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ORIGINAL PAPER

Discussion on methods of soil dehydrogenase determination

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Abstract
In this paper, we discuss the influence of different factors on the measured values of dehydrogenase activity. We focus on the incubation time of the sample and optimal 2,3,5-triphenyl-tetrazolium chloride concentration. We provide a comparison between results obtained from three methods: Casida method, standard method, and optimization method. Some disadvantages of traditional methods were critically discussed. We showed that the results of dehydrogenase activity determination strongly depend on the method used. To minimize these discrepancies, the increase in TPF concentration should be described with the kinetic model which allows determining tangent activity. Michaelis–Menten kinetics can be used to describe the relationship between TTC concentration and tangent activities. We suggest using the value of 4 km as the optimal TTC concentration.

Keywords Modeling · Soil properties · Optimal concentration · Incubation time · Enzyme activity

Introduction

Enzymes produced by soil microorganisms are natural catalysts of many important processes that occur in soil, including decomposition of organic matter, formation, and decomposition of humus, the release of mineral substances and making them available to plants, molecular nitrogen fixation, as well as the detoxification of xenobiotics. For this reason, enzymes may be useful in monitoring the effects of pollution on the soil environment (Tabatabai 1982; Rangaswamy et al. 1994; Małachowska-Jutsz et al. 1997; Trasar-Cepeda et al. 2000; Sannino and Gianfreda 2001; Russel 2005; Xie et al. 2009). The use of enzymes in the study of soils, despite the undoubted successes and achievements evidenced by the thousands of literature references, encounters many difficulties associated with the methodology (Chander and Brookes 1991; von Mersi and Schinner 1991; Friedel et al. 1994; Leirós et al. 2000; Liu et al. 2008). Toxicological studies aim (among other goals) to find the enzymes whose activity can serve as an indicator of "soil health" (Pascual et al. 2000; Gil-Sotres et al. 2005; Russel 2005). It has been shown that a reliable assessment of soil quality contaminated with organic products is possible by testing the activity of lipases, dehydrogenases, catalases, and ureases (Trasar-Cepeda et al. 2000; Sukul 2006; Xie et al. 2009). These activities reflect the changes in the specific properties of the soil complex affected by the presence of contaminants. Research carried out at a laboratory scale indicates the validity of the use of these enzyme proteins as bio-indicators for the removal of hydrocarbons from soil. In these tests, the test sample of soil is incubated with the specific substrate and the activity is determined based on the measurements of the substrate loss or the amount of the generated product. The main advantage of using enzyme assays is that they are relatively simple and commonly available analytical methods. A major drawback is that it does not always provide an outcome corresponding to the degree of soil contamination (Chander and Brookes 1991; Friedel et al. 1994; Leirós et al. 2000; Klimkowicz-Pawlas and Maliszewska-Kordybach 2003).

In this work, we focus on the determination of the dehydrogenase activity of soil microorganisms. Determination of dehydrogenase activity is a quick and relatively simple method to determine the overall activity of microorganisms,

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e.g., in activated sludge or soil (Miksch 1985a, b, 1988; Kumar et al. 2013; Järvan et al. 2014). Dehydrogenase activity can be measured using different tetrazolium salts, e.g., 2,3,5-triphenyl-tetrazolium chloride (TTC) as an artificial terminal hydrogen acceptor in the electron transport chain. This is reduced to red-colored triphenylformazan (TPF). TPF is extracted using organic solvents (e.g., methanol), and the color intensity of the extract is determined by spectroscopic methods. The intensity of the color is directly proportional to the concentration of the produced triphenylformazan.

The use of this method, or similar with another tetrazolium salt, is advisable in the study of soil microorganisms activity (Casida et al. 1964; Ohlinger 1996; Rossel et al. 1996), but the results may be questionable (Chander and Brookes 1991; Mathew and Obbard 2001). The underlying cause was the choice of methodology (Januszek et al. 2015). Various methods often omit the physicochemical properties of soils, in particular, the sorption capacity of the soil complex and organic matter content, which can affect results. Furthermore, different methods have been repeatedly modified leading to an inability to compare the results obtained by various authors. The changes were made mainly in the incubation time (1–40 h), TTC concentration, soil sample weight (1–10 g), incubation temperature (20–37 °C), the use of organic additives (glucose, yeast extract), etc. TTC concentration, which should be high enough to saturate enzymes of the electron transfer chain without being toxic to microorganisms, was often not determined. Also, the range in which the increase in triphenylformazan is linear, which is crucial when the entire kinetics is not known, was not determined in many studies. It is worth pointing out that incubation time and TTC concentration were often chosen arbitrarily or were directly transferred from different experiments, which might lead to misinterpretations (Ross 1971; Januszek et al. 2015).

Therefore, the aim of this study was to discuss the existing methodology (including the Casida method (1964) and the standard method ISO 23753-1:2005) and to conduct a detailed study to determine the optimum incubation time and TTC concentration, highlighting the important relationship between mentioned variables. Research was carried out from 04/2017 to 09/2017 at Silesian University of Technology (Gliwice, Poland).

Materials and methods

Soil characteristics

Soil samples for experiments were taken from a depth 0–20 cm within an uncontaminated area devoted to organic farming. The soil samples were air-dried at 25 °C until the water content was approximately two-thirds of the field capacity (~0.26 g g^{-1} soil). Soil was then sieved through a 2-mm mesh sieve to remove stones and homogenize the sample. All physicochemical soil analyses were carried out in six replicates, and presented values are their arithmetic average.

The texture of the soils was determined with the Casagrande aerometric method separating the sand sub-fraction in sieves with mesh sizes of 1, 0.5, 0.25, and 0.1 mm (Ryżak et al. 2009). The particle size groups were determined in accordance with the classification of the Polish Society of Soil Science (2008) (PTG 2011 2011). Soil pH_{H_2O} and pH_{KCl} in 1 mol KCl dm^{-3} were measured potentiometrically with a combined electrode, according to (PN-ISO 10390:1997 1997). Hydrolytic acidity values and the content of cation exchange capacity (CEC) assayed with the Kappen method (Jaremko and Kalembasa 2014) allowed moisture content of soil to be measured gravimetrically by drying 100 g of soil samples at 105 °C for 24 h (ISO 11465:1993 1993). Total nitrogen was measured using the Kjeldahl method (ISO 11261: 1995 1995), the total organic carbon content was measured using the Tiurin method, and the total phosphorus was determined according to (ISO 11263:1994 1994).

The degree of formazan extraction from the soil

Some soil properties can be the reason for poor TPF extraction from samples which can affect values of obtained results. Therefore, we measure the degree of formazan extraction by the following procedure. For samples containing 5 g of soil, 300 µl of three different TPF solutions in acetone (which quickly evaporates) was added. The concentrations of TPF in soil were: 20, 40, and 60 µg TPF g^{-1} soil. Samples were thoroughly mixed using a glass rod. Samples were left in the dark at room temperature. After 24 h, TPF was extracted using 10 ml of methanol. Samples were prepared in triplicate. The degree of formazan extraction from the soil was determined as a value of the slope of the linear function, which describes the relationship between added and extracted TPF concentrations.

Casida method (1964)

Dehydrogenase activity (DHA) was determined using the method described by Casida et al. (1964). Fresh homogenized soil samples (10 g) were placed in test tubes (16 × 150 mm) and mixed with 2.5 ml of phosphate buffer, 0.2 g $CaCO_3$, and 1 ml substrate (3% v/w TTC). The tubes were incubated at 25 °C for 1, 3, 6, 16, 24, 48, and 72 h. According to Casida et al. (1964), the incubation period was 24 h. A blank sample was similarly prepared with the difference of 1 ml of a 3% TTC solution phosphate buffer being introduced. After incubation, the samples were centrifuged

using MPW-250 at 3000 rpm for 10 min. The supernatant liquid was discarded. The TPF formed was extracted with methanol. 5 ml of methanol was added to each of the tubes and vigorously shaken for a few minutes. The operation was repeated twice (10 ml of methanol was used for extraction). Again the tubes were centrifuged. The obtained supernatant liquid was poured into a clean tube, and the absorbance of the solution was measured for $\lambda = 485$ nm. TPF concentration was calculated using a calibration curve (prepared according to the standard method).

Standard method

DHA was assayed using the standard ISO 23753-1:2005 method. Fresh homogenized soil samples (5 g) were placed in test tubes (16 mm × 150 mm) mixed with 5 ml of substrate TTC. As a optimal TTC concentrations for the sandy loam soil samples, three different concentrations (0.6, 0.8, and 1% TTC solution) were selected (the range 0.6–1% for loam, humic and loamy soil is given in the standards ISO 23753-1:2005, 2005; PN-EN ISO 23753-1:2011, 2005). The blank sample, instead of TTC solution, contained 5 ml of a TRIS buffer solution, the concentration of which was $c = 0.1$ mol dm^{-3}. We introduced some small modifications which do not interfere with the assessment of appropriate choice of time incubation and optimal TTC concentration. The tubes were incubated at 25 °C for 16, 24, 48, and 72 h (the standard method requires 16 h of incubation). TPF was extracted with methanol instead of acetone. 5 ml of methanol was added to each of the tubes and vigorously shaken for a few minutes. The operation was repeated (10 ml of methanol was used for extraction). Again the tubes were centrifuged. The obtained supernatant liquid was poured into a clean tube, and the absorbance of the solution was measured for $\lambda = 485$ nm and the TPF concentration was calculated using a calibration curve.

Optimization method (time and concentration)

Optimum TTC concentration can be determined after a chosen time of incubation as described in Małachowska-Jutsz et al. (2011). However, for purposes of this work, we decide to investigate the kinetics of TPF production in samples with different TTC concentrations. Therefore, eight samples containing 5 g of soil were prepared. For each sample, 1 ml of 0.2% Na_2SO_3 and 1 ml of TTC in different concentrations were added. Obtained TTC concentrations were: 0.001; 0.003; 0.006; 0.008; 0.01; 0.02; 0.03; 0.06 g g^{-1} DW. For mentioned TTC concentration, the time course of the increase in TPF concentration was determined.

After the reagents were introduced into the test tubes, the contents of the tubes were mixed thoroughly with a glass rod (in a manner preventing aeration of a sample) and incubated at 25 °C for 3, 6, 16, 24, 48, and 72 h in the dark. After incubation, the samples were centrifuged (at 3000 rpm for 7 min) and the supernatant was discarded. Then, the produced TPF was extracted in 5 ml of methyl alcohol. The samples were shaken vigorously and then centrifuged again. The absorbance of the supernatant was determined at wavelength 485 nm. TPF concentration was calculated using a calibration curve (prepared according to the standard method). For each combination of different incubation times and TTC concentration, three replications of samples were prepared.

Secant and tangent activity

The rate of TPF production (dehydrogenase activity) can be determined in two different ways. In the traditional approach, the time when changes of TPF concentration are approximately linear is determined. Based on this simplification, the activity in samples is calculated by the division of TPF concentration at the end of this time interval by the value of this interval. Time intervals are often imposed like in standard ISO 23753-1:2005 or the Casida method. This approach leads to the determination of, as we call it in this work, secant activity. The second way to determine the TPF production rate is by measuring concentrations of TPF after different times of incubation. Appropriate models have to be used to describe these data. Parameters of this model can be estimated. Their values can be used to calculate the value of the derivative of the used function when the time is equal to zero. The value of this derivative is the rate of TPF concentration production at the beginning of incubation. We call this value a tangent activity. Values of secant and tangent activity were compared with each other. Furthermore, the values of tangent activity were used to estimate the parameters of the Michaelis–Menten equation. In Table 1, a comparison between all mentioned methods can be found.

TPF concentration changes model

The simple empirical model, which can be used to describe the reduction in TTC and TPF production, can be written as a differential equation:

$$\frac{dC_{TPF}}{dt} = k\left(C_{max} - C_{TPF}(t)\right) \quad (1)$$

where $\frac{dC_{TPF}}{dt}$ is the rate of TPF production [μg g^{-1} DWh^{-1}]; C_{TPF} is a concentration of TPF at a certain time [μg g^{-1} DW];

Table 1 Comparison of three described methods

	Casida method	Standard method	Optimization method
TTC concentration [g g^{-1} DW]	0.003	0.006 0.008 0.01	0.001; 0.003; 0.006; 0.008; 0.01; 0.02; 0.03; 0.06
Sample soil mass [g]	10	5	5
Extrahent volume [ml]	10	10	5
Incubation period [h]	1;3;6;16; (**24**)*; 48; 72	(**16**)* 24; 48; 72	3;6;16; 24; 48; 72
Determination of enzymatic activity	Secant activity	Secant activity**	Secant activity compared with tangent activity**

*Periods are given in the original method

**Defined in chapter 2.6 Secant and tangent activity

C_{max} is a maximal concentration of TPF, which can be produced from a certain amount of available TTC and then extracted from the soil [µg g^{-1} DW]; k is reaction rate [h^{-1}]; and t is time [h].

Equation (1) can be solved for the initial condition $C_{TPF}(0) = 0$. The model can be expressed by the equation:

$$C_{TPF}(t) = C_{max}\left(1 - e^{-kt}\right) \tag{2}$$

Equation (2) is used for parameter estimation. Curve fitting was conducted using a nonlinear least squares method (in MATLAB). We determined the tangent activity, which can be described as the first derivative of the used model (Eq. 2) in time equal to zero. It is given by the equation:

$$C'_{TPF}(0) = C_{max}k \tag{3}$$

where $C'_{TPF}(0)$ is the tangent activity/ rate of TPF production; then, $t = 0$ [µg g^{-1} DW h^{-1}] and $C_{max} = C_{TTC}(0)$.

Optimal TTC concentration

The Michaelis–Menten kinetic model was used to determine the optimal TTC concentration.

It is given by the equation:

$$\frac{dC_{TPF}}{dt} = \frac{V_{max}C_{TTC}}{K_m + C_{TTC}} \tag{4}$$

where $\frac{dC_{TPF}}{dt}$ is rate of TPF production [µg g^{-1} DW h^{-1}]; V_{max} is a maximum rate of TPF production [µg g^{-1} DW^{-1} h^{-1}]; K_m is a Michaelis–Menten constant [g g^{-1} DW]; and C_{TTC} is the TTC concentration time [g g^{-1} DW].

The parameters of M–M equation were fitted to tangent activities using the least squares method.

TTC toxicity test

The toxicity of TTC seems to not be obvious in many cases. Therefore, we prepare an experiment to present the dangers of using high concentrations of TTC. For this purpose, we prepared two samples with different TTC concentrations at the beginning: 0.06 g g^{-1} DW (higher than in the previous experiments) and 0.15 g g^{-1}DW, which was obtained by adding 1 ml of almost saturated solution of TTC. Samples were initially kept at room temperature (20 °C) to increase the possible exposition time of organisms to TTC and to allow it to penetrate the cells, but on the other hand to decrease the TTC reduction rate. Measurements of TPF concentration were taken after 2, 6, 16, 24 h. After 16 h, part of the prepared samples were moved to 37 °C to increase the rate of TPF production. TPF in these samples was measured after 24 and 96 h from the beginning of the experiment. Outcomes obtained in different temperatures and different TTC concentrations were compared.

Parameter estimation

Parameter estimation for each model function was conducted in MATLAB using the least squares method. The goodness of fit was determined by the sum of squared errors (SSE); coefficient of determination (R^2); adjusted coefficient of determination (Adj. R^2—more useful for comparing models with a different number of predictors); and root mean square error (RMSE).

Results and discussion

Physicochemical properties of the soil

Some physicochemical properties of soil such as pH, total organic carbon, total nitrogen, total phosphorus, and particle

Table 2 Physicochemical characteristics of soil (topsoil 0–20 cm)

Measured parameters	Value ± standard deviation, $n = 6$
pH_{H_2O}	6.96 ± 0.02
pH_{KCl}	5.90 ± 0.03
Hydrolytic acidity c mol/kg	1.04 ± 0.43
Cation exchange capacity (CEC) c $molkg^{-1}$	16.76 ± 0.18
Humidity (fresh soil) %	22.78 ± 0.35
Total nitrogen %	0.1763 ± 0.0058
Total organic carbon (TOC) %	2.24 ± 0.01
Total phosphorus %	0.058 ± 0
Sand %	27
Silt %	30
Clay %	43

size distribution are important factors of microorganism enzymatic activity. Therefore, we measured basic properties to provide information about the soil we used in further experiments. Results are shown in Table 2.

Włodarczyk et al. (2002) indicated maximum DHA at pH 7.1, similar to the work of Ros et al. (2003) with pH 7.6–7.8 and Brzezinska et al. (2001) with pH 6.6–7.2. The pH_{H_2O} value of the soil used in our experiment was 6.96 and was conducive to achieving high dehydrogenase activity.

The mean content of organic carbon was 22.4 g kg^{-1} DW (Table 2). DHA is connected with the content of organic matter in soil—the higher the content of organic matter, the higher is a microbial activity (Ross 1971; Xie et al. 2009; Cross and Sohi 2011; Wolinska and Stepniewska 2012; Yuan and Yue 2012). In the analyzed soil, a relatively small amount of nitrogen (1.763 g kg^{-1} DW) and phosphorus (0.58 g kg^{-1} DW) was found (Table 2) and the C/N/P ratio in examined soil was 38/3/1. Liu et al. (2008) indicated that mentioned soil chemical properties can affect enzyme activity. The soil texture can also affect microbial activity. In analyzed soil samples, the clay particles (diameter < 0.002 mm) dominated (43%) (other fractions were silt (0.002–0.06)—30%—and sand (0.006–2 mm)—27%; Table 2), showing relatively high cation exchange capacity (Table 2). Most measured soil properties were optimal or close to optimal for maintaining high enzyme activity. However, small differences in mentioned soil properties, as well as chosen methodology for DHA determination, can strongly affect measured activity. It is worth pointing out that the great number of variables affecting enzyme activity forces us to find a universal way of assessing and comparing DHA values between different samples.

TPF sorption in soil

Different soil properties can affect the sorption of TTC and TPF. Organic matter and other substances with high affinity to TTC can decrease its concentration available to microorganisms. Lower available TTC concentration will cause lower TPF production. This can be interpreted as a low dehydrogenase activity. Therefore, optimal TTC concentration should be used in experiments, ensuring that the enzyme activity sites have been saturated. However, even when this requirement is satisfied, there is still the possibility that some part of the TPF concentration cannot be extracted from the soil. In our experiment, we examined the sorption properties of the used soil. The results are shown in Fig. 1.

The efficiency of the extraction process was approximately 80% (0.8047) and was constant for all used TPF concentrations. Constant and high efficiency should not affect the results of dehydrogenase activity measurements. The degree of formazan extraction may depend on physicochemical soil properties, which are related to adsorption of TPF outside the living cells (this experiment), and on

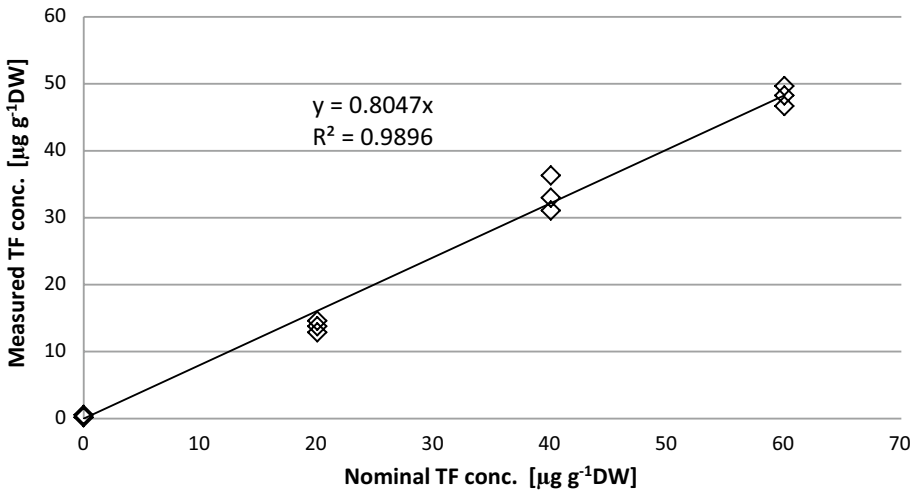

Fig. 1 Relationship between added and extracted TPF concentrations

current soil biocenosis of the soil sample. Different species can produce and accumulate TPF in different ways. Cell density can affect the range of uptake and the influence of TPF (Riss 2004). The extraction of formazan can be difficult in some cases, for example, from yeasts (*Saccharomyces cerevisiae*) due to strong cell walls, which are resistant to degradation. It should be pointed out that during this experiment, it is likely TPF did not penetrate living cells. Therefore, the observed effect is caused probably by the extracellular organic and inorganic matter.

Discussion on the models used

The rate of TPF concentration increase can be described by Michaelis—Menten (M–M) kinetic model (see "Optimal TTC concentration" section). However, to describe the relationship between the increasing product concentration (or decreasing substrate concentration) and the time of incubation, the M–M differential equation has to be solved. The solution can be found in the literature (Maggi and Cecilia 2016); however, in our opinion, it is too complicated to make it useful for common laboratory measurements—which aim is to assess fertility of soils or toxicity of different compounds. Therefore, for determining the derivative (slope) of the function of TPF production at the beginning of incubation time, a large simplification would be much more useful and it is given by Eq. (1). The model describes the situation where the TTC concentration is limiting the rate of TPF production. It is in contrast to the situation with excessive concentrations of TTC described in (Miksch 1985a). We assumed that the highest rate of TPF production and TTC concentration inside the cells is at time "0" and that the concentration of TTC does not increase over time inside the cells. The decrease in TTC concentration during incubation time causes a decrease in measured activity. These are useful simplifications allowing to use a model with only two parameters—Eqs. (1, 2). The interval of approximate linear increase in the TPF production starts at a time "0." Some examples of determination of time period of linear TPF concentration increase can be found in (Miksch 1988). However, the choices of these time periods were made arbitrarily and with the assumption that TTC concentration is still increasing inside the cells (or activated sludge folks) at the beginning of incubation time which results in an increase in TPF production rate. In this sort of cases, the relationship between the time of incubation and current TPF concentration can take more sigmoidal form. Thus, more complex model should be used—including kinetic of TTC concentration changes inside the cells (this kind of model would be more appropriate to describe some data presented by (Miksch 1988)). However, there is also a possibility to use our approach only for the data obtained from cells with stable TTC concentration by omitting measured values at the beginning of the incubation period. Furthermore, in case of the presence of chemicals with reducing potential in the sample, the background TPF concentration should be subtracted from the data.

Instantaneous rate of TPF production can be described by differential Eq. (1). It should be noted that this rate changes continuously until it reaches zero. There are many practical difficulties in determining the instantaneous rate. Therefore, a large majority of researchers focus on the initial phase of TPF production, where changes in the rate of TPF production are negligibly small and TPF concentration increase is almost linear. However, in a very small amount of studies, it has been verified that the increment is linear. Thus, simple division of measured TPF concentrations by the appropriate time of incubation can be the reason for the misinterpretation of results (secant activity). Therefore, we determined the tangent activity, which can be described as the first derivative of the used model (Eq. 2) in time equal to zero.

It should be pointed out that the presented model does not contain a direct relationship with enzyme kinetics—there is no direct relationship with enzyme concentration. Note that $\left(C_{\max} - C_{\mathrm{TPF}}(t)\right) = C_{\mathrm{TTC}}(t)$ so the model (Eq. 1) can be also written as $\frac{\mathrm{d}C_{\mathrm{TPF}}}{\mathrm{d}t} = kC_{\mathrm{TTC}}(t)$ where the differences between it and M–M model are clearly visible (lack of denominator; similar equation can be obtained from M–M model for $C_{\mathrm{TTC}}(t) \ll K_m$). This model can be used only with the assumption that the concentration of organic substrates (available electrons in electron transport chain) is constant over the incubation time. In other words, the concentration of organic substrate does not limit the activity. Therefore, it should be emphasized that presented Eq. (1) is only an *empirical* model (no mechanistic) which well describes experimental data and can be easily solved and used to determine tangent activity (Eq. 3).

Michaelis–Menten model is a mechanistic model derived for a strictly defined reaction catalyzed by the enzymes. The relationship between TTC concentration and rate of TPF production can be simplified and described by Michaelis–Menten kinetics mainly to illustrate this relationship and put it in a strict mathematical form given by Eq. (4). The used model is appropriate for low TTC concentrations because in this form it cannot be used to describe the toxic effect of TTC (in high concentration). In other words, the rate of TPF production is asymptotically increasing to $V_{\max}$ value. This model

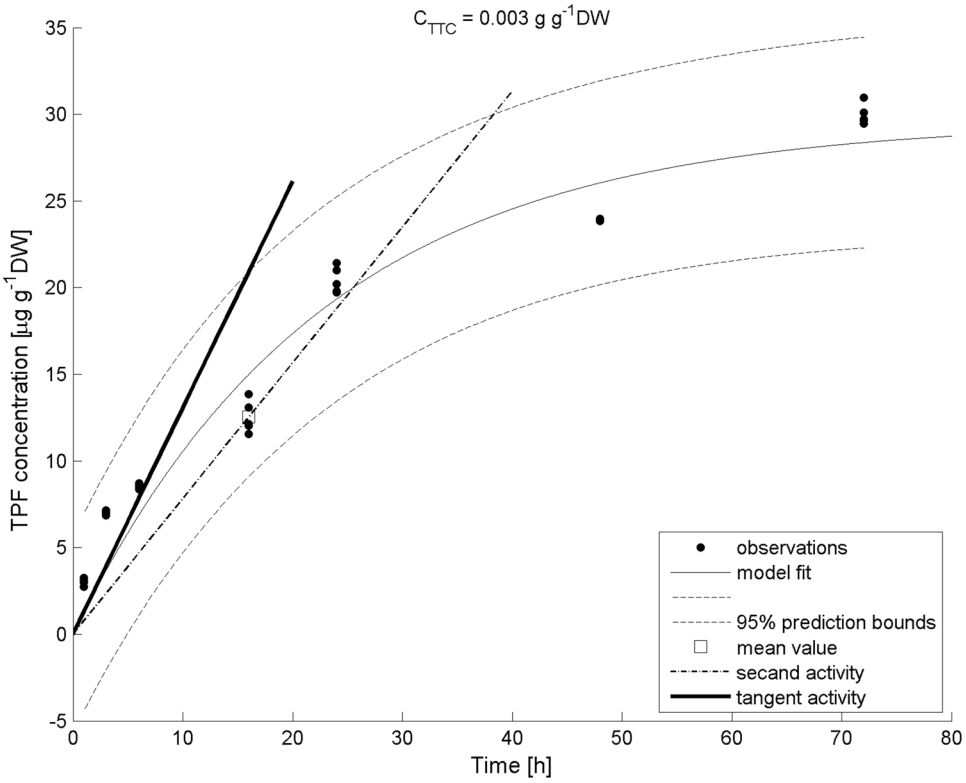

Fig. 2 TPF changes for samples prepared according to the Casida method

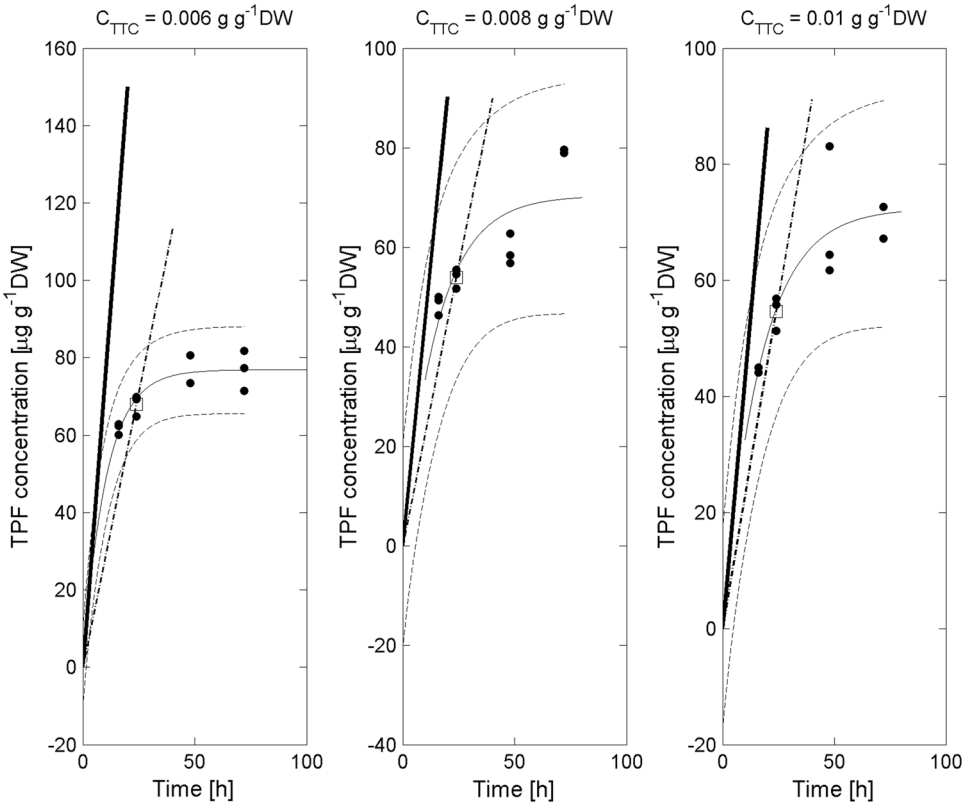

Fig. 3 TPF changes for samples prepared according to the standard method

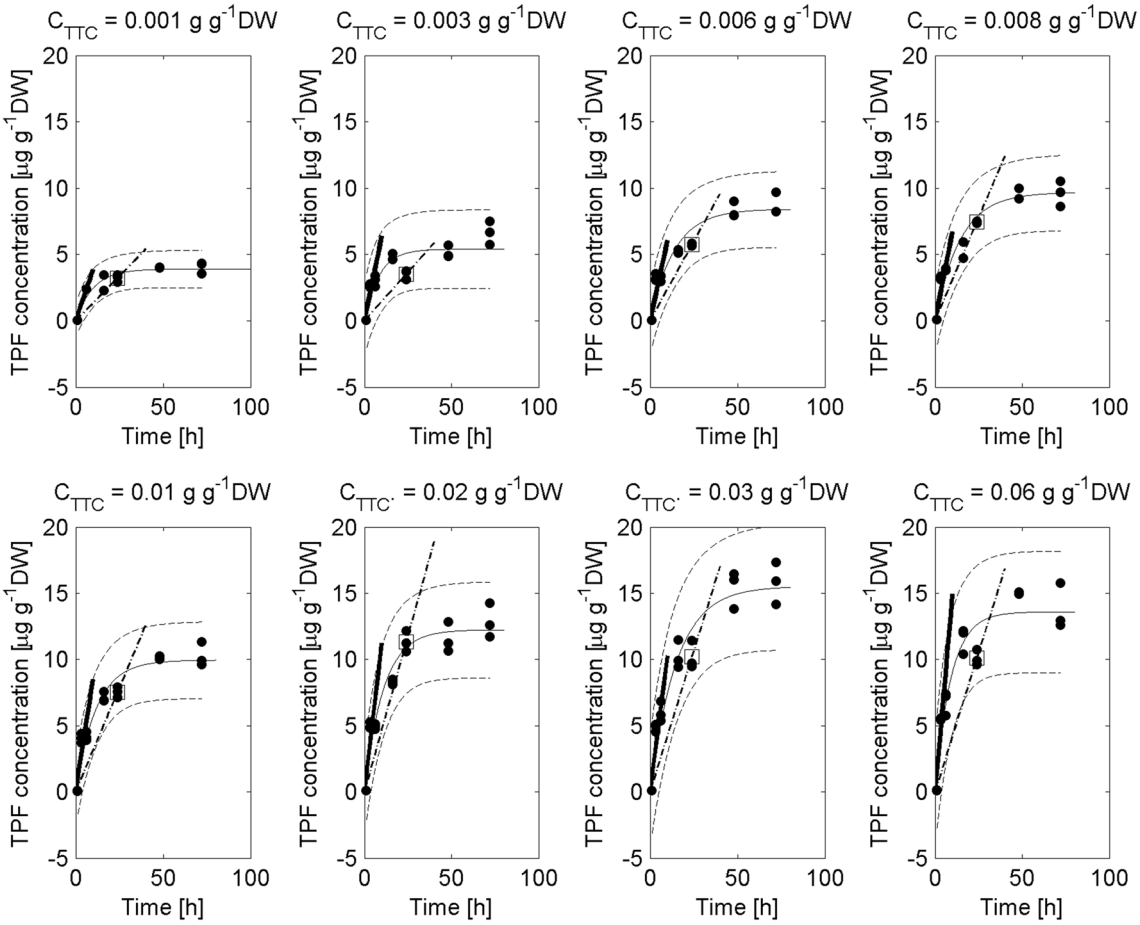

Fig. 4 TPF changes for samples prepared according to the optimization method

Table 3 TPF changes model—estimated parameters for samples prepared according to the Casida method, standard method, and optimization method

TTC g g^{-1}DW	Parameters (95% confidence bounds)		Goodness of fit			
	C_{max}	k	SSE	R^2	Adj. R^2	RMSE
Casida method						
0.003	29.6 (27.19, 32.02)	0.04412 (0.03516, 0.05308)	162.8253	0.9448	0.9431	2.2213
Standard method						
0.006	76.88 (73.2, 80.56)	0.09754 (0.07698, 0.1181)	110.1262	0.8019	0.7799	3.4980
0.008	70.37 (60.98, 79.77)	0.0641 (0.03738, 0.09082)	442.3262	0.6481	0.6090	7.0105
0.01	72.44 (64.09, 80.79)	0.05955 (0.03912, 0.07999)	313.8130	0.8018	0.7797	5.9049
Optimization method						
0.001	3.91 (3.465, 4.355)	0.09869 (0.055, 0.1424)	2.8551	0.9121	0.9053	0.4686
0.003	5.403 (4.586, 6.219)	0.1182 (0.05047, 0.1859)	18.2173	0.7766	0.7634	1.0352
0.006	8.425 (7.464, 9.386)	0.07186 (0.04551, 0.09821)	15.0617	0.8961	0.8896	0.9702
0.008	9.681 (8.7, 10.66)	0.06933 (0.04604, 0.09262)	13.7348	0.9279	0.9231	0.9569
0.01	9.952 (9.055, 10.85)	0.08523 (0.06043, 0.11)	19.3341	0.9218	0.9177	1.0088
0.02	12.24 (11.16, 13.33)	0.09176 (0.06467, 0.1188)	28.4960	0.9138	0.9090	1.2582
0.03	15.52 (13.86, 17.18)	0.0661 (0.04535, 0.08686)	46.8152	0.9101	0.9051	1.6127
0.06	13.6 (12.31, 14.89)	0.1099 (0.07307, 0.1467)	49.7519	0.9000	0.8947	1.6182

cannot describe the possible decrease in this rate in high TTC concentration. However, as would be shown in further parts of this work, the Michaelis–Menten model was suitable for obtained data.

Results obtained from the Casida method, standard method and optimization method

The results of the experiment are shown in Figs. 2, 3, 4 and Table 3. The goodness of fit was satisfying in most cases with the coefficient value of determination higher than 0.9. The curvature of fitted function was determined by parameter "*k*." Values of this parameter were similar in each experiment. They were between 0.04 and 0.12 with the Casida method and the optimization method, respectively. This parameter gives us information about the rate of reaction; in particular, it is the proportionality factor between TTC concentration available for microorganism and the rate of TPF production. The parameter k is equal to the reaction rate determined for the unit value of the substrate concentration. Therefore, tangent activity in $t = 0$ is proportional to parameter k and TTC concentration. *k* value does not depend on the concentration of the TTC or incubation time. Thus, it does not vary much between experiments as shown in Table 3. It can be said that it meets the requirement for the universality of the parameter for the evaluation of ADH (at least in case of using different methods for the determination of ADH). Further considerations in this work are based on the k parameter (tangent activity).

C_{max} parameter value corresponds to maximum TPF concentration, which can be produced by organisms in the soil sample. This concentration relates to TTC concentration in samples and the degree of formazan extraction from the soil. C_{max} is a stoichiometric parameter, and its value depends only on substrate concentration—available TTC concentration and available electrons originating from catabolic pathways. It should be pointed out that C_{max} does not influence the catalytic properties of dehydrogenases and vice versa. Equation 3 gives a link between the rate of reaction of TPF production and C_{max}. However, it should be noted that the rate of this reaction depends on organic substrate concentration (assumed to be constant), TTC concentration (which in this case has the same value as C_{max} ($C_{TTC}(0) = C_{max}$)), and the properties of enzymes (activity, concentration, and saturation which are hidden in Eqs. (1–3) behind the constant "*k*"). The same values of C_{max} can be obtained in different samples with different activities after different times of incubations (different *k* values). The relationship between TTC concentration and the rate of TPF production is described in the next section.

The values of C_{max} were different in each conducted experiment. The highest was observed when standard methodology was used, the lower was observed with the Casida method, and the lowest was observed with the optimization method. C_{max} in Casida, standard, and optimization methods is different in samples with the same amount of TTC added. For TTC concentration, 0.003 g g^{-1} soil C_{max} was equal to 29.6 with the Casida method and 5.403 with the optimization method. The differences were also observed in samples with a TTC concentration of 0.006 g g^{-1} soil: 76.88 and 8.425 in standard and optimization methods, respectively. In each of the three methods, a different solvent was used for the preparation of the TTC solution (Casida method: phosphate buffer, standard method: TRIS buffer, optimization method: distilled water). This can explain the differences between parameter values obtained in each of the experiments. Nevertheless, some common dependencies like constant *k*-value and the influence of TTC concentration on C_{max} value are noticeable. The relationship between C_{max} and TTC concentration can be observed clearly in results from the optimization method (Table 3). The higher the TTC concentration, the higher the C_{max} is. In the standard method, the C_{max} values for different TTC concentrations were similar, which could lead to the conclusion that, for example, the saturation point of TPF concentration was reached and more TPF could not be produced. When the dehydrogenase

Table 4 Comparison between activities determined by tangent and secant linear functions

TTC g g^{-1} soil	Casida method		Standard method		Optimization method	
	Secant	Tangent	Secant	Tangent	Secant	Tangent
0.001	–	–	–	–	0.1346	0.3859
0.003	0.7833	1.3060	–	–	0.1467	0.6386
0.006	–	–	2.8305	7.4991	0.2387	0.6054
0.008	–	–	2.2474	4.5107	0.3097	0.6712
0.01	–	–	2.2782	4.3141	0.3139	0.8482
0.02	–	–	–	–	0.4724	1.1234
0.03	–	–	–	–	0.4259	1.0262
0.06	–	–	–	–	0.4210	1.4938

activity is measured using traditional methods (Casida and standard method), time of incubation, which produces TPF concentration equal or close to C_{max}, should be avoided. This is because the relationship between time and TPF concentration produced is highly nonlinear. In this case, the rate of TPF production cannot be calculated as a ratio between TPF concentration and an arbitrarily chosen time of exposition (e.g., 24 h in Casida method or 16 h in standard method). This approach leads to the calculation of secant activity, which can be very different (smaller) to tangent activity at the beginning of the incubation period. The graphical interpretation is shown in Fig. 2.

It should be pointed out that the C_{max} depends on TTC concentration in the sample. The time of linear increase in TPF concentration (defined for example as a time interval in which 5% of available TTC concentration is transformed into TPF) depends only on k value. However, the mathematical description of this relationship can be defined only using arbitrarily chosen criterion.

Secant and tangent activity: Which one to choose?

The secant and tangent activities obtained in three different experiments are presented in Table 4. In all cases, the tangent activity was much higher than the secant activity. As shown in Figs. 2 and 3, the time of incubation proposed with the Casida method (24 h) and standard method (16 h) was too long and missed the time interval where the increase in TPF concentration is linear. We observed similar results given by the optimization method, where for comparison we arbitrarily chose the incubation time of 24 h. This may lead to misinterpretation of results as the true value of the activity is understated. To skip the determination of mentioned time intervals, it is possible to use all obtained data with the simple kinetic model (Eq. 2) and calculate the tangent activity. Using tangent activity is more appropriate as it defines the activity at the beginning of incubation where the decrease in substrate (TTC) concentration does not significantly affect the reaction rate. Moreover, the tangent activity, and even the rate of linear increase in TPF concentration, often cannot be determined directly from experimental data. This is because the TPF concentration in these two cases is so small that it is difficult to measure it with good accuracy. It is possible to increase TPF concentration by increasing the TTC concentration at the beginning. However, it is limited according to Michaelis–Menten Eq. (4). The main advantage of the presented approach is the determination of tangent activity from the simple kinetic model which can prevent formation errors resulting from improperly selected and imposed incubation times. Proposed model (Eq. 1) can be easily solved, and the solution can be fitted to experimental data. However, because more data points are needed compared to the Casida and standard method, it is more time- and cost-consuming.

Optimal TTC concentration: How to choose?

The optimum concentration of TTC is variable and depends on the current composition, morphology, and the physiological condition of the studied biocenosis. Determination of this concentration is a compromise between conflicting requirements: on the one hand to ensures sufficient concentration of TTC to reach intracellular structures and exhibit dehydrogenase activity and on the other hand that the applied concentration is not toxic. It is therefore advisable to determine the optimum concentration of this compound for a particular biocenosis and type of soil, depending on the content of organic substances (Małachowska-Jutsz et al. 1997). The discussion on optimal TTC concentration can be found in (Miksch 1985a, b); however, the author does not provide a mathematical description of the phenomena. Numerous studies have shown that it is necessary, and as a result, a standard was published in 2005 by the International Organization for Standardization, which describes the currently valid methodology for determining the dehydrogenase activity of soil microorganisms (ISO 23753-1:2005 2005; PN-EN ISO 23753-1:2011 2011). This standard, like the methodology, takes into consideration the need to apply a specified concentration of TTC, depending on the type of soil and organic matter content, especially for humic substances. The concentrations of TTC recommended in this norm for certain types of soils correspond with

Table 5 Values of Michaelis–Menten equation parameters

Parameters (95% confidence bounds)		Goodness of fit			
V_{max}	Km	SSE	R^2	Adj. R^2	RMSE
1.468 (1.027, 1.909)	0.006786 (0.0003984, 0.01317)	0.1579	0.8190	0.7888	0.1622

The curve was fitted to tangent activities determined in the optimization method

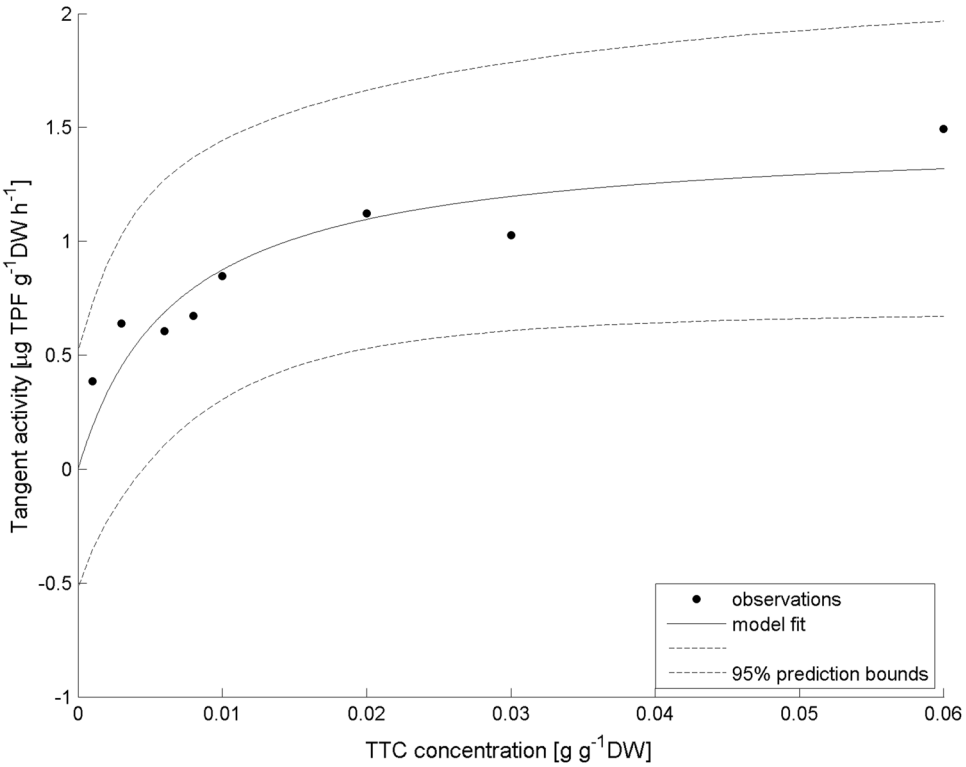

Fig. 5 Michaelis–Menten fit

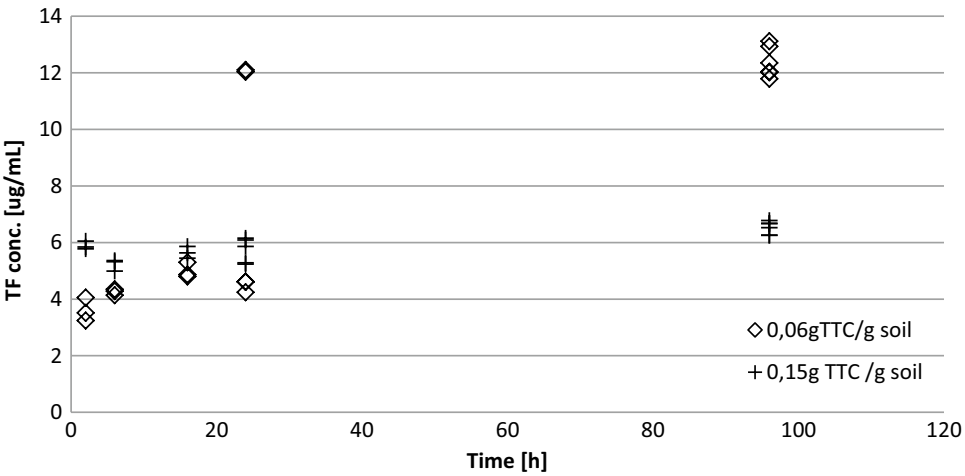

Fig. 6 TTC toxicity test

the concentrations indicated by Małachowska-Jutsz et al. (1997), as the optimum concentrations for the soils to be tested. However, a mathematical description of the determination of optimal TTC concentration was still missing. The relationship between tangent activity and TTC concentration is presented in Table 5.

For low TTC concentrations, which would not be toxic, this relationship can be described by Michaelis–Menten equation (Fig. 5 Michaelis–Menten).

As shown in Fig. 5, the highest TTC concentration results in the highest dehydrogenase activity. However, activity asymptotically approaches the maximum value. In high TTC concentration, the differences between activities can be negligibly small and we can assume that activity reaches a certain level; further increase in TTC concentration will not increase activity. The dehydrogenase enzymes are fully saturated with substrates (TTC), and their real activity can be measured. In lower TTC concentration, the rate of TPF production is lower because the TTC concentration is insufficient and limits the process (Miksch 1985a). For even higher concentrations of TTC, the toxic effect can be observed as a decrease in activity (Miksch 1985a). To describe these phenomena, the kinetic model with inhibition by the substrate or product should be used. However, this was not observed in our experiments. The toxic effects were noted only in the TTC toxicity test (Fig. 6). The main purpose of this test was to produce a toxic effect by extending the exposure time of microorganisms to the presence of TTC by reducing the temperature in the initial incubation phase and slowing down the TTC transformations.

It is known that the rate of enzyme catalysis generally increases with an increase in temperature until an unfavorable temperature, at which enzymes become denaturized and activity reduces (Wolinska and Stepniewska 2012). It has been shown that increasing the temperature from 24 to 37 °C results in an increase in dehydrogenase activity in samples of lower TTC concentration (0.06 g TTC g^{-1}) (Fig. 6), while no changes have been noted in samples with the addition of 0.15 g TTC g^{-1}, indicating a toxic effect of TTC. It should be noted that toxic effects were obtained in high TTC concentration (0.15 g TTC g^{-1})—higher than the value of 20 km.

The TTC concentration can affect the rate of TPF production, which can be approximately described by Michaelis–Menten kinetics. Therefore, we suggest choosing an optimal TTC concentration equal to 4 km. In this concentration, the tangent activity would be equal to 80% of its maximum value V_{max} and still far away from a level that could be toxic. The percentage of V_{max} can be used in the calculation of activity. For example, the activity of dehydrogenase measured using TTC at 4 km should be divided by 0.8 to calculate the actual activity in the sample. A higher concentration would not increase tangent activity significantly. For example, the value of 5 km will result in tangent activity equal to 83% of V_{max}. Much higher TTC concentration could also be toxic.

Conclusion

The dehydrogenase activity strongly depends on the method used to measure it—even when activity is measured in the same soil sample. Therefore, it is difficult to compare results from different experiments.

It is possible to minimize the influence of some important factors affecting the results. The main two factors are the time of incubation and TTC concentration in the sample. Unfortunately, time intervals in which increase in TPF concentration is linear are often smaller than the incubation time proposed in the standard and Casida methods. This may lead to misinterpretation of results. Nevertheless, it is possible to describe the increase in TPF concentration with the simple kinetic model. This approach allows the bypassing of time intervals in which the TPF concentration increment has a linear charter. It allows the possibility of calculating tangent activity from all obtained data. Furthermore, tangent activities depend on TTC concentration, which can be described by Michaelis–Menten kinetics. This relationship seems to be useful to determine the optimal TTC concentration. We suggest using the value of 4 km, which should result in tangent activity equal to 80% of maximal possible activity. The presented methodology provides a restricted mathematical description of determining optimal TTC concentration.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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