The lack of humidity (soil strength) in long term cause decreasing of intercellular partial pressure of CO_2 (p_i) which is directly related with increasing of ^{13}C isotope (Farquhar et al., 1989).

However, plants are able to adapt their photosynthetic pathways as a response to changes in leaf water status. Some (non-tropical) species are able to dramatically change their photosynthetic metabolism from C_3 to CAM. This phenomenon is displayed on shift of carbon isotopic composition about 10-15‰(Farquhar et al., 1989). Thus the ^{13}C values increase and this change is recorded in plants tissues. The switch of photosynthetic metabolism is reversible and it depends on water status.

Water-use efficiency Water-use efficiency of C_3 species is dependent on transpiration efficiency, what is amount of carbon biomass produced per unit water transpired by the crop (Farquhar et al., 1989). It is possible to express the ratio of CO_2 assimilation rate to transpiration rate in equation (14),

$$\frac{A}{E} = \frac{p_a(1 - \frac{p_i}{p_a})}{1.6\nu} \tag{14}$$

where ν is the water vapor pressure difference between the intercelular spaces and the atmosphere, $\frac{p_i}{p_a}$ is ratio of partial pressure CO_2 in intercellular spaces to that in atmosphere. From equation (14) is clear, that the smaller the $\frac{p_i}{p_a}$ is, the higher $\frac{A}{E}$ is. Transpiration efficiency characterized by $\frac{A}{E}$ is negatively related with Δ and that means changes in the carbon isotope composition. When the $\frac{A}{E}$ increase (due to decreasing of $\frac{p_i}{p_a}$), the Δ decrease, therefore the ^{13}C isotopes are more represented (Farquhar et al., 1989). Hence, the water-use efficiency is in direct positive relation with $\delta^{13}C$. The greater water-use efficiency is (that points at bad water conditions), the higher amount of ^{13}C isotopes are represented in plants tissue. Likewise the increasing of precipitation and humidity cause decreasing of $\delta^{13}C$ (Gröcke, 1998).

Salinity Because of tight relationship between water availability and salinity it is clear that it plays important role in $\delta^{13}C$ values. The somatal closure is typically associated with increase of salinity (Farquhar et al., 1989). Accordingly, when the salinity is high, the

water-efficiency rises and then the δ^{13}C increases. It is because the salinity reduce stomatal aperture therefore $\frac{p_i}{p_a}$ decreases and δ^{13}C can reach high positive values as 11‰ (Gröcke, 1998). Interesting knowledge is a relation of concentration CO_2 to increasing salinity. From Farquhar et al. (1989); Warren et al. (2001) is clear relation between the water-use efficiency and the amount of CO_2, but Gröcke (1998) mentioned that an increase in CO_2 enhance the salinity effect on δ^{13}C. This is undirected relation between salinity and CO_2 concentration.

9.2 Influence of CO_2 concentration and partial pressure

Carbon isotope composition of the atmosphere is not a constant, but varies with space and time as does CO_2 content (Korner et al., 1988). Influence of increasing atmospheric CO_2 have minimal effect on δ^{13}C values in plant tissue (Gröcke, 1998). There are studies which the influence of atmospheric CO_2 observe. The results were often negative (for example see Nguyen Tu et al. (2004)). It is true, that the rate of discrimination is influenced by partial pressure of CO_2 - the higher pCO_2, the higher the isotope discrimination is (Nguyen Tu et al., 2004). It is because of sufficiency of CO_2 is ensured by higher pCO_2 and that is why the isotope discrimination is higher. If there are lack of CO_2, that means low pCO_2 pressure, the discrimination is lower.

However, not all plants are sensitive to pCO_2 variations. Not only one study have result, that the pCO_2 or CO_2 concentration has no or minimal effect on isotopic composition (Nguyen Tu et al., 2004; Hamilton et al., 2001). Better tool for observing concentration of CO_2 in atmosphere is based on number of stomata and stomatal parameters. Stomatal density is used for determination if the leaf is sun or shade leaf of for determination environmental conditions in general (Lockheart et al., 1998).

On the other hand, there are studies, where the influence of CO_2 is. In Bocherens et al. (1994) is mentioned, that equations linking δ^{13}C values of plants to those of inorganic-carbon source are function with negative slope of the ratio $\frac{p_i}{p_a}$ (p_i - pCO_2 inside the leaf, p_a - pCO_2 atmospheric). Therefore any parameter which leads to variation in this ratio will provoke variations in δ^{13}C values of the plant and the influence of environmental factors is summarized in Fig. 11. Depend on this statement is that reducing partial pressure of CO_2 in the intercellular leaf space (caused by closure of stomata as response on water-

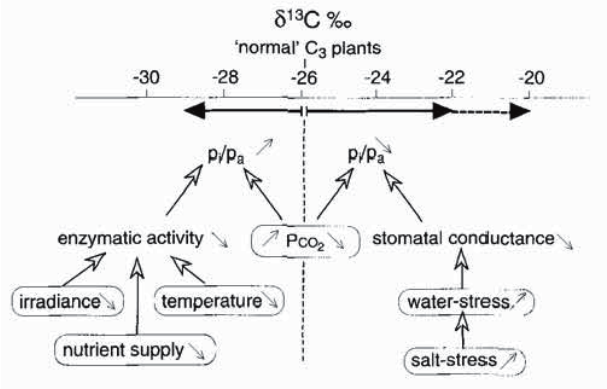

Figure 11: Effects of factors influencing the variation of $\delta^{13}C$ values in C_3 plants, established from Tieszen (1991), (Bocherens et al., 1994).

stress, increasing altitude (Korner et al., 1988) or decreasing atmospheric pCO_2) leads to a decrease of $\frac{p_i}{p_a}$, that mean more positive $\delta^{13}C$ values (Bocherens et al., 1994).

Also in Gröcke (1998) is mentioned, that the CO_2-recycling under a forest canopy causes $\delta^{13}C$ values of plant become more negative. This effect is probably enhanced by light intensity influence, which has greater effects on $\delta^{13}C$ values variability, than the temperature itself (Gröcke, 1998).

9.3 Light intensity influence

The light intensity is very closely related with a vertical variability of carbon isotopic composition (see chapter 9.6). There are studies about differences in ^{13}C content between sun and shade leaves. This also relates with anatomy of leaves, when the variations in the chemical composition of leaves may account for differences in bulk carbon isotope values. In general, the less negative $\delta^{13}C$ values of the sun leaves clearly indicate a reduction in the effect of fractionation against ^{13}C in compare with the shade leaves. Because one of factors influencing the $\delta^{13}C$ value is $\frac{p_i}{p_a}$ ratio (Lockheart et al., 1998), therefore differences between sun and shade leaves are directly related to p_i - lower p_i is reflected by less negative $\delta^{13}C$ values. The explanation of this phenomenon can be:

1) less stomata on the sun leaves, it reduce CO_2 uptake and thus lower fractionation,

2) in the sun leaves is higher photosynthetic rate,

3) stomatal closure due to greater elementar factor as wind or temperature, decrease of stomatal conductance.

For better choosing, which explanation is more correct, it is appropriate to know stomatal density (see Lockheart et al. (1998)).

9.4 Latitudinal and altitudal influence

Korner et al. (1988) observed increasing δ^{13}C with altitude. There are trends, that with altitude decrease level of O_2 and the partial pressure of CO_2 is changing. This induce the increase of ^{13}C values and the plant tissues are ^{13}C-enriched (Korner et al., 1991). δ^{13}C signature of conifers wood and altitude were positively related with increase in δ^{13}C of 2.53‰ per kilometer of altitude. Also there was weak positive relationship between gradient in CO_2 partial pressure into the leaf and altitude (Warren et al., 2001). ^{13}C isotope discrimination consistently decreased (δ^{13}C increased) with altitude where the average change amounts to $+1,2$ ‰ per 1000 m of altitude (Korner et al., 1988). That means, the further from the equator, the greater ^{13}C content is in plant tissue.

Lowland sites between the equator and the polar end of higher plant distribution reveal a significant latitudinal decrease in discrimination: δ^{13}C values from the higher latitudes have greater ^{13}C content of plant tissue (Korner et al., 1991). Both variability can be caused by changing environmental conditions in different latitudes and altitudes, as water availability or temperature.

9.5 Fractionation of C_3,C_4 and CAM

As was written above in chapter 2.1.1, plants have different types of metabolism and thanks that, the different isotope fractionation is observed. C_3 pathway is well distinguished from C_4 or CAM because of its low δ^{13}C values. The values are from $-12,4$ ‰ to -37 ‰, the median is -27 ‰. In contrast the C_4 plants, thanks another enzyme (PEP) with smaller discrimination rate, have δ^{13}C values around -14 ‰. CAM pathway has the same enzyme as C_4, that is why the values of δ^{13}C are very similar - around -11 ‰. Therefore C_4 and CAM cannot effectively distinguish, unlike the C_3 from C_4 or CAM are very well distinguishable (John et al., 2007).

Also differences in bulk δ^{13}C of plant parts are observed as common. Plant roots are enriched relative to leaves in case of C_3 and for C_4 are similar of slightly depleted related to leaves (Hobbie and Werner, 2004). Differences related to leaf morphology are observed

as well - deciduous species are generally more depleted in ^{13}C than evergreens (John et al., 2007). The thicker the assimilating tissue, the smaller is ^{13}C discrimination (Korner et al., 1991).

9.6 Seasonal, spatial and growth variability in $\delta^{13}C$ values

Spatial (vertical) variability Recent plants are tested and observed for their vertical variability in $\delta^{13}C$ values. In pluvial tropical forest and in temperate beech forest the stratification of $\delta^{13}C$ was demonstrated (Bocherens et al., 1994). It is clear, that many factors influence the variability. There are vertical and seasonal patterns thank them is explained the variability. The vertical patterns point at the fact, that $\delta^{13}C$ values are not the same in tree canopy and the base. Main factor is specific leaf area, which decrease with height in the canopy in all species. Therefore the photosynthetic rate (A) increase from the base to the top of the crown. Also respiration rate (R_D) increase with canopy height (Ubierna and Marshall, 2011). The result was, that in both cases there was $\delta^{13}C$ enrichment of canopy ($-25‰$) against $\delta^{13}C$ of underbush near the forest floor ($-35‰$) (Bocherens et al., 1994; Koch, 1998). The $\delta^{13}C$ enrichment of canopy was observed as a trend (Ubierna and Marshall, 2011).

Seasonal variability of carbon isotopic composition Seasonal variability is factor influencing on $\delta^{13}C$ values. Recent trees were observed and there are seasonal trends. The $\delta^{13}C$ values were measured lower from May to June. Values are different depending on species, but within species or concrete tree displayed ^{13}C enrichment from May to June and rapid ^{13}C depletion from July to August (Ubierna and Marshall, 2011). Maximum carbon isotope discrimination values were observed also in arid plants. The values tended to be greatest in cooler winter and spring months. The lowest ^{13}C values were in autumn as well as were observed in tropical and temperate plants. The most likely explanation is, that the carbon used for producing new growth after rain is derived from stored carbon. The stored carbon has been laid down at the end of last season and therefore plants at the beginning of growing season seem to have lower $\delta^{13}C$ values (Ehleringer et al., 1992). On biochemical site, there are evidence, that carbon isotopic composition of glucose, galactose, arabinose and xylose are variable in relation with season. All compounds have

different number of carbon atoms however the isotopic variation have the same trend - ^{13}C depletion during October and enrichment during May (Dungait et al., 2010). The seasonal $\delta^{13}C$ variation of conifers wood were also observed and points that ^{13}C depletion differs between species (Warren et al., 2001).

In general, it seems that the main influence on seasonal and spatial variability could have the photosynthetic rate (connected with differences between species), respiration rate, stomatal conductance, ratio in source of carbon and also water-use efficiency (Farquhar et al., 1989; Ubierna and Marshall, 2011). Another reason of this variability can be the rate of drying soil or ratio of CO_2 concentrations in the leaf intercellular spaces to that in the atmosphere (Ehleringer et al., 1992). This topic is not observed very well, the variability of data interpretation can be often.

Temperature From majority of paragraphs above the great influence of temperature is clear, but not direct influence. There are no results which would proof direct influence of temperature (Gröcke, 1998), rather the temperature is important cofactor and helps another factors change $\delta^{13}C$ values.

Growth variability Also the growth variability is mentioned in couple of studies. It seems, that there is possibility of ^{13}C enrichment in growing leaves in contrast with mature leaves. It can be caused by factors of expanding leaves - low photosynthetic rate and leaf mass area. Therefore it can be observed the respiratory enrichment in expanding leaves. The respiratory enrichment also depend on the substrate of respiration (Ubierna and Marshall, 2011). This topic has not been very observed yet, the results have to be discussed.

9.7 Variation in compounds

For qualitative analysis of carbon isotope is very important to know, what compound is measured. It is because of different enrichment or depletion of compounds. Lipids or lignin, both can be isotopically lighter (depleted) than the whole tissue (John et al., 2007; Bocherens et al., 1994). In order for ^{13}C it is alkanes $<$ lipids $<$ bulk. Alkanes related to bulk are depleted about $4-6$ ‰ in case of C_3 plants ($8-10$ ‰ for C_4 plants)

(Hobbie and Werner, 2004). In contrast, cellulose and other carbohydrates are heavier about $1-2$ ‰ than whole tissue (John et al., 2007). Leaf proteins are depleted in contrast with seed proteins which are enriched ^{13}C (Bocherens et al., 1994).

10 Paleoclimate and paleoenvironmental reconstructions

In many studies are improved paleoclimate models based on different datasets. For the quality of paleoclimate model is necessary to have as wide data as is possible. In data spectrum, from the isotopic data is appropriate to have isotopic analysis of oxygen, carbon and hydrogen. All these elements can be measured in different way within the individual elements - see chapter 7. In text below is example of paleoclimate and paleoenvironmental reconstruction from published studies. The main topic is interpretation of carbon isotope analysis data. In Fig. 11 is showed, what can influence the δ^{13}C value, directly or indirectly.

In case of ^{13}C-enrichment of fossil sample there are at least 5 different interpretations of paleoenvironment and factors influenced the isotope composition.

^{13}C-enrichment of atmospheric CO_2 According the chapters above, one of the possible cause of ^{13}C-enrichment is ^{13}C-enrichment of atmospheric CO_2 (Nguyen Tu et al., 2002). For better reconstruction of atmospheric conditions is necessary to have other data source, for example isotopic composition of oxygen (for temperature information), carbon isotopic composition of sediments or directly carbonatites, data from ice core etc.

Atmospheric CO_2 levels Another possible explanation is on a short time scale a decrease of atmospheric CO_2 levels, which probably induced ^{13}C-enrichment in plant tissues (Nguyen Tu et al., 2002). This effect is caused by lack of atmospheric CO_2 required for biosynthetic processes. The carbon from CO_2 is substituted by recycled carbon in plant tissues.

Atmospheric O_2 level The next interpretation is increase of atmospheric O_2 level, which induce the rising of δ^{13}C (Nguyen Tu et al., 2002). Tissue from plants grown at

reduced oxygen partial pressure are further depleted in δ^{13}C. Decreasing ambient oxygen concentration lowers the level of photorespiration and the growth is changing (Smith et al., 1976).

Temperature, water stress, salinity The ^{13}C-enrichment can be interpreted also as increasing of temperature, which is connected with CO_2 level, partial pressure or photosynthetic rate. It can be also interpreted as water stress conditions or increasing of salinity (Nguyen Tu et al., 2002). Fossil plants growing in stressed ecological conditions generally have more positive δ^{13}C values (Gröcke, 1998). If the composition of sediment indicate saline conditions and δ^{13}C value as well (higher δ^{13}C value - $-20‰$), the saline conditions may be linked to this positive shift (Gröcke, 1998). Additionally the light conditions play also important role in photosynthesis rate (Nguyen Tu et al., 2002). All these interpretations have to consider all factor influencing on plant and the carbon saving. It have to consider the environmental factor as well as the biosynthetic factors and plant physiology. There are many factors, which should not be forgotten to be considered during the interpretation of data.

11 Discussion

The carbon isotope composition it seems to be very powerful tool for paleoenvironmental and paleoclimate reconstructions. However, all methods have some problems, the carbon isotope analysis and interpreting of data as well. The first problem can be bad choice of analyze. It is very important to think out, which of analysis is appropriate for concrete sample. All-important is also to know the sample, what it is. If it is plant crop, then what part of crop it is. The more information we have about the sample, the better interpretation can be. Therefore, as a mistake can be lack of information about sample.

Environment When we have results of isotope analysis, we have to know which values are common for which environment - each environment have own conditions and different levels of factors influenced the δ^{13}C values. Very good is to have some information about environment from another data sources - from sediments, ice cores or drills. After that we can try to interpret measured data.

Compounds and degradation Big problem can be preservation of isotopic abundances in fossil plants because of degradation of organic matter may lead to changes in δ^{13}C values. It is known, that values for specific compound in plants are different and each compound is more or less preserved in different environments. Plant remains, which have endured carbonization prior to burial, have their δ^{13}C values preserved (Bocherens et al., 1994). This is one way, how preserve plant δ^{13}C values. Another preservation way can be early diagenesis, which involves degradation and loss of cellulose, than there is selective preservation of lignin-like macromolecular substances in wood tissue (Bocherens et al., 1994). But this type of preservation changes δ^{13}C values significantly. The problem of preservation is, that carbohydrates have low resistance to diagenesis (Gröcke, 1998), therefore δ^{13}C values are measured on compounds, which are degradation-resistant. One of degradation-resistant compound can be leave waxes of cuticle in general. The measured values of fossil plants are ^{12}C-enriched by approximately 1-2‰ in comparison with recent plants due to preferential loss carbohydrates during the preservation processes (Gröcke, 1998). This should be considered, when interpreting measured data, this is another possible mistake.

Space and time Another think is, what the isotopic composition record? Global or local trends, long-time or short-time changes in environment and mass or individual changes? Most of these question can be answered, if we have sufficient information. But if we have small amount of samples or only one species of plant, we can not reconstruct palaeoenvironment without another external data (another isotopic analysis, analyze of sediments, etc.) or some global trend. Recent studies have indicated, that not only local environmental factors control fossil δ^{13}C plant values, but also the global signature can be recorded (Gröcke, 1998). The border between global and local trend have to be detected by another source of data.

Environmental factors In concrete interpretation is necessary to be careful on details as seasonality, vertical variation, differences in δ^{13}C between species, altitude and latitude etc. It is very difficult to interpret only one main factor which caused δ^{13}C variation, because in most recent cases it is combination of many of them. In chapters above there is linked, that for example variation only of temperature or variation only of pCO_2 can

not be interpreted from variation δ^{13}C values, because their direct influence is not proven in fossil plants nor recent. And it is important that when sometimes something seems to be only one main influence, something is wrong, because always the δ^{13}C variation is caused by two or more factors (temperature, spatial variation, light, water availability, pCO_2, type on photosynthetic pathway, measured compound, etc.).

Metabolism Differences in δ^{13}C values between C_4, C_3 and CAM plants are well known. In fossil samples have to be considered, when each photosynthetic pathway arise in evolution. These different pathway required individual access in interpretation. Each pathway is typical for different environment and it is linked with different factor in different environments as was said above.

12 Conclusions

For interpretation of measured data from carbon isotopic analysis is very important to consider all possible influencing factors, which can cause δ^{13}C variability. Between this factors can be included external factors, effect in leaf tissue during fixation CO_2 and cellular pathways for incorporating carbon atoms. As external factors consider variability of temperature, water availability and humidity, light intensity, partial pressure of CO_2 and O_2, salinity, altitude and latitude, seasonality, source of nutrients and atmospheric composition. Effects during fixation of CO_2 are dependent mainly on type of photosynthetic pathway (C_3,C_4 or CAM) and enzymes which the CO_2 fixate (RUBISCO, PEP and their rate of discrimination). But it is important to consider stomatal density, stomatal conductance as well as partial pressure of CO_2 in the leaf intercellular spaces. The last part of factors are contained inside the cell - biosynthesis pathways of compounds as carbohydrates, alkanes, lipids and waxes. These compounds are synthesized in pathways composed of four reactions at least and each reaction can discriminate the heavier carbon isotope. Therefore the longer the biosynthetic pathway is, the ^{13}C-depleted the compound is. There are also exception as cellulose and carbohydrates, which are ^{13}C-enriched. However, if we consider all this, the data interpretation can be correct.

13 Acknowledgments

I am grateful to RNDr. Jiří Kvaček, CSc. for consultations, corrections and for connections with Mgr. Petr Pokorný, PhD. and Nikolai Pedentchouk, PhD. I thank Mgr. Petr Pokorný, PhD. for suggesting locality Valča and for treatment and provision of samples from Valča. Also I thank him for consultation about locality Valča. Many thanks to Nikolai Pedentchouk, PhD. for consultation methods of mass spectrometry and literature recommendation. Thanks to National museum in Prague for providing SEM to observe samples. I am grateful to Ms. Hana Šimsová for language correction.

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15 Attachments

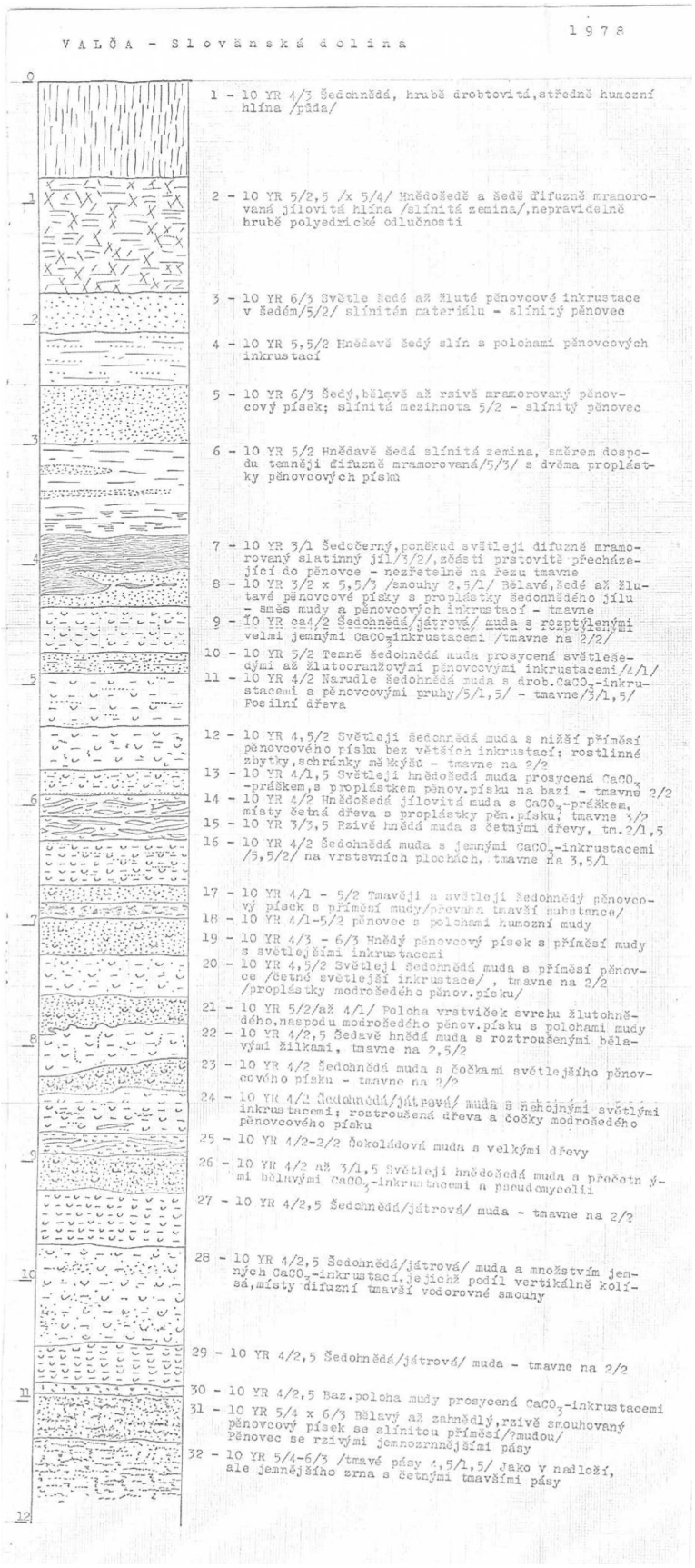
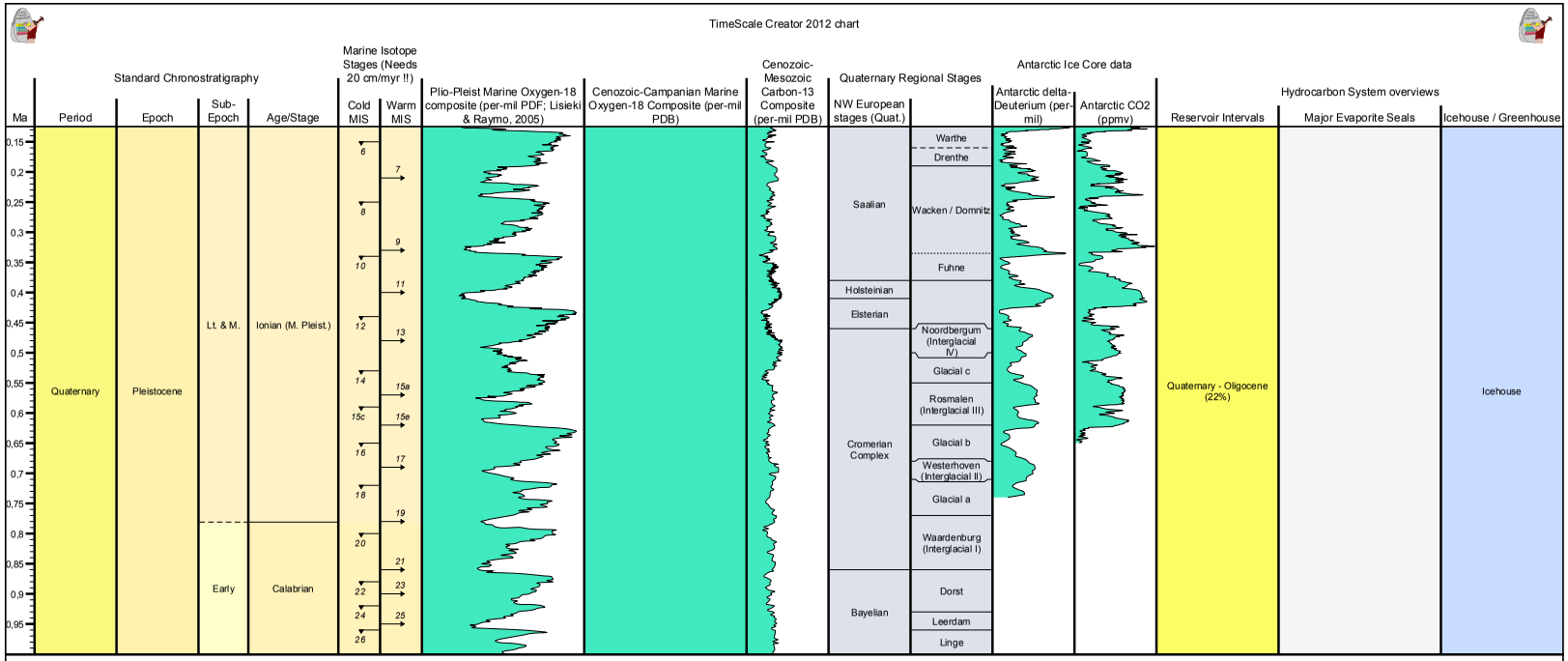


Figure 12: The profile of locality Valča documented and described by Vojen Ložek in 1978, not published.



Selected sources of data for main column sets

Standard Chronostratigraphy :: *International Commission on Stratigraphy ICS. Click Geol. TimeScale Foundation GSSP for boundary (GSSP) definitions, status and nomenclature.*

Sub-Epoch :: *Special set (not official) as used by Exxon, etc.; but with Paleocene divisions as suggested by ICS Paleocene subcommission (not Exxon's usage)*

Marine Isotope Stages (Needs 20 cm/myr !!) :: *MIS numbering, including Pliocene extension, from Crowhurst (2002) -- [see Delphi Project, Shackleton et al., Cambridge University -- http://131.111.44.196/coredata/v677846.html) for relatively placement to their O-18 curve]. Warm MIS (odd numbers), and Cold MIS (even numbers) listed in separate columns to avoid over-crowding.*

Cold MIS :: *Even numbers are "glacial" or cold events. MIS numbering, including Pliocene extension, from Crowhurst (2002) -- [see Delphi Project, Shackleton et al., Cambridge University -- http://131.111.44.196/coredata/v677846.html) for relatively placement to their O-18 curve].*

Warm MIS :: *Odd numbers are "interglacial" or warm events. MIS numbering, including Pliocene extension, from Crowhurst (2002) -- [see Delphi Project, Shackleton et al., Cambridge University -- http://131.111.44.196/coredata/v677846.html) for relatively placement to their O-18 curve].*

Plio-Pleist Marine Oxygen-18 composite (per-mil PDF; Lisieki & Raymo, 2005) :: *Lisiecki, L. E., and M. E. Raymo (2005), A Pliocene-Pleistocene stack of 57 globally distributed benthic d18O records, Paleoceanography, 20, PA1003, doi:10.1029/2004PA001071.*

Cenozoic-Campanian Marine Oxygen-18 Composite (per-mil PDB) :: *Derived from Cramer (2009) -- See Saltzman and Thomas (GTS2012 Carbon-isotope chapter); but only every 10th item from 9-point averaging of Benthic foraminifer compilation (29000 data points in original) is shown here; NOTE: Cramer had two sets -- original and "adjusted" (which removed many of the original)*

Cenozoic-Mesozoic Carbon-13 Composite (per-mil PDB) :: *CENOZOIC (0-70 Ma) = Derived from Cramer (2009) -- See Saltzman and Thomas (GTS2012 Carbon-isotope chapter); but only every 10th item from 9-point averaging of Benthic foraminifer compilation (29000 data points in original) is shown here; NOTE: Cramer had two sets -- original and "adjusted" (which removed many of the original)); LATE CRET (70-100 Ma) = Composite English Chalk by Jarvis et al. (2006) plus Camp-Maastr boundary from Voight et al. (2010); EARLY CRET (100-145 Ma) = Gale et al. (2011; Cret. Res.), Herrle et al. (2004, EPSL), Renard et al. (2005) and Follmi et al (2006, Paleoceanography); JURASSIC (145-200 Ma) = mainly Jenkyns et al. (2002) enhanced by Glowniak and Wierzbowski (2007) for mid-Oxf, Kemp et al. (2005) for early Toarcian, and Palfy et al (2001) for Tri-Jur boundary; TRIASSIC = latest Perm from Payne et al'04; early Triassic from Galfetti et al.'07 who used N.Amer. ammon. zones; mid-Triassic from Payne'04; then Korte et al'05 for Carnian-Rhaetian ("bad" points removed from Korte'05, but his entire point-set is not yet inserted here)*

Quaternary Regional Stages :: *P. Gibbard (2004, and references therein; pers. commun. to J.Ogg, 2006)*

NW European stages (Quat.) :: *P. Gibbard (2004, and references therein; pers. commun. to J.Ogg, 2006)*

NW European substages :: *P. Gibbard (2004, and references therein; pers. commun. to J.Ogg, 2006)*

Antarctic delta-Deuterium (per-mil) :: *Jouzel et al. (2004). Accessed from NCDC Paleoclimatology Program.*

Antarctic CO2 (ppmv) :: *0-11 ka = Taylor Dome (Indermuhle et al, 1999a); 11-27 ka = Taylor Dome (Smith et al, 1999); 27-60 kyr = Taylor Dome (Indermuhle et al, 1999b). 64-417 kyr = Vostok (Barnola et al., 2003); 417-649 kyr = Dome C (Siegenthaler, 2005). Accessed from NCDC Paleoclimatology Program, and spliced together.*

Reservoir Intervals :: *With % of world's trapped reserves: Ulmashek and Klemme, 1990 -- Lowell Waite (author) and Roger Gilcrease (compiler), 2002. Phanerozoic Cycles and Events (NV PXD Global Stratigraphic Chart 02.DSF), March 27, 2002 (printed by Pioneer Natural Resources; permission provided by L. Waite).*

Major Evaporite Seals :: *Major evaporite packages (seal facies) from Sun, 1994 -- as summarized by Lowell Waite (author) and Roger Gilcrease (compiler), 2002. Phanerozoic Cycles and Events (NV PXD Global Stratigraphic Chart 02.DSF), March 27, 2002 (printed by Pioneer Natural Resources; permission provided by L. Waite).*

Icehouse / Greenhouse :: *after Fisher, 1981, as summarized by Lowell Waite (author) and Roger Gilcrease (compiler), 2002. Phanerozoic Cycles and Events (NV PXD Global Stratigraphic Chart 02.DSF), March 27, 2002 (printed by Pioneer Natural Resources; permission provided by L. Waite).*