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Chapter 8

Dehydrogenase Activity in the Soil Environment

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Additional information is available at the end of the chapter

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1. Introduction

The main purpose of the chapter is clarify description of the role of intracellular enzyme-dehydrogenase in the soil environment, as well as presentation of soil factors, influencing an enzymatic activity, by either stimulation or inhibition effect on soil dehydrogenase activity (DHA).

The most common laboratory procedure used for DHA determination is the method developed by Casida et al. (1964). According this method, specific dyes such as the triphenyltetrazolium chloride (TTC), that can specify the flow of electrons are useful indicators of electron transport system (ETS) activity. By the reduction of colorless, water soluble substrate (TTC) by dehydrogenases present in the soil environment, an insoluble product with red color (triphenylformazan-TPF) is formed. TPF can be easily quantified calorimetrically at the range of visible light (485 nm). This test however, reflected positive answer only at neutral range of pH and in presence of calcium carbonate for buffering soil system. Briefly, if the red colors of soil samples prepared for spectrophotometer analyses are more intensive, the measured level of DHA is higher. Consequently, soil samples without red colors or those with light red colors are characterized by lower DHA values.

Determination of DHA in the soil samples gives us large amount of information about biological characteristic of the soil. It was confirmed that although oxygen and other electron acceptors can be utilized by dehydrogenases, most of the enzyme is produced by anaerobic microorganisms. In other words, soil DHA strongly increases under anaerobic conditions.

Several environmental factors, including soil moisture, oxygen availability, oxidation-reduction potential, pH, organic matter content, depth of the soil profile, temperature, season of the year, heavy metal contamination and soil fertilization or pesticide use can affect significantly DHA in the soil environment. In the current chapter we would like to concentrate on precise description of mentioned factors effect on soil DHA level. Presented

results of laboratory experiments were conducted on different soil types, representing dominant types of arable soils in Poland, in order to demonstrate changeability and variability of DHA at diverse soil environment.

2. Role of dehydrogenase activity in the soil environment

There are lots of enzymes in soil the environment, such as Oxidoreductases, Hydrolases, Isomerases, Lyases and Ligases. Each of them play key biochemical functions in the overall process of material and energy conversion (Gu et al., 2009).

Soil dehydrogenases (EC 1.1.1.) are the major representatives of the Oxidoreductase enzymes class (Gu et al., 2009). Among all enzymes in the soil environment, dehydrogenases are one of the most important, and are used as an indicator of overall soil microbial activity (Quilchano & Marañon, 2002; Gu et al., 2009; Salazar et al., 2011), because they occur intracellular in all living microbial cells (Moeskops et al., 2010; Zhao et al., 2010; Yuan & Yue, 2012). Moreover, they are tightly linked with microbial oxidoreduction processes (Moeskops et al., 2010). What is important dehydrogenases do not accumulate extracellular in the soil.

Dehydrogenases play a significant role in the biological oxidation of soil organic matter (OM) by transferring hydrogen from organic substrates to inorganic acceptors (Zhang et al., 2010). Many specific dehydrogenases transfer hydrogen to either nicotinamide adenine dinucleotide or nicotinamide adenine dinucleotide phosphate (Subhani et al., 2001). Throughout mentioned co-enzymes hydrogen atoms are involved in the reductive processes of biosynthesis. Due to this fact, the overall DHA of a soil depends on the activities of various dehydrogenases, which are fundamental part of the enzyme system of all living microorganisms, like enzymes of the respiratory metabolism, the citrate cycle, and N metabolism (Subhani et al., 2011). Thus, DHA serves as an indicator of the microbiological redox-systems and could be considered a good and adequate measure of microbial oxidative activities in soil.

Brzezińska et al. (2001) found that active dehydrogenases can utilize both O_2 and other compounds as terminal electron acceptors, although anaerobic microorganisms produce most dehydrogenases. Therefore, DHA reflects metabolic ability of the soil and its activity is considered to be proportional to the biomass of the microorganisms in soil. However, the relationships between an individual biochemical property of soil DHA and the total microbial activity is not always obvious, especially in the case of complex systems like soils, where the microorganisms and processes involved in the degradation of the organic compounds are highly diverse (Salazar et al., 2011).

3. Soil factors stimulating dehydrogenase activity

Among different environmental factors with special emphasis on enzymatic activities in the soil environment it is possible to screen some, which have positive impact on DHA. The most important soil factors stimulating soil DHA are described below.

3.1. Soil moisture

Life in the soil environment, as well as land use is related to alternate cycles of humidification and drainage (Wolińska & Bennicelli, 2010). Water availability strongly affects on soil microbial activity, community composition (Geisseler et al., 2011), and consequently on soil enzymatic activities. As soils dry, the water potential increases, and as well microbial activity as intracellular enzyme activity slows down (Geisseler et al., 2011). In the case of wet soils, increased moisture could bring into soil solution soluble OM, what might be responsible for increase of bacterial population number (Subhani et al., 2001). What is important, we should have consciousness that any compound, which alters the number or activity of microorganisms, could on the other hand affect on soil biochemical properties, and ultimately also on soil fertility and plant growth (Subhani et al., 2001).

A basic hydrophysical characteristic of soil is water retention, that can be described as a dependence between soil water content and soil water potential. Soil water content in the function of the soil water tension is described by pF curve, which provides information about the ability for water retaining by the soil pores at any given water tension, or conversely, how tightly a water is held between soil aggregates (Wolińska & Bennicelli, 2010).

The Figure 1 demonstrates diminishing trend for DHA behaviour at different soil moisture, described as water potential values. During this experiment gig set of soils (n=315), including all representatives among the most typical Polish mineral soils (*Eutric Cambisol*, *Eutric Histosol*, *Eutric Fluvisol*, *Mollic Gleysol*, *Orthic Podzol*, *Rendzina Leptosol*, *Haplic Phaeozem*) were investigated. However, each of soil unites displayed DHA reducing trend with increase of soil pF value, what means that maximum values of DHA in the soil profiles are indirectly connected with maximum soil moisture (pF 0).

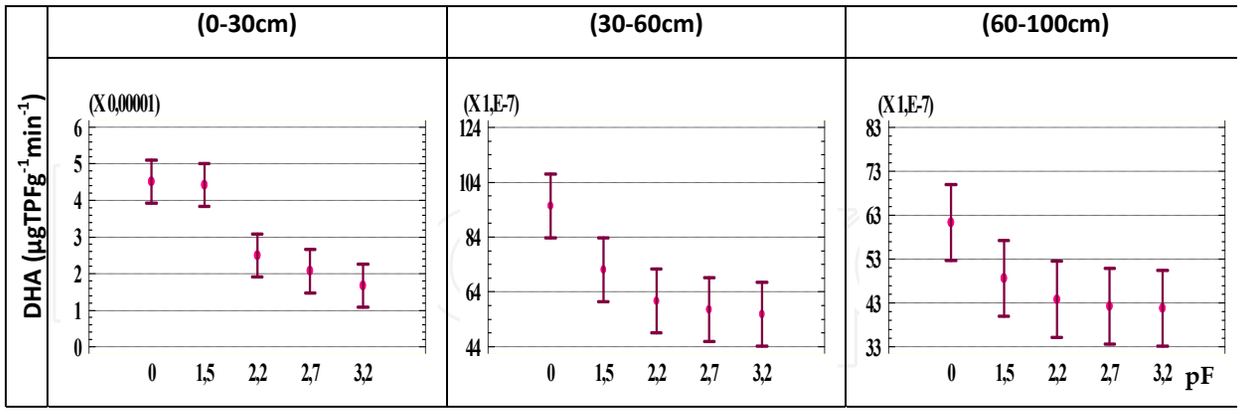

Figure 1. DHA (µgTPFg^{-1}min^{-1}) dependence from water potential (pF) at different mineral Polish soil types, during reoxidation (n=315), according to Wolińska (2010)

Statistical relationships between DHA and soil water content, described as pF value in the range of pF0 – pF3.2, determined by Wolińska & Bennicelli (2010) are presented in Table 1.

Founded significant negative relationships between DHA and pF are confirmed by our above mentioned observations, that DHA is strongly affected by soil moisture. These strong

correlations are undoubtedly connected with the fact that the metabolism and the survival of soil microorganisms are also strongly impacted by the availability of water (Uhlirova et al., 2005), what is essential for microbial survival and activity. Consequently, low water availability can inhibit microbial activity by lowering intracellular water potential, and thus by reducing of hydration and enzymes activity (Wall & Heiskanen, 2003). Periods of moisture limitation may affect microbial communities through starvation. Thus, the most common environmental stress for soil microorganisms is perhaps drought (Wolińska & Stępniewska, 2011).

It was shown in many studies that DHA is significantly influenced by water content and dropped with the decrease of soil humidity. For example, Gu et al. (2009) observed higher DHA level (even by 90%) in flooded soil, rather than in non-flooded conditions. The higher DHA values in flooded conditions agreed also with results presented by Zhao et al. (2010) and Weaver et al (2012).

DHA response	Depth (cm)	pF
Rendzina Leptosols	0-20	-0.98***
	50-60	-0.95**
Eutric Fluvisol	0-20	-0.97***
	50-60	-0.22 n.s.

*, **, *** - indicate significance at the 5, 1 and 0.1% level, respectively, n.s. – not significant differences

Table 1. Statistical significance of differences between DHA and pF described by correlation coefficient (R) (95% LSD method, n=15), according to Wolińska & Bennicelli, 2010

The decline of DHA with an increase of pF value, could be also explained by the fact, that flooding of soil with water significantly increased the electron transport system (Wolińska & Stępniewska, 2011). Dehydrogenases however, are responsible for electron transport in the soil environment. It was also reported that DHA is higher in flooded, anaerobically soils, than aerobically incubated soils (Trevors, 1984; Subhani et al., 2001).

3.2. Soil aeration state (redox potential and oxygen diffusion rate)

Oxygen diffusion rate (ODR) is usually considered to be the most critical proximal regulator of microbial activities (Hutchinson, 1995). Moreover, it is often assumed that a decrease of soil water content (higher value of pF), cause a significant (P<0.001) increase of ODR and redox potential (Stępniewski et al., 2000; Wolińska & Bennicelli, 2010). The available literature shows that low ODR level, ranged below its critical values (35 μg O_2 $m^{-2}s^{-1}$), is favorable and optimal for DHA (Stępniewski et al., 2000; Brzezińska et al., 2001; Wolińska & Bennicelli, 2010).

We confirmed that dehydrogenases are sensitive enzymes, indirectly depended on the soil aeration status (Wolińska & Bennicelli, 2010). Based on performed measurements we found, that pF constitutes a significant factor, determining ODR in the soil environment, as well as its DHA level (P<0.01). The reoxidation processes, occurring in the direction from pF 0 to pF

3.2, were the reason of DHA inhibition and stimulation of ODR level in the *Rendzina Leptosols* and *Eutric Fluvisol* soil samples (Fig. 2). We also stated that soil DHA at pF 3.2 was lower by about 60.86%, in comparison to the activity estimated at pF 0.

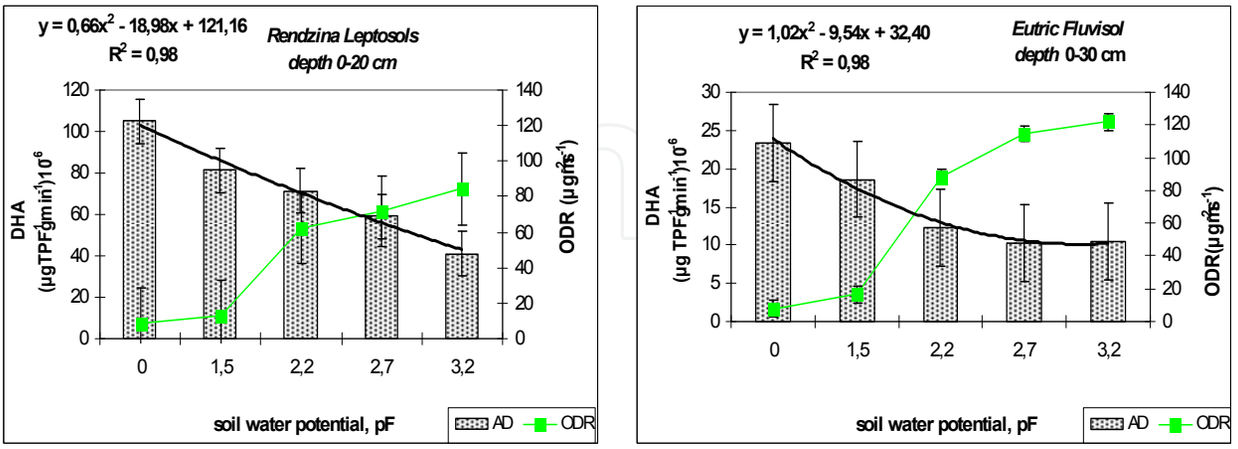

Figure 2. The response of soil DHA to varied aeration factors (pF and ODR), at surface layers of *Rendzina Leptosols* and *Eutric Fluvisol* , during reoxidation process (according to Wolińska & Bennicelli, 2010). Averaged values of three replicates with standard deviations are presented

The Figure 3. demonstrates that low oxygen diffusion rate (2.8-25 µg O_2 m^{-2} s^{-1}) was optimal for DHA, what was also confirmed by correlation coefficient (Wolińska & Bennicelli, 2010; Wolińska, 2010). Our results and founding's are compatible with work of Stępniewski et al. (2000), Brzezińska et al. (2001), and Yang et al. (2005).

Statistical relationships between DHA and ODR, determined for two soil types (*Rendzina Leptosols* and *Eutric Fluvisol*) by Wolińska & Bennicelli (2010) are presented in Table 2. At every case negative correlations DHA-ODR were determined.

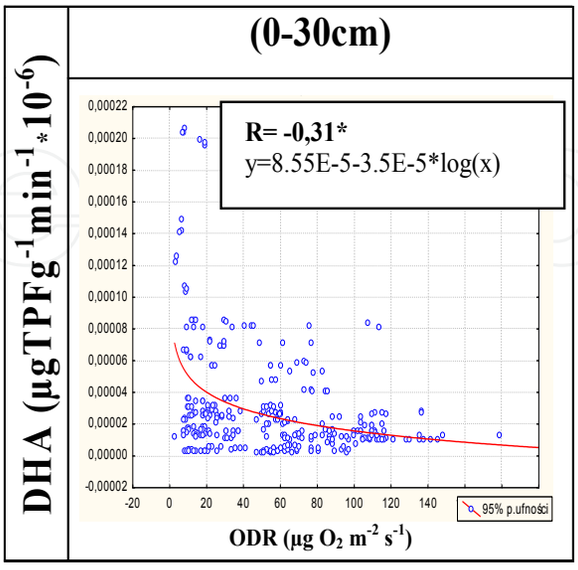

Figure 3. Relationship between DHA (µgTPF$g^{-1}$$min^{-1}$ * 10^{-6}) and ODR (µg m^{-2} s^{-1}), in surface layer of different mineral Polish soil types (n=315, *P*<0.05), according to Wolińska (2010)

DHA response	Depth (cm)	ODR
Rendzina Leptosols	0-20	-0.90**
	50-60	-0.84*
Eutric Fluvisol	0-20	-0.96**
	50-60	-0.16 n.s.

*, **, *** - indicate significance at the 5, 1 and 0.1% level, respectively, n.s. – not significant differences

Table 2. Statistical significance of differences between DHA and ODR described by correlation coefficient (R) (95% LSD method, n=15)

Redox potential (Eh) is the next, important, environmental factor, which expresses the tendency of an environment to receive or to supply electrons in solution (Stępniewski et al., 2005). The well-oxygenated soils are characterized by high values of Eh (600-800 mV), in quite well-oxygenated soils Eh ~ 500-600 mV, whereas in anaerobic conditions drop of Eh below 300 mV or even lower values were observed (Pett-Ridge & Firestone, 2005; Stępniewski et al., 2000).

It is well known, that Eh play a crucial role in regulating microbial activity as well as community structure (Pett-Ridge & Firestone, 2005; Song et al., 2008), and affecting on soil enzymatic activity, especially DHA. Brzezińska et al. (1998) indicated that among all aeration parameters, Eh plays the most important role in determining soil DHA level. Similar conclusions were also reported by Włodarczyk et al. (2001) and Menon et al. (2005).

We founded significant negative relationships between DHA and Eh (Fig. 4) at surface layers of *Mollic Gleysols*, *Eutric Fluvisols*, *Rendzina Leptosols* and *Haplic Phaeozems*, where determined correlation coefficients equaled as follows: r=-0.91*, r=-0.43*, r=-0.47** and r=-0.48** (Wolińska, 2010).

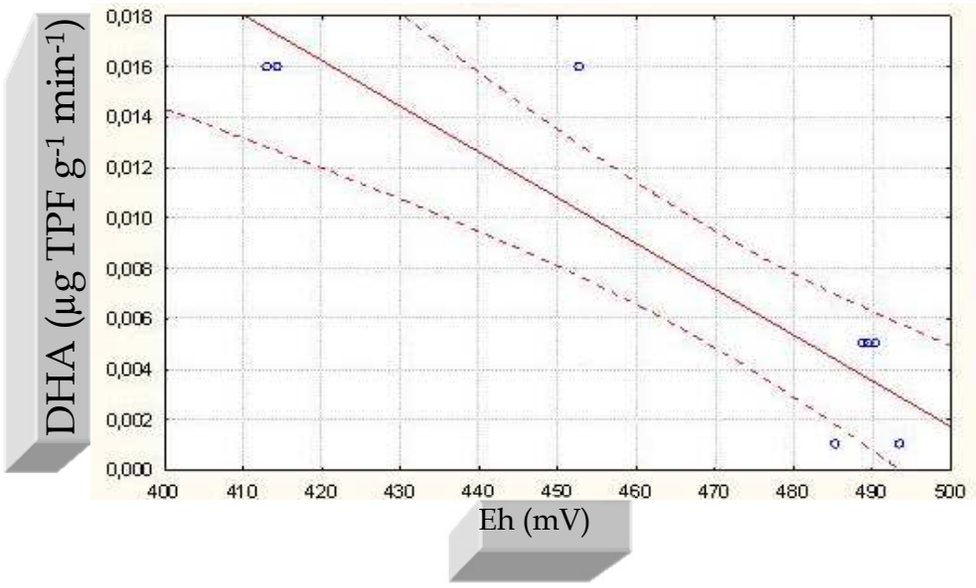

Figure 4. Relationship between soil DHA level and Eh at *Mollic Gleysol* (n=9, r=-0.91*), according to Wolińska (2010)

Negative correlations DHA-Eh were also described by Brzezińska et al. (1998), who determined r=-0.75***, r=-0.83*** and r=-0.87*** for temperature 10, 20 and 30ºC, respectively, and by Stępniewski et al. (2000), and Nyak et al. (2007).

Mentioned relationships DHA-Eh have significant negative character, what means that increase of soil DHA level is indirectly connected with decrease of Eh values, as most of microorganisms, which are responsible for DHA prefer rather anaerobic conditions, and belong to obligate anaerobes. What is more, anaerobic conditions are consequence of flooding and decrease of oxygen availability in soil environment. Competition for oxygen limits aerobic processes and the subsequent oxygen deficiency creates local anaerobic microsites, which stimulates growth of anaerobic bacteria (Wolińska & Stępniewska, 2011), and finally DHA. Also, in the absence of oxygen in the soil a decline of Eh and the reduction of oxidized forms (nitrate, Mn^{4+}, Fe^{2+} and SO_4^{2-}) takes place. Bohrerova et al. (2004) reported that the most common ions forming the redox couples of soil include NO_3^-/NO_2^-, Fe^{3+}/Fe^{2+}, and Mn^{4+}/Mn^{2+}. In the literature data, it was also assumed that DHA is strongly affected by both Fe as Mn presence in the soil (Brzezińska et al., 1998; Włodarczyk et al., 2002).

3.3. Organic matter content

Soil organic matter (OM) has important effects not only on soil enzymes activities but first of all on microorganisms activities. Soil OM has been considered as an indicator of soil quality (similarly like dehydrogenases,) because of its character of nutrient sink and source that can enhance soil physical and chemical properties, and also promote biological activity (Salazar et al., 2011). Interestingly, not only amount of OM in the soil is important but most of all its quality, as OM affects the supply of energy for microbial growth and enzyme production (Fontaine et al., 2003).

It is evident that soil enzymatic activity is strongly connected with soil OM content. The higher OM level can provide enough substrate to support higher microbial biomass, hence higher enzyme production (Yuan & Yue, 2012). Several authors reported positive correlation between DHA and OM content (Chodak & Niklińska, 2010; Moeskops et al., 2010; Romero et al., 2010; Zhao et al., 2010; Yuan & Yue, 2012).

Zhang et al. (2010) indicated also that as well DHA and $CaCO_3$ correlated with OM content, and what is more DHA, OM and $CaCO_3$ were correlated with each other in their spatial distribution, suggesting that abundant OM content contributed to the formation of pedogenic calcium carbonate.

Salazar et al. (2011) hypothesized that activities of dehydrogenases in different forest ecosystems are involved in the carbon cycling, and they also reported their positive relationships. Dehydrogenases, are highly associated with microbial biomass (MB), which in turn affects on decomposition of OM and the release of $CaCO_3$ (Zhang et al., 2010).

We also investigated effect posed by total organic carbon (TOC) and response of DHA in the agricultural used *Mollic Gleysol*, taken from Kosiorów village (SE part of Poland). We

determined significant (P<0.0001) correlation between TOC-DHA (Fig. 5). Mentioned strong relationship was also confirmed by high value of correlation coefficient (r=0.99***). In our laboratory conditions the optimal value of TOC content for reaching maximal values of soil DHA was its level above 25%.

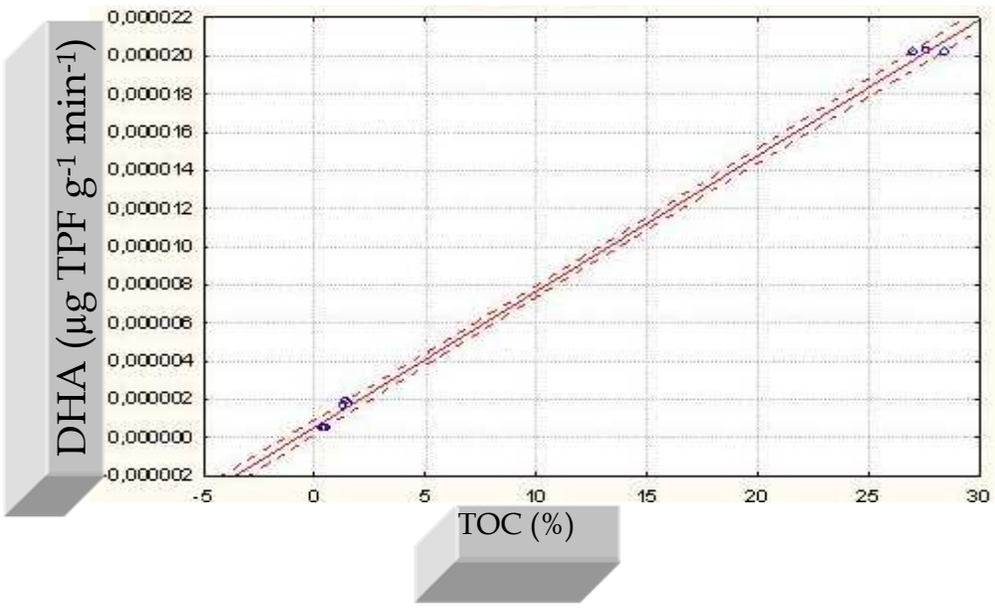

Figure 5. Relationship between DHA and TOC content in the *Mollic Gleysol* (n=9, r=0.99***), according to Wolińska & Stępniewska (unpublished data)

Analogically to our investigations also Koper et al. (2008) found and reported strong significant relationships between DHA and organic carbon content in *Haplic Podzol* soil samples, and they described mentioned correlations by r coefficient ranged between 0.56* and 0.98*.

The study of Kumar et al. (1992) indicated that DHA displayed the close, positive correlations not only with OM content but also with fungal population abundance in four forest stands (two at low and two at higher attitudes).

High correlation coefficient reported for enzymatic activities and TOC level suggested an important role of these enzymes in transformations of basic components of soil OM (Wolińska & Stępniewska, 2011). There is in general agreement with previous results indicated by Pascual et al. (2000), who found that soils characterized with low microbial and biological activity (e.g. low microbial carbon and low respiration rate), also display the lowest values of DHA.

Summarizing, the higher content of OM, the more active the soil microorganisms. Microorganisms accelerate the degradation of OM, which is reflected in soil respiration and release of carbon dioxide from the rizosphere (Zhang et al., 2010), thus DHA is positively correlated with OM content. Similarly, increase of DHA with higher microorganisms number was reported (Fontaine et al., 2003).

3.4. pH

The literature data, currently available, referring to the connections between DHA and soil pH are still ambiguous.

Generally, enzyme activities tend to increase with soil pH (Błońska, 2010; Moeskops et al., 2010) – please put a space before Moeskops. Błońska (2010) determined significant positive correlation (r=0.50***) DHA-$pH_{(water)}$ in the pH range 3.67-5.88.

Fernandez-Calviño et al. (2010) noted significantly positive correlations among soil DHA and pH in the range of 4.1 (pH_{KCl}) and 4.9 (pH_{water}), suggesting that acidity suppressed potential enzyme activity.

Adequately, a study by Levyk et al. (2007) demonstrated that acidic conditions in the pH range between 1.5–4.5 resulted with strong DHA inhibition in relation to alkaline soils, whereas Ghaly & Mahmoud (2006) noted that under acidic conditions with pH less than 6.5, the rate of TTC - specific substrate for DHA, did not decrease.

According to Frankenberger & Johanson (1982), the weakening of enzymatic activity in soil with the increase of soil acidity is the effect of destroying ion and hydrogen bonds in enzyme active centre.

On the other hand, study performed by Włodarczyk et al. (2002) indicated maximum DHA at pH 7.1, similarly to the work of Ros et al. (2003), where optimum for DHA was noted for pH 7.6-7.8. Also Brzezińska et al. (2001) reported that the best pH conditions for DHA ranged between 6.6-7.2.

Natywa & Selwet (2011) noted positive correlation between DHA and pH in soils under maize growth at pH range from 5.17 to 7.27.

Trevors (1984) concluded that very little DHA is observed below pH 6.6. and above pH 9.5. According to Nagatsuka & Furosaka (1980) the optimum range for DHA is contained between 7.4–8.5. However we should realize that many heterogeneous soil types might not be included in mentioned above range.

Our investigations, performed on *Mollic Gleysol* sample (from Kosiorów village) indicated however, that DHA also reached high level at lower pH values–between 5.5-5.73 (Fig. 6). Significant inhibition of DHA (even by 95%) we scarcely noted when soil pH was above 5.75.

It is often assumed that pH may affects soil enzymes level in three different ways (Shuler & Kargi, 2010):

1. by changing in the ionic form of the active sites of the enzymes, which consequently affect the enzyme activity and hence the reaction rate,
2. by altering the three-dimensional shape of enzyme, and
3. by affecting the affinity of the substrate to the enzyme.

Thus, the pH factor is considered to be the best predictor of DHA in the soil environment (Quilchano & Marañon, 2002; Moeskops et al., 2010).

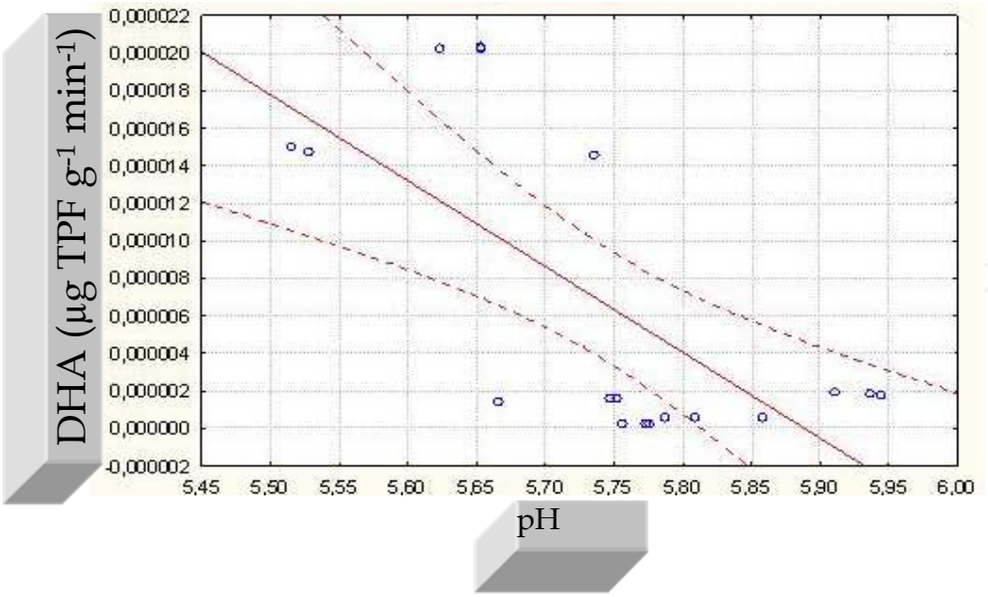

Figure 6. Relationship between DHA and pH values in the *Mollic Gleysol* (n=18, r=-0.70**), according to Wolińska & Stępniewska (unpublished data)

3.5. Temperature

Many researchers have studied effect posed by temperature incubation on soil DHA and/or on soil microorganisms abundance (Subhani et al., 2001; Ghaly & Mahmoud, 2006; Trasar-Cepeda et al., 2007). Taking into account the important fact that DHA is found inside the viable soil microbial cells only, its activity must be the highest at a temperature close to optimum temperature for microorganisms growth and their development (Wolińska & Stępniewska, 2011).

It is known that, the rate of enzyme catalysis generally increases with increase in temperature until the unfavorable temperature, at which enzyme becomes denaturized and hence its activity reduces (Wolińska & Stępniewska, 2011).

Our investigations were concentrated on investigations of DHA changeability at temperature range 5-30ºC, what reflect natural changes of soil temperature during seasons. Surface layer (0-20 cm) of *Mollic Gleysol* was used for experiment. Soil samples were incubated at the following temperatures: 5, 10, 20 and 30ºC. DHA was measured after 30 h incubations at proper temperature and after ethanol extractions. Absorbance was tested at λ=485 nm (UV-1800 Shimadzu). Received results are presented in Fig. 7.

We found growing, linear trend for DHA with increase of temperature at the range from 5 to 30ºC, what we described by R^2=0.97. The differences between DHA level estimated at 5 and 30ºC were significant (P<0.01), analogically like between 5 and 20ºC (P<0.05). The lowest values of DHA at 5ºC were found, where DHA equaled 1.259 (µg TPF g^{-1} min^{-1}*10^{-6}), whereas the same soil sample incubated at 30ºC reached DHA level of 3.149 (µg TPF g^{-1} min^{-1}*10^{-6}), what was by c.a. 60% higher in relation to DHA level from 5ºC. Quite high DHA level (2.741 µg TPF g^{-1} min^{-1}*10^{-6}) was also estimated at 20ºC, where mentioned value was only by 13% lower than maximum DHA, from 30ºC.

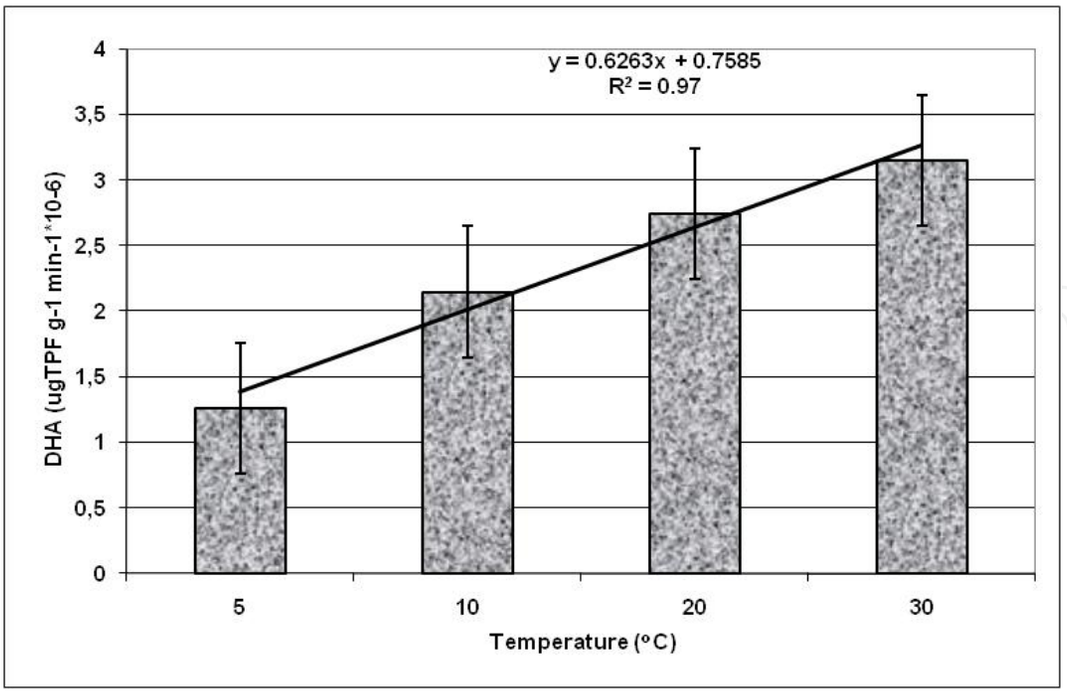

Figure 7. The dependence between DHA and temperature incubation in the *Mollic Gleysol*, according to Wolińska & Stępniewska (unpublished data). Averaged values of three replicates with standard deviations are presented

Casida et al. (1964) indicated that incubation of soil samples at 37ºC increased of soil DHA above the value normally observed at lower temperatures.

Trevors (1984) described positive significant correlation among DHA and temperature in the range from 5 to 70ºC and determined r coefficient on the level of 0.99*. Moreover, study by Trasar-Cepeda et al. (2007) reported that increased temperatures up to 57-70ºC enhanced the product formation in the reaction catalyzed by soil dehydrogenases increased with, explained by the fact that specific substrate (TTC), used for DHA determination, is chemically reduced at high temperatures.

Analogically, Subhani et al. (2001) noted positive correlation in soil samples incubated at 10, 25 and 40ºC (under constant moisture – flooded conditions), what confirmed by r=0.82*.

As suggested by Cirilli et al. (2012) optimum temperature for soil DHA is 30ºC, what is in agreement with our findings. Similarly, Brzezińska et al. (1998) indicated that under laboratory conditions DHA demonstrated the highest activity at 28-30ºC.

3.6. Season of the year

Seasonal variations in both microbial biomass and soil enzymatic activities reflect the combine effects of temperature, moisture, substrate availability and other environmental factors. Dehydrogenases belong to the enzymes displaying strong fluctuations in their activities caused by season of the year, as they are in close relationships with dynamic of microbial activity.

Yuan & Yue (2012) stated the highest DHA level in autumn season and the lowest value of DHA in winter time. The study performed by Piotrowska & Długosz (2012) indicated that DHA level in *Luvisols* revealed significantly higher values in April (by 96%) than in August, probably due to intensive winter wheat growth with an increased secretion of substrates such as polysaccharides, organic acids, which may have affected the growth and activity of microorganisms.

Similarly, our investigations demonstrated the highest level of DHA in *Eutric Fluvisol* sample taken in May (0.0087 μg TPF g^{-1} min^{-1}), than in the same soil type taken in October, where DHA was reduced by 42.5 % (Fig. 8). Quite high level of DHA (lower by 14.9% from its maximum reaching in May) we also noted in July. Moreover, we did not found significant differences (P>0.05) in DHA values during autumn season, where DHA remained on similar level equaled 0.000598 (μg TPF g^{-1} min^{-1}) and 0.0005 (μg TPF g^{-1} min^{-1}), for September and October, respectively.

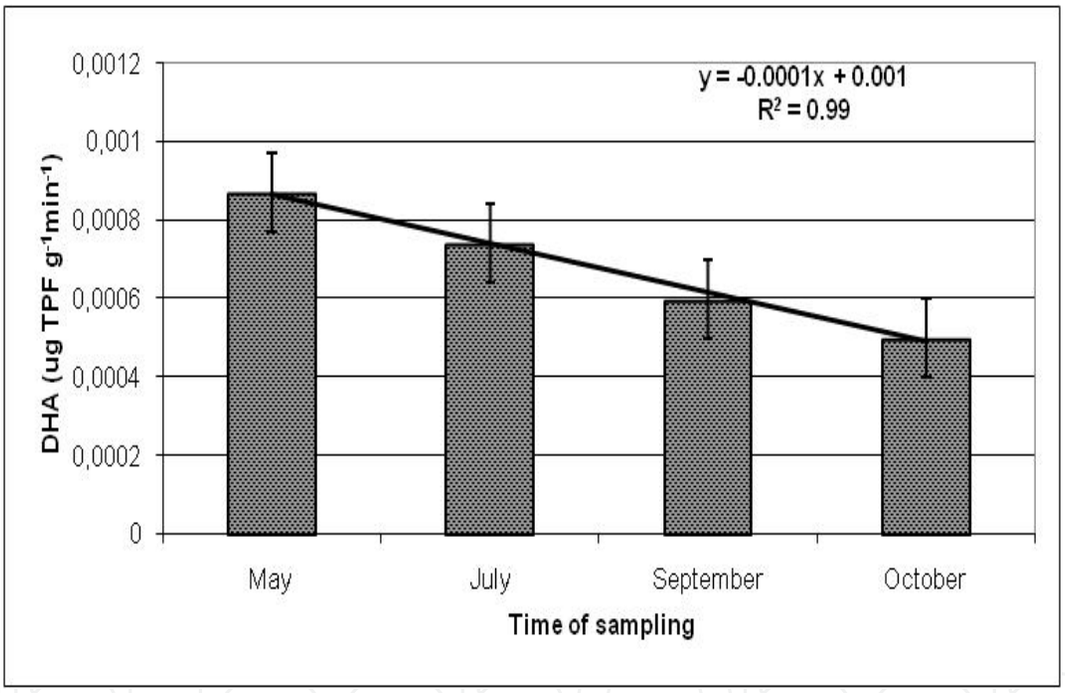

Figure 8. Effect of the season of the year on soil DHA in the *Eutric Fluvisol*, according to Wolińska & Stępniewska (unpublished data). Averaged values of three replicates with standard deviations are presented

Analogical trend like our observations, related to high Oxidoreductases activity at the time form spring to autumn was noted by Januszek (1993). A study by Włodarczyk (2000) performed on *Orthic Luvisol* sample, showed that DHA demonstrated seasonal pattern and reached the highest values in September, whereas the lowest in winter time. Similarly effect noted Tripathi et al. (2007), who indicated maximum DHA in September and its reduction in January.

Spring season is strongly connected with increase in microbial activity, intensification of oxido-reduction reactions and temperature change, what is indirectly impacted with DHA,

and is the reason of slight DHA increase during this time. Moreover, taking into account that DHA is present inside viable microbial cells, its activity must be the highest at temperature 20-30ºC (temperature characteristic for summer and early autumn), close to the optimum temperature for microbial growth, activity and development (Wolińska & Stępniewska, 2011).

4. Soil factors inhibiting dehydrogenase activity

Some of environmental factors have ability to affect negatively on DHA, by reducing its activity. In the role of enzyme inhibitors usually different molecules are involved, which by binding to enzymes activation sites are the reason of prohibition the enzymes from catalyzing its reaction, and finally decrease their activity. The most important soil factors inhibiting soil DHA are described below.

4.1. Depth of the soil profile

Depth of the soil profile is one of the most known and popular environmental factor reducing soil DHA level. It is well known that the highest microorganisms abundance is in the surface layer of the soil profile (till to the depth of 30 cm), at the deepest part of the soil the number of microbial cells is limited, and consequently also DHA level display diminishing trend.

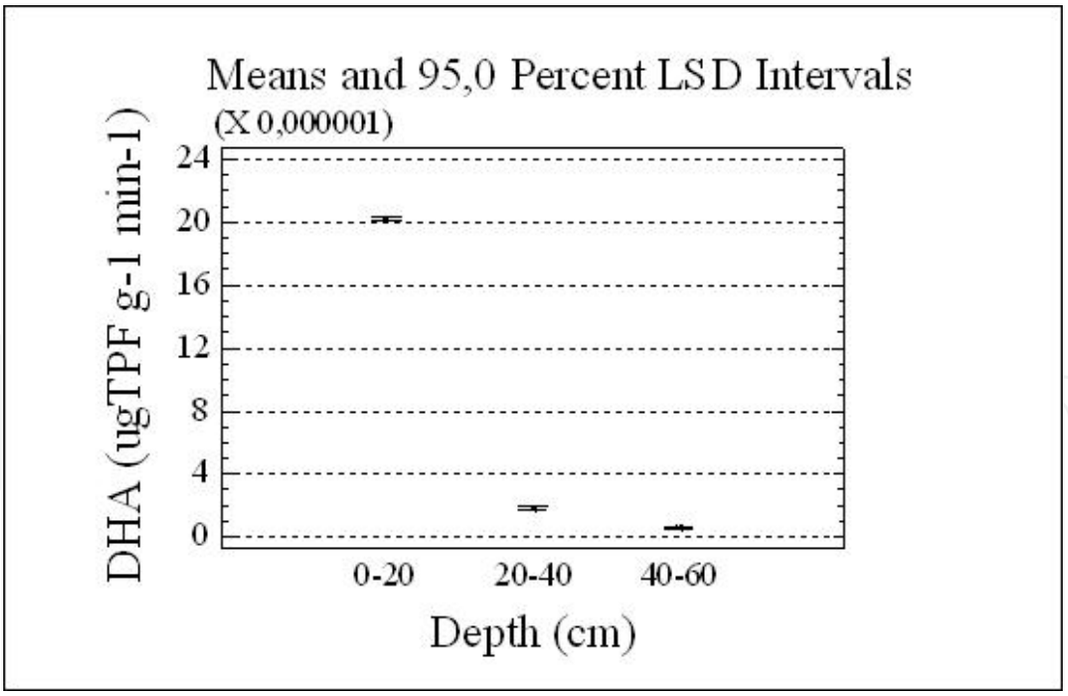

Figure 9. DHA (μgTPFg^{-1}min^{-1}) at different depth of the *Mollic Gleysol* profile (n=18, *P*<0.001), according to Wolińska founding (unpublished data)

The confirmation of the above statement might be the Fig. 9, where effect of depth on DHA in *Mollic Gleysol* is presented. The highest level of DHA we noted in surface layer (0-20 cm),

whereas at the deepest part of the soil profile (40-60 cm) DHA was reduced by 95%, in relation to the surface layer. This trend is undoubtedly connected with presented in literature data and mentioned above spatial distribution of soil microorganisms (Agnelli et al., 2004; Levyk et al., 2007; Wolińska, 2010), and its preference to inhabiting the surface layers, where optimum conditions for its growth and development are guaranteed.

Our results are in agreement and might be supported by the findings of Xiang et al. (2008), who observed that DHA was roughly 4-fold higher in surface (till to 5 cm depth), than in subsoil (90-100 cm). It was also suggested by study of Gajda (2008), that values of DHA noted in the anthropogenic soil, till depth to 4 cm were by c.a. 30% higher in relation to the deeper part of the soil profile.

Brzezińska (2006), reported even 9-fold increase of DHA in the surface layer of the soil, than in the subsurface parts and 25-fold higher enzymatic activity in surface than in subsoil.

Generally it is possible to state, that both diversity, abundance as distribution of microorganisms are more even under oxic (surface layers) conditions, relative to anoxic (deeper layers) conditions (Fierer et al., 2003; Wolińska & Stępniewska, 2011).

4.2. Fertilization and pesticide amendment

Organic and inorganic fertilizers are commonly used to increase nutrient availability (Macci et al., 2012). The balanced fertilization of major elements (N, P, K) for plant nutrient could be beneficial for the growth of plant aboveground parts and roots (Chu et al., 2007), and also for improvement of soil structure (Macci et al., 2012).

However, fertilization could affect on the population of soil microorganisms and consequently soil enzymatic activities. It is often assumed, that inorganic fertilizers had relatively less effect on soil enzymes activity than organic fertilizers (Chu et al., 2007; Xie et al., 2009; Romero et al., 2010). Macci et al. (2012) noted, that DHA usually reached higher level in the organic treatments.

As was suggested by Chu et al. (2007) and Xie et al. (2009) long-term balanced fertilization greatly increased DHA level in the soil environment, rather than nutrient-deficiency fertilization. Zhao et al. (2010) indicated, that soils with higher fertility are more capable of maintaining the original biological functions (i.e. have a higher functional stability).

On the other hand, Moeskops et al. (2010) compared the effect of organic and conventional farming practices on soil enzymatic activities. On the organic farms, soil fertility was maintained mainly with composted OM, in contrast to conventional farmers, who combined fresh manure and chemical fertilizers, and typically applied large amounts of pesticides. As a consequence, a strong negative impact of intensive fertilizer and also pesticide use on DHA was demonstrated (Moeskops et al., 2010).

Soil DHA is an indicator of soil quality and microbial activity and also is the most frequently used to determining the influence of various pollutants (like pesticides or excessive fertilization) on the microbiological quality of soils (Xie et al., 2009; Tejada et al., 2010).

Despite the fact that pesticides are important tools in agriculture that help to minimize economic losses caused by weeds, insects and pathogens, they also are recognized as a source of potential adverse environmental impacts (Tejada et al., 2010). It is often assumed that less than 0.3% of the pesticide reaches its target pest, the remaining 99.7% is released to the environment, representing a potential hazard for non-target organisms (Muñoz-Leoz et al., 2011).

Stepniewska et al. (2007) noted the relationship between soil DHA and Fonofos (Stauffer Chemical Co., Westport, USA) concentration in the *Mollic Gleysol*. In the investigated samples influence of pesticide on soil enzymatic activity started to be observed after one week of incubation, but since 14th day to the end of experiment this effect was significant and noticeable (Fig. 10). Generally, 1μg g^{-1} dose of Fonofos was responsible for about 26% inhibition of soil DHA, whereas ten times higher factor reduced activity for 46.6% at 21st day of incubation time, later fall of enzymatic activity ranged from 22.5% to 30% in relation to the control samples was considered.

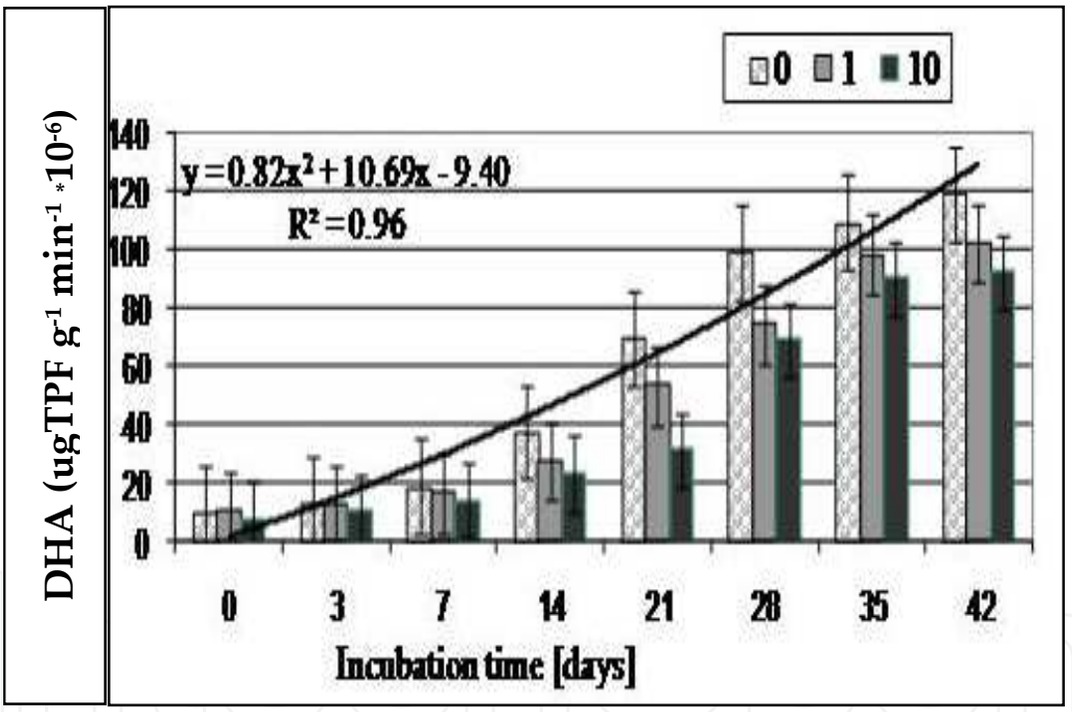

Figure 10. Dynamic of DHA during incubation at 20ºC (0 - control, 1 - 1μg g^{-1} Fonofos supplement; 10 - 10 μg g^{-1} Fonofos supplement), according to Stępniewska et al., 2007. Averaged values of three replicates with standard deviations are presented

Our results suggest a negative effect of Fonofos on soil DHA in the first stage after application (1-7 day), later an initial, almost linear growth of DHA was observed and the final day of incubation resulted in significant extension of DHA, presumably because the process of Fonofos decomposition in the soil environment was almost completely finished.

Tejada et al. (2010) indicated that MCPA herbicide rate of 1.5 l ha^{-1} (manufactures rate recommended) was the reason of 39.3% soil DHA inhibition, what suggest that the MCPA

caused toxic effect on soil enzymatic activity. A field half-life of MCPA ranged from 14 days to 1 month, dependently on soil moisture, pH and microorganisms abundance. The most important soil factor in predicting MCPA effect on soil enzyme activities is pH, as at acidic conditions persistence of pesticide may last even 5 years, whereas at alkaline pH only 6 days. Moreover, decrease of soil moisture and reduction of microbial abundance influence on elongation of MCPA decomposition process.

We also studied effect posed by MCPA (Organika Sarzyna, Poland) on soil DHA behavior (Fig. 11). The following MCPA dosage were introduced into the soil samples: 0.165; 0.30 and 3.3 mg MCPA per g of soil. Non-amended with pesticide soil sample was marked as 0 and used as a control. As a result of realized experiment we found linear inhibition of DHA by increasing MCPA doses (R^2=0.99). Decrease of DHA level at 3.3. mg g^{-1} MCPA dose by c.a. 38.5%, in comparison to the control sample, was noted. However, registered inhibition was not significant (P>0.05). Our conclusions are comparable with results presented by Tejada et al. (2010).

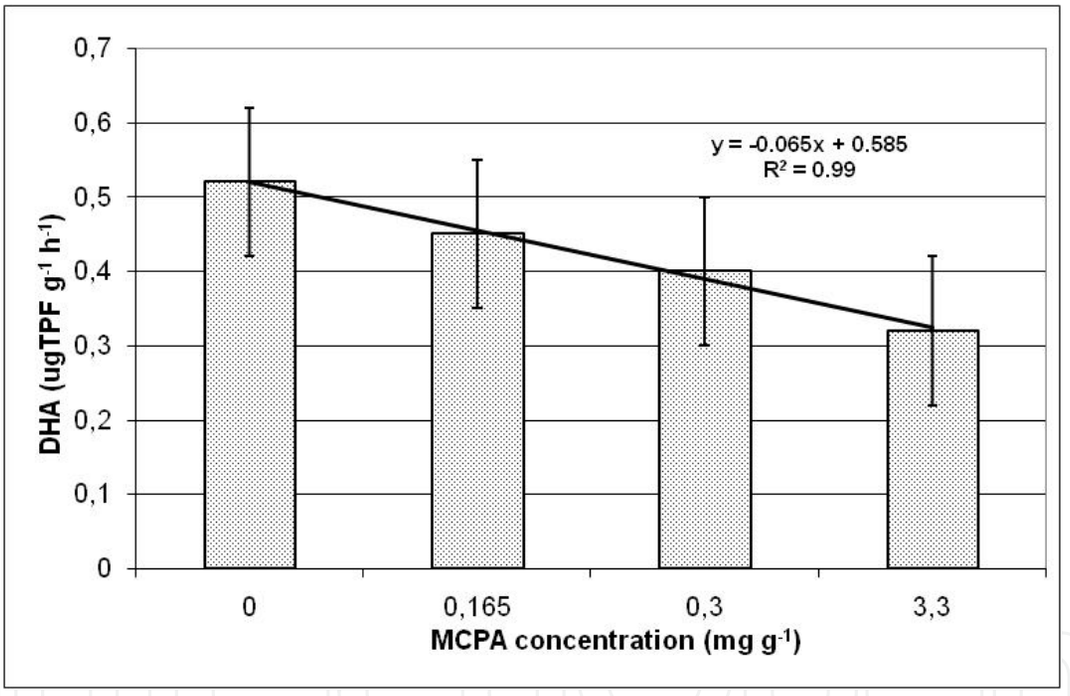

Figure 11. Effect of MCPA pesticide on DHA level in *Eutric Fluvisol*, according to Stępniewska founding (unpublished data). Averaged values of three replicates with standard deviations are presented

Other pesticide, which we take into account in our investigations was Glyphosate – commonly used by Polish farmers (in the form of RUNDUP), a broad spectrum, non-selective, systemic and post-emergence herbicide, widely popular in soil cultivation, forestry, rights-of-way and aquatic systems to prevent grass and weeds competition with plant seedlings (Bennicelli et al., 2009). At low doses it is used as a plant growth regulator.

Glyphosate (Monsanto Co., USA) is a polar substance that is highly soluble in water (12 g l^{-1} at 25°C), and insoluble in most organic solvents. In soil is moderately persistent; its half-life

is reported between 1 to 174 days (Bennicelli et al., 2009). Glyphosate in soil is transformed to aminomethylphosphonic acid (AMPA), which is non-persistent metabolite. As a effect of mentioned transformations and in presence of dehydrogenases (microorganisms), Glyphosate give CO_2 and H_2O (Forlani et al., 1999). Glyphosate degradation in soil is mainly the reason of microbial activity, while the chemical decomposition and photolysis play a minor role (Bennicelli et al., 2009). As was reported by Zabaloy et al. (2008), Glyphosate, as an organophosphonate can be used as a source of P, C or N by either gram-positive as gram negative bacteria.

The purpose of our study was to research the influence of Glyphosate on soil DHA in the *Mollic Gleysol* (from Wieprz river valley), *Eutric Fluvisol* (from Vistula river valley) and *Terric Histosol* (from Bystrzyca river valley), taken from surface layer (0-20 cm). Soil samples were enriched with Glyphosate, as follows: with 1 μg (first combination), and 10 μg (next version), and 0 μg (control) of pesticide per 1g of soil. Thus prepared samples were incubated in thermostatic chamber at 20ºC. Received results are presented in Fig. 12.

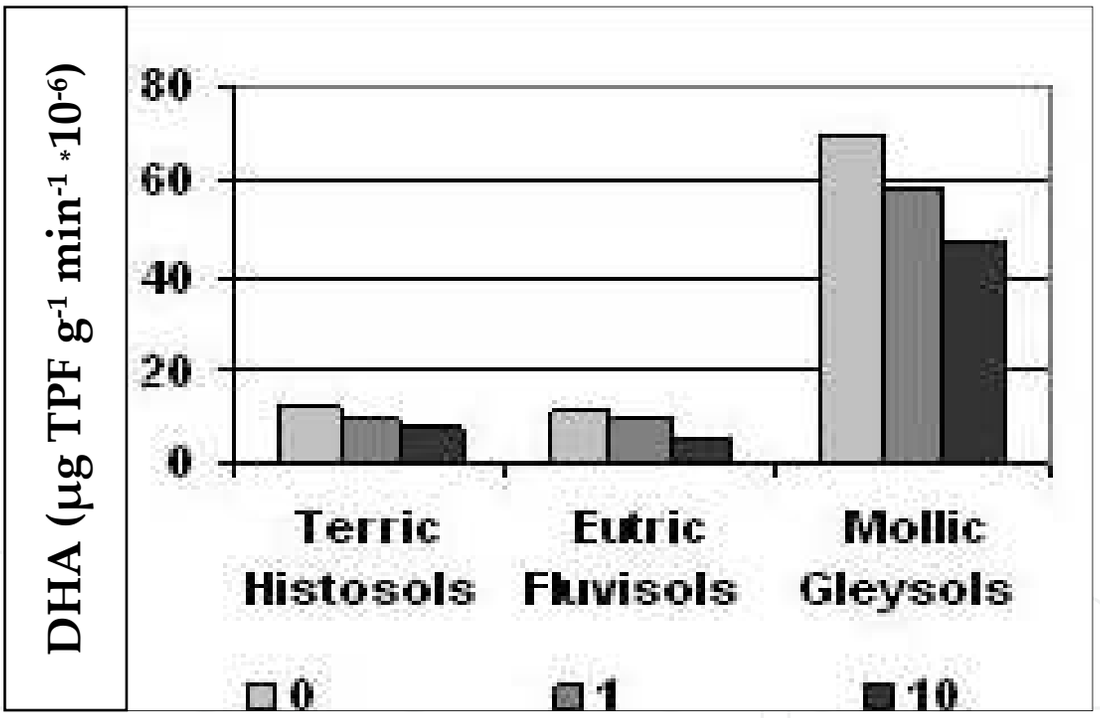

Figure 12. Mean DHA in *Terric Histosols*, *Eutric Fluvisols* and *Mollic Gleysols*, in three combinations of Glyphosate doses: 0-control; 1-1μg g^{-1}; 10-10μg g^{-1} (according to Bennicelli et al., 2009)

We found that both 1 and 10 μg of Glyphosate additions to soils caused a decrease of DHA, dependently on the pesticide doses. The strongest effect of Glyphosate was observed in *Terric Histosols* and *Eutric Fluvisol* (10 μg g^{-1} of soil),where reduction of DHA by 33-47%, relative to control soils (non-amended with Glyphosate), was noted. The most resistant to

Glyphosate supplement seemed to be *Mollic Gleysol*, in 10 μg g^{-1} of soil dose, where DHA dropped by c.a. 24%.

Suggested by us inhibition effect, may be supported by founding's of Zabaloy et al. (2008), who in typical Ardiudoll from Argentina observed reduction of DHA for about 48%, as an effect of Glyphosate contamination, in comparison to control sample.

Results, suggesting inhibitory pesticide effect on DHA level are also in agreement with those obtained by other plaguicides such as: chlorpyrifos (Kadian et al., 2012), or vermicompost (Romero et al., 2010). Moreover, Muñoz-Leoz et al. (2011) noted that DHA was inhibited by 14%, as a effect of application 5 mg kg^{-1} tebuconazole fungicide dosage.

Conversely, others have found also different results. For example, Tejada et al. (2011) noted insignificant (by 10%) growth of DHA, when the Prochloraz fungicide applied to the soil increased, possibly because the fungicide is commonly used by bacterial communities, as a source of energy and nutrients. Also Andreá et al. (2003), noted that DHA was slight higher after month from Glyphosate application. In that case authors reported, that Glyphosate stimulated DHA, which means that the herbicide might stimulate the soil oxidative processes.

4.3. Heavy metals presence

Heavy metals, even though they are natural constituents of soil, could have long-term hazardous impacts on the health of soil ecosystems, and adverse influences on soil biological processes (Pan & Yu, 2011). Generally, it was assumed that heavy metals can reduce enzyme activity by interacting with the enzyme-substrate complex, denaturing the enzyme protein or interacting with the protein-active groups, they could also affect the synthesis of enzyme microbial cells (Pan & Yu, 2011).

Xie et al. (2009) noted that Cu of 100 mg kg^{-1} could suppress DHA significantly, while Cd of 5 mg kg^{-1} had relative greater influence on soil microbial diversity, what suggest that the effect of each soil pollutant on soil microbes and their enzymatic activities was specific. On the contrary, a study by Fernandez-Calviño et al. (2010) indicated adverse effect of Cu on DHA ($r=-0.24$, $P<0.01$). Threshold Cu concentrations at which changes in the enzyme activities became evident were 150-200 mg total Cu kg^{-1} and 60-80 mg bioavailable Cu kg^{-1}.

A study by Pan & Yu (2011) undertaken with brown soil, showed that DHA was significantly lower by 37.8% and by 51.1% in Cd and Pb treatments, than in control. Moreover, mentioned researchers noted that the effect of Cd and Pb combined on DHA were higher, than Cd or Pb alone.

We also investigated effect posed by Cd (2 and 20 mg kg^{-1}) on soil DHA (Fig. 13). Incubation of soil material with mentioned Cd doses lasted 42 days. After that DHA was determined via Casida et al. (1964) method.

We observed that Cd presence at concentration of 2 mg kg^{-1} had stimulating effect on soil DHA level, and we noted increase of DHA by 8.8%, in comparison with control sample (without Cd contamination). However, 10-fold higher Cd amendment (20 mg kg^{-1})

consequence with strong DHA inhibition, by as follows: 29.4% and 35%, in relation to control and 2 mg kg^{-1} sample, respectively. Observed inhibition effect was probably caused by Cd interaction with enzyme-substrate complex, what resulted with strong decrease of DHA level.

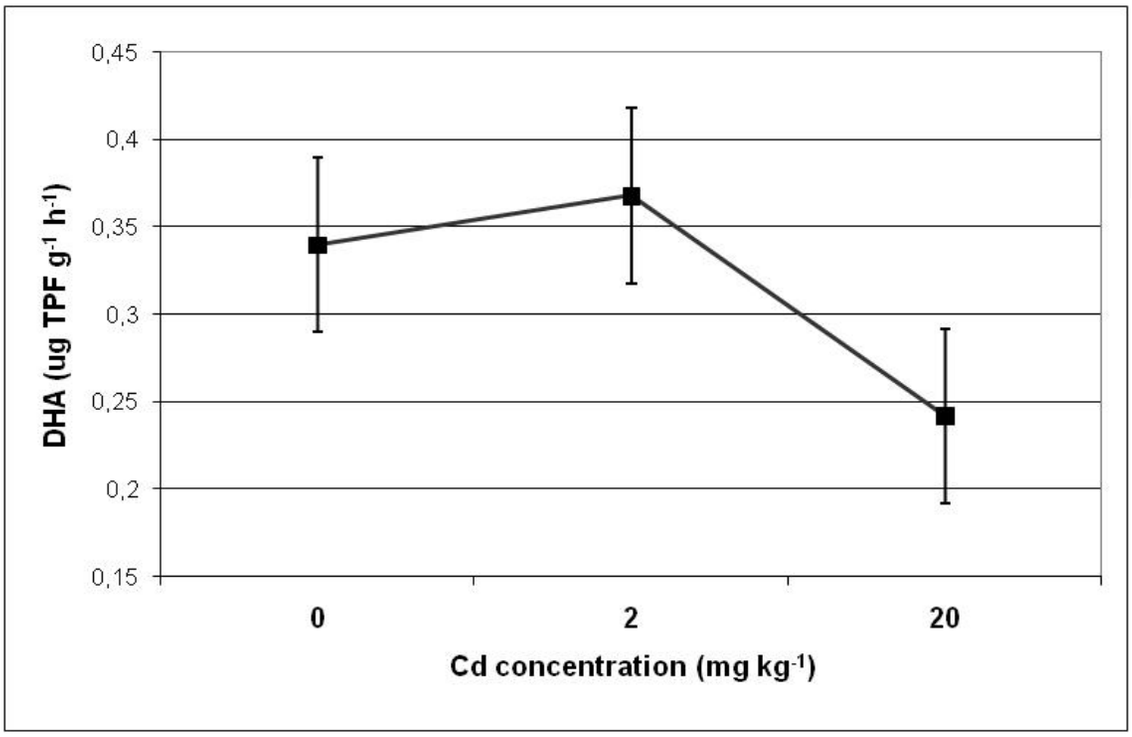

Figure 13. Effect of Cd on DHA in *Eutric Fluvisol* (according to Stępniewska & Wolińska, unpublished data) from Cd introduction into the soil. Averaged values of three replicates with standard deviations are presented

Our results, may be supported by findings of Moreno et al. (2001), who by investigating the influence of Cd on DHA stated, that Cd content strongly affected on DHA, by reducing its activity, and this effect is noticeable even after 3 hours.

Negative effect of heavy metals on DHA was reported also by Kizilkaya et al. (2004), who organized the following order of this inhibition: Cu > Cd > Co. Analogically, strong reduction of DHA by Cd contamination was indicated by Welp (1999), who tested the effect of Co, As, Hg, Cd, Pb and Cu on soil DHA, and demonstrated that the strongest effect was displayed by Hg and Cd.

Stępniewska & Wolińska (2005) found that the application of trivalent and hexavalent chromium compounds had a noticeable negative effect on soil DHA (Fig. 14). The soil sample (*Haplic Luvisol*) was amended with Cr (III), as a $CrCl_3$ and with Cr (VI), as a $K_2Cr_2O_7$ in the concentration range from 0 to 20 mg kg^{-1} and 0-100 μg kg^{-1}, for Cr (III) and Cr (VI), respectively. The differences in Cr (III) and Cr (VI) doses resulted from the fact that Cr (VI) is highly toxic and much mobile form of Cr, and is considered to cause much stronger effect on living organisms, than Cr (III).

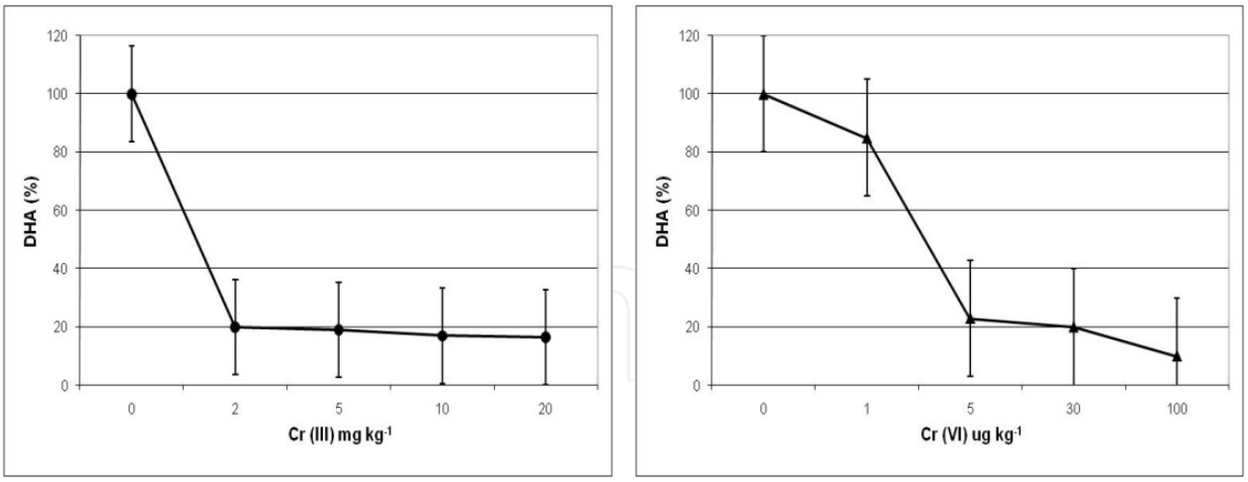

Figure 14. The variations of DHA in *Haplic Luvisol* at different Cr (III) and Cr (VI) concentrations (according to Stępniewska & Wolińska, 2005). Averaged values of three replicates with standard deviations are presented

Non-amended soil samples were used as a control, and their enzymatic activity were estimated as 100%. Effect posed by Cr content was calculated as a decrease of its level, in relation to the control value. We found that the lowest values of DHA were the effect of increasing Cr(III) and Cr(VI) doses. *Haplic Luvisol* seemed to be very sensitive on Cr contamination. DHA was reduced to 18-20% in the samples enriched in Cr (III) forms. Surprisingly, the more dangerous form of Cr (VI) was less harmful for DHA in the *Haplic Luvisol*, because enzymatic activity remained on the level of 84%, with a 1μg kg^{-1} addition and decreased to the value of 14% with the highest supplement of Cr (VI). One possible explanation for this fact is that. the more dangerous form of Cr (VI) was reduced to the less toxic form of Cr (III) by microorganisms, living in the soil (Stępniewska & Wolińska, 2005).

In the same way we investigated effect of Cr forms on *Eutric Cambisol* (Stępniewska & Wolińska, 2004). Received results are shown in Fig. 15. We stated that excess of Cr forms in soil disturb homeostatic metabolism of microbes, what reflect their enzymatic activity. DHA demonstrated a tendency to decrease with increase of Cr concentration. The lowest content of both Cr (III) and Cr (VI), at the level of 2 mg kg^{-1} reduced soil DHA to 51-66%, respectively. But at the same time the highest Cr (III) and Cr (VI) supplement at the level of 20 mg kg^{-1} limited DHA to 6-15%, in relation to the control.

Inhibition of DHA by applied Cr compounds was also reported by Wyszkowska et al. (2001), who noted that decrease of enzymatic activity in soil should be considered as very unfavourable in terms of soil fertility, because soils of good quality and high content of soil OM show high enzymatic activity.

The decrease of soil DHA by several metallic elements (Al, Be, Cu, U) was also discussed by Antunes et al. (2011), whereas a study by Nowak et al. (2002) found that DHA decreased by up to 85% at 5 mM selenic acid (IV) presence.

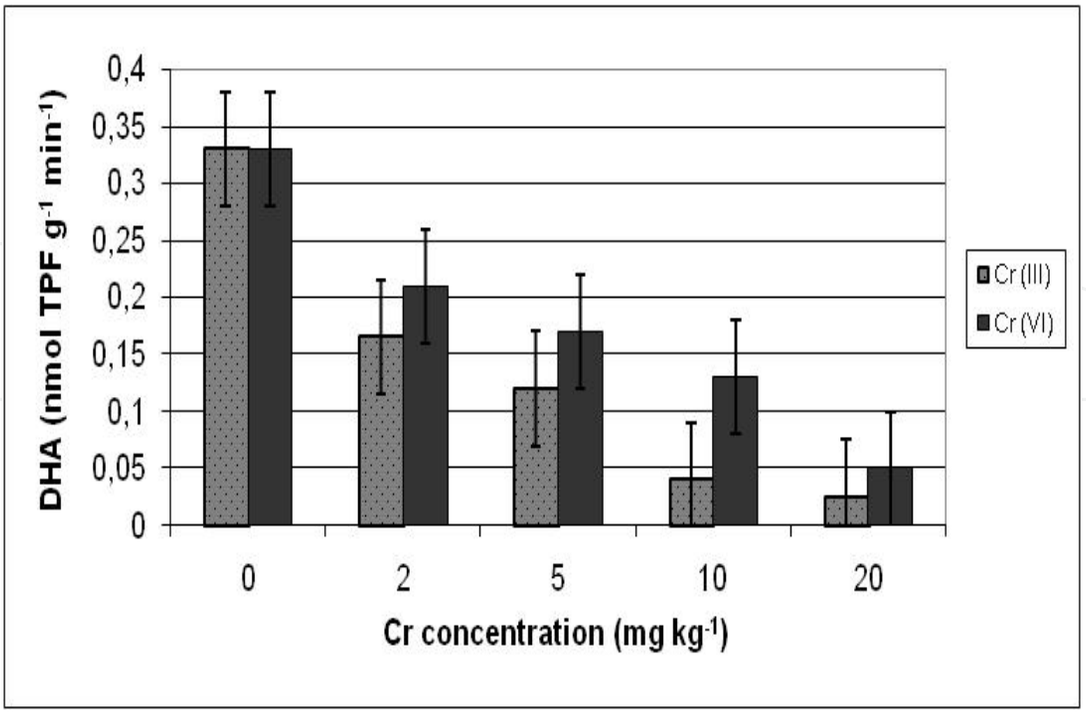

Figure 15. The variations of DHA in *Eutric Cambisol* at different Cr (III) and Cr (VI) concentrations (according to Stępniewska & Wolińska, 2004). Averaged values of three replicates with standard deviations are presented

5. Conclusions

Soil is a part of the terrestrial compartment, and supports all terrestrial life forms. Thus, without proper soil protection policies, numerous problems may arise, like reduction of soil fertility, erosion, groundwater contamination, insufficient water holding capacity and loss of biodiversity. To asses soil quality, it is essential to measure all potential changes in biological soil properties, because they are highly sensitive to any environmental perturbations and stresses. A usual approach to diagnose soil quality, is to use a soil microbial indicators, which are very sensitive and respond quickly to environmental alterations.

Among different soil indicators, DHA is one of the most adequate, important and one of the most sensitive bioindicators, relating to soil quality and fertility. Moreover, their routine measurement is simple and low-cost under laboratory condition. However, we should not remind about limitations, resulting from laboratory conditions, when we are able to measure and estimate only potential DHA, similarly like we are able to cultivate only small percentage of soil microorganisms, on artificial media.

Soil enzymes are strongly associated with microorganisms. Soil enzymatic activity plays an important role in catalyzing reactions indispensable in life processes of soil microorganisms, decomposition of organic residues, circulation of nutrients, as well as forming organic matter and soil structure. Thus, it is possible to say that without proper soil enzymes system, soil life processes will be disturbed.

DHA is related to quantitative changes in microorganisms populations, as only strictly intracellular enzymes can truly reflect microbial activity, because with respect to the degradation processes of extracellular soil enzymes, they will be quickly mineralized by other enzymes (i.e. proteases), unless they are either adsorbed by clays or immobilized by humic molecules.

It should be also remind, that overall soil DHA level depends most of all from the activities of various types of dehydrogenases, which are fundamental part of the enzyme system of all living soil microorganisms, i.e. the respiratory metabolism, the citrate cycle, and N metabolism.

Due to this fact, DHA is proposed as the best indicator of the microbiological redox-systems, and could be considered as good and adequate parameter of microbial oxidative activities in soil. Furthermore, soil DHA is also used as a measure of any soils disruption posed by pesticides, heavy metals, or other soils contaminates and improper management practices.

As DHA is strictly connected with living microbial cells, its activity depends from the same environmental factors, which influence on microorganisms abundance, activity and life processes. Consequently, when entertaining soil DHA behavior in the soil environment, we should be not only limited to DHA, but it is necessity to consider on the most important soils factors and conditions, affecting measuring by us DHA level.

In the presented chapter we described the most important soil parameters, affecting DHA, which poses ability either for stimulation or inhibition its activity.

To sum up the forgoing observations it was demonstrated, that DHA display increasing trend under anaerobic conditions, what suggest that the facultative and anaerobic member of soil microbial community become more important in soil respiration processes. Thus, soil DHA was reported to be negatively correlated with soil water potential, oxygen diffusion rate, and redox potential, what means that DHA reached higher values at lower soil water potential, lower oxygen diffusion rate and lower redox potential conditions. Analogically, negative correlation we also found in the case of soil depth–what was connected with spatial stratification of microorganisms abundance and its preference for inhabiting the surface layers of the soil profiles. Inhibiting effect on DHA level have also pesticides and soil contamination with heavy metals.

Important parameter affecting soil biological activity is pH. Our investigations demonstrated, that optimal pH range for DHA is between 5.5-5.73, what was confirmed by correlation coefficient (r=-0.70*).

Soil DHA depends also from the season of the year, similarly like dynamics of microbial activity, and reached the highest level in May, as spring season is strongly connected with increase in microbial activity, and intensification of oxido-reduction reactions, what is indirectly linked with DHA.

Positive relationships we noted between DHA and two parameters: TOC and temperature, what means that DHA reached higher values at soils with higher TOC content (what is also

preferred by soil microorganisms), analogically like increase of temperature to 30°C (temperature close to optimum for microorganisms growth and development) resulted in DHA stimulation.

Presented and discussed above results are based on our several years studies, however additional investigations are needed and recommended to determine the relative contribution of the different environmental effects on soil DHA. However, the discussion highlights the strong interactions between the soil environment, soil enzymes (dehydrogenases especially) and soil microorganisms.

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