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Temperature sensitivity of soil dehydrogenase activity determined by the TTC method: An exponential modelling approach

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Abstract

Soil dehydrogenase activity (DHA) is widely assessed using the Casida method, which quantifies the optical density of triphenyl-formazan (TPF) formed after inoculating soil with triphenyl-tetrazolium chloride. However, how incubation temperature shapes the underlying reaction dynamics remains poorly resolved. We measured DHA in rhizospheric soil from a semi-arid site in Rajasthan, India, under two fertilization regimes (an unfertilized control, T_1 , and a treatment with recommended nutrients plus biofertilizer, T_5), sampling TPF formation over extended incubation times (up to 96 h) at 28 °C for both treatments and additionally at 15 °C and 40 °C for T_5 . An exponential model, $TPF(t) = TPF_{max}(1 - e^{-t/\tau})$, was fitted to each time series, yielding a saturation TPF level (TPF_{max}) and time constant (τ). The model fitted all series well (adjusted $R^2 = 0.952-0.993$) and outperformed a linear approximation in three of four cases. For T_5 , TPF_{max} peaked at 28 °C and declined sharply at 15 °C and 40 °C, while τ decreased monotonically with temperature (49.9 h at 15 °C to 29.8 h at 40 °C), showing temperature governs both the amount and speed of TPF formation, not rate alone. Combining both parameters, predicted 24-h DHA at 28 °C fell to ~65% at 40 °C and ~22% at 15 °C. Biofertilizer treatment (T_5) raised DHA 1.8-fold over the control at 28 °C, with the control showing a faster but lower-magnitude reaction. These findings identify 28 °C as the optimal incubation temperature for this soil and confirm the exponential model as a robust tool for resolving TPF formation kinetics, supporting efficient sampling-time selection in future DHA assays.

Keywords: Dehydrogenase, TPF, incubation temperature, rhizosphere, exponential modelling, biofertilizer

Introduction

As primary members of the family of oxido-reductase enzymes, soil dehydrogenases are to be mentioned (EC 1.1.1) (Gu *et al.*, 2009) ^[4]. They appear within all living microbial cells (intracellularly), and are one of the most significant enzymes among all inhabiting the soil environment. Soil dehydrogenases are therefore also used as a suitable overall indicator for microbial activity in soil (Gu *et al.*, 2009; Salazar *et al.*, 2011; Tabatabai, 1982) ^[4, 18, 20]. They are also closely related to microbial oxido-reduction reactions (Moeskops *et al.*, 2010) ^[14]. The additional important property of not accumulating extracellular in the soil makes them further suitable as microbial activity indicator. The variety of soil enzymes like ligases and lyases, as well as oxido-reductases, isomerases or hydrolases attribute each of them certain special biochemical roles or functions in the soil's energy and matter conversion process (Gu *et al.*, 2009) ^[4].

The relevant role in biological soil organic matter oxidation by soil dehydrogenases results from the hydrogen-transfer towards inorganic acceptors, from the inhabiting organic material (Zhang *et al.*, 2010) ^[25]. The transfer of hydrogen to nicotinamide adenine dinucleotide phosphate or either nicotinamide adenine dinucleotide occurs by dedicated dehydrogenases where the transferred hydrogen atoms take part in the biosynthetic reduction processes throughout these indicated co-enzymes (Subhani *et al.*, 2001) ^[19]. By this, any given soil's entire DHA is framed by multiple individual dehydrogenase activities of different enzymes present in this soil's living micro-organisms and hence can become considered as biological indicator of the microbial redox-system and used as measurement proxy to quantify the overall micro biological oxidation activities in the soil environment (Subhani *et al.*, 2001) ^[19].

Soil dehydrogenase enzyme produced by diversity of rhizospheric micro-organisms are the natural catalysts of different metabolic strategies that occur in the rhizosphere i.e., biodegradation of agrochemicals, decomposition of humus and organics matter, accumulation of organic matter for metabolic activities, release of essential macro-as well as micro-nutrients and offering them for plant's usage through molecular nitrogen fixation (Xie *et al.*, 2009; Małachowska Jutcz and Matyja, 2019) ^[24, 13]. There are reports showing that a biological indicator for judgement of soil quality parameters with organic matter and xenobiotic compounds is possible by evaluating the different enzymes activities i.e., soil dehydrogenase, lipases, cellulase, xylanase, catalase and ureases (Małachowska Jutcz and Matyja, 2019) ^[13].

Active dehydrogenases is able to utilize O₂ as well as other compounds as terminal electron acceptors, although anaerobic microorganisms produce most dehydrogenases (Brzezińska *et al.*, 2001) ^[1]. Because of this, the soil's metabolic ability is represented by the DHA and its activity level (or DHA value) may be used as suitable proxy for the soil's microorganism's biomass. Nevertheless, when dealing with the complex soil environment, with the highly diverse microorganisms and degradation processes, the relations between the overall microbial activity and the underlying particular biochemical characteristics are sometimes covered from the obvious (Salazar *et al.*, 2011) ^[18]. It has been reported that several soil factors, like soil moisture, pH, temperature and, clearly also organic matter content, as well as the soil's aeration conditions (rate of oxygen diffusion and redox potential) are stimulating dehydrogenase activity (Wolinska and Stępniewska, 2012) ^[22]. Various studies have investigated the change of soil DHA results caused by variations of the incubation temperature (at DHA measurement) or/and the soil's microorganisms quantity level (Subhani *et al.*, 2001; Ghaly and Mahmoud, 2006; Trasar-Cepeda *et al.*, 2007) ^[19, 5, 21]. As the soil Dehydrogenase Activity takes place within the cells of viable soil microbes, an optimum temperature range for highest activity can be expected, which should correlate with the temperature for optimum growth conditions of the respective microorganisms (Wolinska and Stępniewska, 2011) ^[23]. As the enzyme catalysis rate correlates positive with rising temperature, until a level becomes reached where enzyme-denaturation takes place (Wolinska and Stępniewska, 2012) ^[22], a similar temperature pattern for the DHA results may be expected.

Further, the physico-chemical properties of the rhizospheric soil may alter the results depending on the environmental factors. Organic matter content as well as the soil's sorption capacity shall be mentioned here as to become considered. Further-more, variations and/or modifications in the DHA determination strategies are creating a difficulty in comparing results from various experiments (researchers/DHA measurement methods) when changes in TTC concentration, soil sample type/weight (1-10 g) or incubation time (1-40 hrs) and incubation temperature (20-37°C), and use of various organic matter additions (glucose, dextrose, yeast extract, etc.) take place. Sometimes a linear increase of the TPF over time, starting with the TTC inoculation, is assumed at least for certain duration (incubation time range). This (variable and unknown) range where a linear increase in triphenyl formazon can be considered, and becomes relevant in case the entire kinetic

of enzymes is unknown, and was not described in various research. It is worth pointing out that the parameters incubation time, incubation temperature and/or selected TTC concentration were often selected based on former research experiments, or had been selected according other boundary conditions. Such data may create the risk of comparing and interpreting results which were measured on different pre-conditions (Januszek *et al.*, 2015) ^[9]. Last but not least, the selected TTC concentration level is preferable sufficient elevated for saturating the active site of enzymes in the ETC system, but shall not be harming (toxic) to the diversity of rhizospheric micro-organisms; a relevant parameter often not determined (Małachowska-Jutcz and Matyja, 2019) ^[13].

There are also various mathematical models mentioned for the TPF formation during the incubation time, either based on the exponential function or modified models as the Michaelis-Menten kinetic model (Małachowska-Jutcz, A., and Matyja, K., 2019) ^[13], which are in contrast to the assumption of an (initial) phase where TPF increase in a linear way. Having a suitable mathematical model allows also to interpolate TPF levels between different incubation durations and therewith compare results more accurate, especially if the TPF formation process does not follow a linear increase. Therefore, one objective of the presented work was conducting a detailed modelling approach to determine the dynamics of the TPF formation at different incubation time and temperatures and comparing against result according the existing Casida method (Casida *et al.*, 1964) ^[2].

Materials and Methods

Collection and Physico-chemical characterization of soil samples

The study was conducted at the Agriculture Research Station, Mandor, Agriculture University, Jodhpur, Rajasthan, India (25°23.115' N latitude, 72°30.726' E longitude; 150 m above sea level). The region is characterized by an arid climate with sandy soils typical of western Rajasthan. Soil samples were collected following the harvest of pearl millet during the 2022-2023 cropping season. Rhizospheric soil samples were collected from a depth of 0-20 cm, representing the active root zone, from adjacent experimental plots under two treatments (T₁ and T₅). The samples were collected using standard soil sampling procedures, ensuring minimal disturbance and contamination, and were transported to the laboratory for further analysis. Treatments T₁ (Control): Soil without the application of Recommended Dose of Nutrients (RDN) or biofertilizers; T₅ (Treatment): Soil collected from plots treated with Recommended Dose of Nutrients (RDN), plant growth-promoting rhizobacteria (PGPRs) as biofertilizers, and an appropriate quantity of Farm Yard Manure (FYM).

The samples got air-dried at 25 °C to a water content of around 2/3 of field capacity (~ 0.26 g g⁻¹ soil). For removing stones and to homogenize the samples, the soil became sieved using a 2mm mesh. Physico-chemical soil parameter analyses were carried out in four replicates; numbers presented are the arithmetic average of the replicates (table 1). Soil texture was classified according the Casagrande aerometric method by separating the sand sub-fraction in sieves with mesh sizes of 1, 0.5, 0.25, and 0.1 mm (Małachowska-Jutcz and Matyja, 2019) ^[13]. Particle size groups were determined as per Polish Society of Soil

Science classification (PTG, 2011). Measurement of soil pH H₂O and pH KCl in 1 mol KCl dm⁻³ was done potentiometrically according to (PN-ISO 10390:1997 1997), with a combined electrode. The Kappen method (Jaremko and Kalembsa, 2014) [10] was used to determine the cation exchange capacity (CEC). Hydrolytic acidity values and CEC permitted gravimetric moisture content determination according ISO 11465:1993 (drying of 100 g soil sample for 24 hrs at 105 °C). The Kjeldahl method (ISO 11261:1995) was applied to measure total nitrogen and Tiurin method, as per standard protocol suggested by Mebius (1960) [15], to measure overall organic carbon content. Overall phosphorus level was determined from Soil Science Lab of IARI New Delhi, according to (ISO 11263:1994).

Table 1: 'Physico-chemical characteristics of soil (0-20 cm)

Parameter	Value ± Standard deviation, n = 4
pH _{H2O}	7.12 ± 0.01
pH _{KCl}	5.10 ± 0.02
Hydrolytic acidity c mol/kg	1.08 ± 0.37
Cation exchange capacity (CEC) cmol kg ⁻¹	13.76 ± 0.18
Moisture (%)	17.78 ± 0.35
Nitrogen (%)	0.1463 ± 0.0011
Organic carbon (%)	0.19 ± 0.01
Phosphorus (%)	0.048 ± 0
Sand (%)	84
Silt (%)	5
Clay (%)	11
Class	Loamy Sand

Estimation of soil dehydrogenase activity (DHA) at different temperature and incubation periods

For both soil probes, DHA was measured according the standard protocol published by Casida *et al.*, (1964) [2], with additional sampling at different incubation times. The reagents were mixed in the test tubes using a glass rod (in a way avoiding sample-aeration). For soil probe T₁ the incubation temperature was set to 28 °C and selected incubation time were 2, 4, 8, 12, 22, 32, 72 and 96 h, lightning condition was dark during incubation. This T₁ results were used to get base data and to select a suitable parameter estimation process. For soil probe T₅ we selected with 4, 8, 24, 48, 72 and 96 hours in the dark slightly different incubation times and, besides the 28 °C incubation temperature, two additional temperatures maintaining 40 °C on the high side and 15 °C on the low side during the incubation time.

Following the incubation duration, the probes were centrifuged for 7 minutes (at 3000 rpm), the supernatant was discarded. Afterwards, the produced TPF was then extracted in 5 ml of methyl alcohol. After shaking the samples properly, they were centrifuged a second time. The light absorbance of the supernatant was then determined with a Systronics Spectrophotometer Model 482-SI at the optical wavelength of 485 nm. The final TPF concentration of the probe was then determined according a calibration curve which was prepared beforehand according to established standard procedure. For each sample defined by soil-probe (treatment), incubation-temperature and incubation-time, three replicates have been analyzed, resulting in 24 probes for T₁ and 54 probes for treatment T₅.

All analysis work was done at at the Basic Science Laboratory (BSL) at Agriculture University Jodhpur, Rajasthan, India.

Function modeling and parameter optimization

According to Małachowska-Jutysz and Matyja, (2019) [13] the formation of TPF concentration can be approximated by the differential equation:

$$d/dt \text{ TPF}(t) = k * (\text{TPF}_{\max} - \text{TPF}(t)) \quad (1)$$

The change (increase) of TPF at any given time (t) after incubation can be described as delta between a final saturation level (TPF_{max}) and the actual TPF level (TPF(t)), multiplied with a constant factor (k). Solving this differential equation for the initial condition TPF_{t=0} = 0 and substitution of $\tau = 1/k$ gives:

$$\text{TPF}(t) = \text{TPF}_{\max} (1 - e^{-t/\tau}) \quad (2)$$

with "e" denoting Euler's number (2,71828...) and "τ" a characteristic constant value.

By this, the generated TPF amount can be described as a function of incubation time by an exponential function with TPF_{max} as saturated TPF value (after "infinite" incubation time) and the characteristic time constant τ, indicating the incubation time (duration) after which the TPF value reaches ~63% (value: 1-e⁻¹) of the saturation level TPF_{max}. Variation of the two unknown parameters (TPF_{max}, τ) and comparing against the measured data lead to an optimum combination to approximate the measured values best, as per selected model.

For finding the best parameter combination the "Coefficient of determination" (R²) was used as quality metric (optimization target: R² → max).

$$R^2 = 1 - (\text{SS}_{\text{res}} / \text{SS}_{\text{tot}}) \quad (3)$$

With

SS_{res}: Sum of Squares of the Residues; Σ(measured values-modeled values)²

SS_{tot}: Sum of Squares Total; Σ(measured values-measured mean value)²

This coefficient varies between *zero* (model is the mean value as a constant) and *one* (model shows perfect fit to the measured data). The "measured values" are the average of the replicates for each sample time, the "modeled values" are the model outputs (for a particular parameter combination) at each sample time and the "measured mean value" is the mean of all "measured values" (for each particular treatment series).

Search range for TPF_{max} was selected between 80% and 140% of highest measured TPF value for each series, with an increment (resolution) of 0.2 μg. For the time constant τ, the search range was set between 10 and 70 hours, with an increment of 0.1 h resulting in 601 different τ values and between 223 and 895 different TPF_{max} parameter values for the particular optimization runs (e.g. 537895 parameter combinations for T₅-28 °C). The optimization of the parameter combination (search for maximum R² value) was done with the mathematics software OCTAVE.

The achieved R² values were also adjusted (R²_{adj}) for sample size and number of model parameters according the following definition (Wherry):

$$R^2_{\text{adj}} = 1 - \frac{n-1}{n-p} * (1 - R^2)$$

With n : Sample size (9 for T_1 and 7 for T_5 , each including $t = 0$); p : Number of model parameters (2, for the selected exponential function)

Statistical Analysis

The calculated soil parameters (Table 1) showing the mean $\pm$ Standard Deviation values out of four individual replicates per sample (calculated with MS EXCEL 2016). Various pooled treatment data were subjected to analysis of variance (ANOVA) using SPSS (version 15.0). The significant difference between mean of different treatments was analysed with Tukey's Honestly Significant Difference (HSD) test. Least Significant Difference (LSD) test at $p < 0.05$ was applied to test the resulting separation of the treatment means.

Results

Different soil physico-chemical properties *i.e.* pH, Hydrolytic acidity, moisture, cation exchange capacity (CEC), total nitrogen and phosphorous, total organic carbon, particle percentage distribution are essential factors of the microbial enzymatic activity (Soil biological Indicator). This basic physical-chemical properties were measured and are provided as suitable information about the collected rhizospheric soil in the table 1.

The pH value of rhizospheric soil collected for the experiment was at 7.12 and this was optimum for generating maximum soil dehydrogenase activity. Similarly, mean content of total organic carbon was $1.90 \text{ g kg}^{-1} \text{ DW}$. Soil dehydrogenase enzyme activity highly correlated with presence of total organic carbon content in the rhizospheric

soil. In physico-chemical properties of soil, a relatively low amount of nitrogen and phosphorous ($1.463 \text{ g kg}^{-1} \text{ DW}$ and $0.58 \text{ g kg}^{-1} \text{ DW}$, respectively) was found and also depicted in the table 1. Soil dehydrogenase enzyme activity might be also affected by the soil texture. In this regard we have estimated soil texture of rhizospheric soil. In estimation, rhizospheric soil samples, the sand (diameter $0.005\text{--}2 \text{ mm}$)-dominated (84%), silt ($0.002\text{--}0.005 \text{ mm}$)-5% and clay ($< 0.002 \text{ mm}$)-11%.

The aim of the different additional incubation times for the DHA data was to verify the exponential development over time of the DHA activity process, and to test the quality of a model fit to the proposed exponential function that can be achieved for the two different soil types and at the different incubation temperatures for treatment T_5 . All optimization runs converged for each series (treatment) towards an individual single maximum for R^2 within the defined search range, indicating a suitable model with a defined single optimum parameter set. Figure 1 shows measured TPF values (TPF_{mean} - mean from 3 replicates) at different incubation times, along with the 95% confidence range (from Student's two tail t-test) for treatment T_1 and T_5 (sub-figure A1 for treatment T_1 , sub-figures B1, C1 and D1 for treatment T_5). Also the model predicted data (line curve) and the model predicted maximum TPF value (TPF_{max} , dashed line) is shown. The outcome of the parameter optimization process is shown in the respective contour plots (A2... D2), showing parameter combinations with constant R^2 values and the parameter combination resulting in the highest R^2 (* symbol).

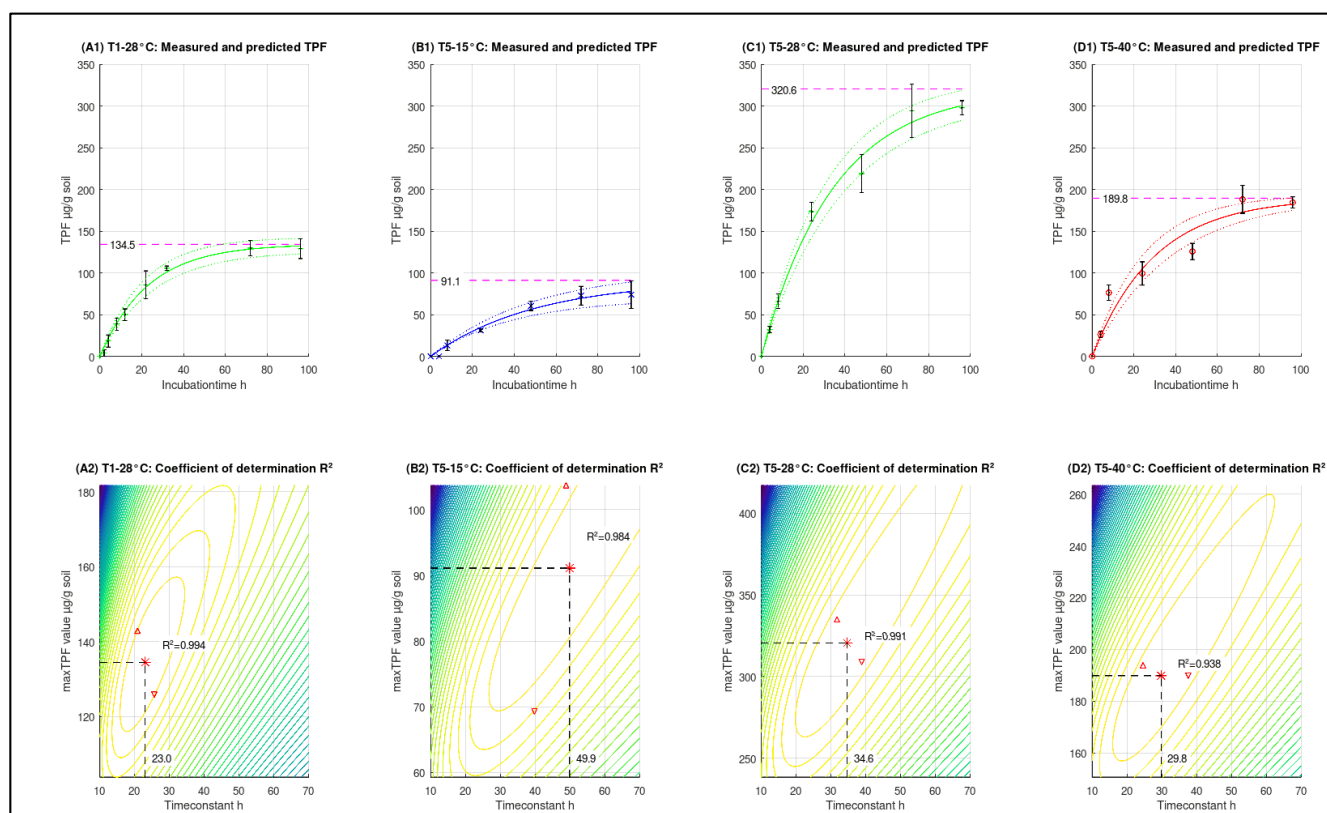


Fig 1: Upper row (A1.. D1) shows measured TPF values (mean of 3 replicates) at different incubation times, along with the 95% confidence range (from Student's two tail t-test) for treatment T_1 and T_5 (black markers and error-bars). Sub-figure A1 for treatment T_1 at 28 °C incubation temperature, sub-figures B1, C1 and D1 for treatment T_5 with incubation temperatures of 15 °C, 28 °C and 40 °C respective. Line curve in the sub-figures show best-fit model predicted values and the predicted maximum TPF value (dashed line) for each data series. Dotted line curves show best TPF model fit when upper/lower confidence values had been used for optimization run, instead of the mean values from the replicates.

Lower row of sub-figures (A2.. D2) show the respective contour plots for R^2 (coefficient of determination) for each treatment, as function of the two variation parameters TPF_{max} and time constant τ (in hours). The dashed lines indicate the optimum variation parameter combinations for maximum R^2 , as well as the resulting R^2 values, always for the mean value data. Additionally, the found parameter combinations for best fit with the upper bound data (Δ symbol) and the lower bound data (∇ symbol) are shown.

In order to find reasonable ranges for the optimum model parameters, the model parameter search was repeated using the values on the 95% confidence range of the TPF_{mean} values ($TPF_{mean} \pm$ confidence interval). The dotted curves in the figures A1 ... A4 indicate the resulting model values when using the upper/lower confidence boundaries of the measured values as input for the parameter modeling. The herewith found parameter combinations are indicated in the contour plots as Δ symbols for results on the upper bound and as ∇ symbols for the lower bound results.

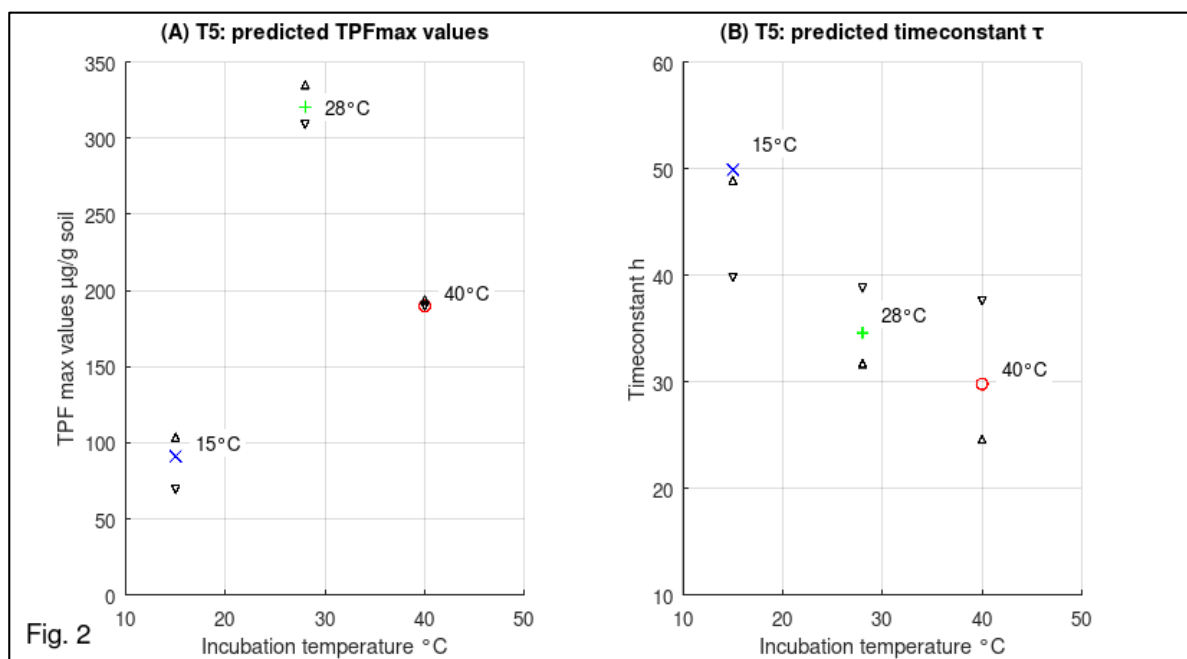


Fig 2: Sub figure (A) show model-predicted TPF_{max} values for soil treatments T₅ with incubation temperature on horizontal axis. Also, model-predicted TPF_{max} values for the treatments when mean values $\pm$ confidence range was used in parameter optimization runs, with ' Δ ' symbol for "mean + confidence range" and the ' ∇ ' symbol for "mean-confidence range" data.

Sub figure (B) show model-predicted time constant values (τ) for treatments T₅ with incubation temperature on horizontal axis. Also, model-predicted τ values for the treatments when mean values $\pm$ confidence range was used in parameter optimization runs, with ' Δ ' symbol for "mean + confidence range" and the ' ∇ ' symbol for "mean-confidence range" data.

Results from treatment T₅ show highest TPF_{max} for 28 °C incubation and lowest TPF_{max} at 15 °C, indicating highest TPF levels are achieved between the 15 °C and 40 °C incubation temperature. For the time constant, there is a clear trend to faster reaction velocity with higher temperatures, indicated by lower values of the time constant

parameter at higher temperatures. According to the optimum model parameters, the reaction velocity is fastest at 40 °C for this treatment, with a time constant of 29.8 h and shows slowest reaction at 15 °C incubation, with 49.9 h until 63% of TPF_{max} is reached. In comparison of treatments T₁ vs. T₅, where we used same incubation temperature (28 °C) we can see a TPF_{max} prediction with a level of 42% for T₁, compared to T₅, but with a faster reaction velocity (time constant of 23.0 h for T₁ compared to 34.6 h for T₅, 28 °C) showing that both parameters are not only temperature but also treatment depending. All the data are depicted in the table 2 and table 3.

Table 2: Measured and predicted DHA (24h secant activity) and predicted maximum DHA, at beginning of incubation (tangent activity).

Treatment	Incubation temp. ° Cels	DHA measured µg TPF/g soil/h	Confidence Range (95%) µg TPF/g soil/h	DHA secant pred. † µg TPF/g soil/h	DHA tangent pred. ‡ µg TPF/g soil/h
T ₁	28	3.90*	± 0.76	3.63**	5.85
T ₅	15	1.30	± 0.07	1.45	1.83
T ₅	28	7.23	± 0.47	6.68	9.27
T ₅	40	4.14	± 0.58	4.37	6.37

* Measured after 22 hours, ** prediction for 24 hours

† Predicted secant values are derived from model data, based on 24h incubation time

‡ Predicted tangent values are derived from model data, based on first derivative at $t = 0h$

Table 3: Model predicted maximum TPF levels, time constants and Coefficient of determination for TPF creation process

Treatment	TPF _{max} (μg)/(g soil)	Time-constant (h)	K (1/h)	SSE	R ²	R ² _{adj}	RMSE
upper [^]	142.9	20.8	0.0481	165.2	0.993	0.992	4.86
T ₁ -28 °C	134.5	23.0	0.0435	128.4	0.994	0.993	4.28
lower [˘]	125.9	25.6	0.0391	377.1	0.980	0.978	7.34
upper [^]	103.7	48.9	0.0204	154.5	0.983	0.979	5.56
T ₅ -15 °C	91.1	49.9	0.0200	104.8	0.984	0.981	4.58
lower [˘]	69.3	39.8	0.0251	165.2	0.965	0.958	5.75
upper [^]	335.0	31.7	0.0315	1280.1	0.988	0.985	16.00
T ₅ -28 °C	320.6	34.6	0.0289	843.0	0.991	0.989	12.98
lower [˘]	309.0	38.8	0.0258	961.5	0.988	0.986	13.87
upper [^]	193.8	24.6	0.0407	2490.6	0.929	0.915	22.32
T ₅ -40 °C	189.8	29.8	0.0336	1966.1	0.938	0.925	19.83
lower [˘]	189.8	37.6	0.0266	1497.6	0.947	0.936	17.31

[^] Results from "mean + confidence range" data

[˘] Results from "mean-confidence range" data

The enzymatic activity in soil DHA is usually expressed as (μg TPF) (g soil)⁻¹(hours incubation time)⁻¹. Following the Casida method, the DHA is therefore the TPF level after 24 h incubation time, divided by 24 h. This is equivalent to the “secant activity” described by Małachowska-Jutysz, A., and Matyja, K., (2019) [13]. The “tangent activity” referred-to in that paper equals to the first derivative at the beginning of incubation, or the TPF_{max} value divided by the time constant τ . Having a suitable mathematical model permits to compare

the model output with the measured data and/or predicted values. Figure 3 shows the predicted DHA values and the results achieved with the measured data (as per Casida method). The predicted DHA is highest at the beginning of the incubation time and then reducing, as expected by the used model. The DHA levels at time = 0 are naturally also corresponding to the values achieved with the “tangent method” (see Małachowska-Jutysz, A., and Matyja, K., 2019 → tangent activity) [13].

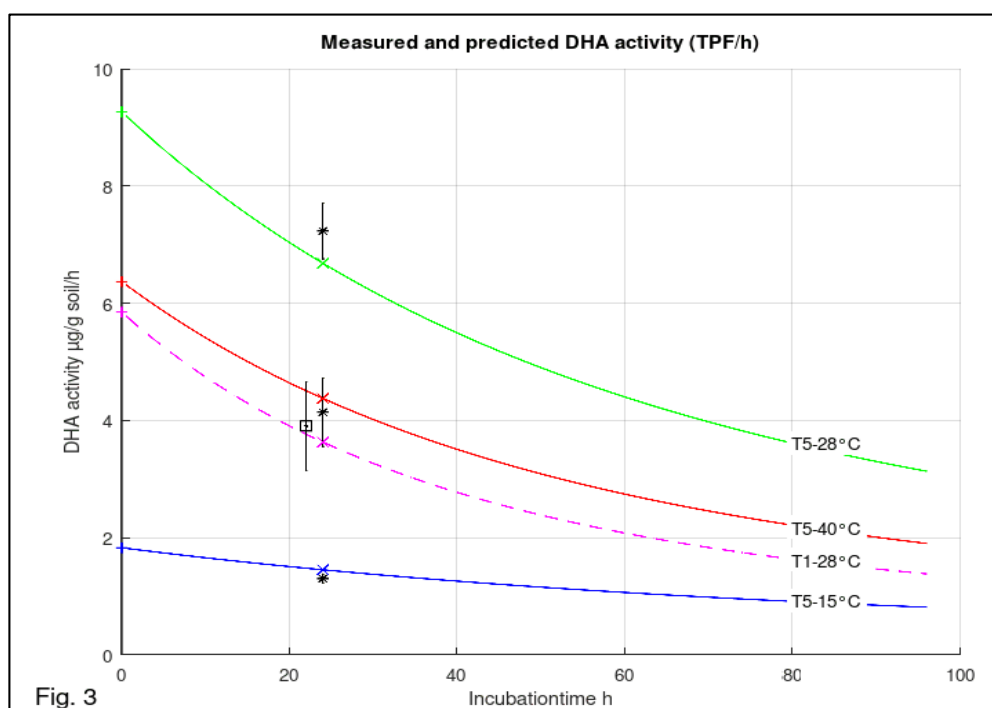


Fig. 3: Shows measured DHA activity according Casida (black ' * ' markers for T₅ treatments, after 24h incubation and black ' □ ' marker for treatment T₁, after 22h incubation) along with resulting confidence ranges as error-bars. Solid line curves show model predicted DHA activity for T₅ and dashed line for T₁ (TPF values/incubation-time, in μg TPF/g soil/h). The values at incubation-time = 0h correspond to “tangent method” according Małachowska-Jutysz, A., and Matyja, K., (2019) [13]. The ' x ' markers denote the model predicted DHA values at 24 h, also for the soil treatment T₁.

We finally tested the exponential model against a linear function for the TPF formation. For this, the mean of the measured data from T₁ and T₅ up to 22 h respectively 24 h incubation time were used and a linear regression coefficient was calculated. We then compared the adjusted R² resulting from this linear model (R²_{adj lin}) against the respective ones from the exponential model (R²_{adj exp}), and used this to judge the model quality; higher R²_{adj} indicate better fit of the

model data to the measured values. With our data we achieved better model fit (higher R²_{adj}) for 3 out of the 4 treatments with the exponential model. The exception was T₅-28 °C, where the linear model achieved a marginally higher R²_{adj} (0.996) than the exponential model (0.989), likely because TPF had not reached saturation within the 24 h range used for the linear comparison. (Table 4).

Table 3: Linear regression parameter, linear model predicted TPF levels after 24h, predicted DHA values and model fit quality parameter R^2_{adj} (for linear model and exponential model)

Treatment	linear Regression gain* ($\mu\text{g TPF}/(\text{g soil})/\text{h}$)	TPF _{pred} @ 24h ($\mu\text{g}/(\text{g soil})$)	DHA _{pred} @ 24h ‡ ($\mu\text{g}/(\text{g soil})/\text{h}$)	R^2_{adj} lin	R^2_{adj} exp
T ₁ -28 °C	4.044	97.06	4.044	0.986	0.993
T ₅ -15 °C	1.300	31.20	1.300	0.947	0.981
T ₅ -28 °C	7.354	176.50	7.354	0.996	0.989
T ₅ -40 °C	4.726	113.43	4.726	0.718	0.925

* Linear regression was forced through zero (at $t = 0$), hence offset parameter equals zero

‡ As DHA value equals TPF value after incubation time, divided by incubation time, the linear model predicted value is identical to linear regression gain parameter

Underlined numbers indicate better model fit (higher R^2)

Discussion

In our estimation of physic-chemical soil properties, most of these were found to be optimal for higher soil dehydrogenase enzyme activities efficiency. However, minor differences in described rhizospheric soil properties and selected appropriate approach for soil dehydrogenase enzyme estimation can affect the efficiency of the measured enzyme activities (Liu *et al.*, 2008; Xie *et al.*, 2009; Cross and Sohi, 2011) [12, 24, 3]. The presented experiment, carried out on one soil type in two different fertilization treatment conditions, reveal that the soil treated with recommended (bio-) fertilizer amount shows 1.8-fold increase in Dehydrogenase activities, compared to our control (T₁). This result is not a surprise, as DHA is reflecting the amount of enzymatic activity in soil, where elevation is expected with the application of bio-fertilizer. We also see a dependency of the TPF level (and the DHA results) on the incubation temperature with a maximum at 28 °C (the mid-one of the selected temperatures), where the DHA is 1.7-fold higher compared to the 40 °C, and 5.6-fold higher compared to 15 °C incubation temperature, at the same soil. As the variants on incubation temperature for T₅ were carried-out on the same soil sample the differences come from the change in the temperature of the analysis (the measurement) procedure. They may result from either in slower reaction dynamics or a lower reaction amount, or a combination of both. While in the first case the TPF is expected to still reach the same level after longer incubation, only taking more time, the second case would mean a comparable reaction dynamic at a lower reaction intensity, indicating the TPF will not reach same amount, even after long incubation.

To answer these questions, we turned to mathematically modeling the TPF formation process as exponential function and investigated the temperature dependency, reflected by the achieved model parameters. The findings reveal a *faster reaction* with higher temperature (ref. Fig 2 B), where reaction speed increases by ~20% at 40 °C incubation, indicated by smaller time-constant parameter τ (change from 34.6h to 29.8h), but slows down by ~30% at 15 °C incubation temperature, always with 28 °C as reference. In terms of *reaction intensity*, we see a peak at 28 °C, where model predicted TPF saturation level TPF_{max} (after "infinite" incubation time) reduces to ~60% at 40 °C and to ~28% at 15 °C incubation temperature (Fig. 1). The combination of reaction dynamics and intensity result into a model predicted maximum DHA (determined by the TPF level after 24h incubation) at 28 °C, with a drop to ~65% (from 6.68 to 4.37 $\mu\text{g TPF}/(\text{g soil})/\text{h}$) at 40 °C and a drop to ~22% (from 6.68 to 1.45 $\mu\text{g TPF}/(\text{g soil})/\text{h}$) at 15 °C incubation temperature (see Table 1).

All model predictions are well supported by the measured results, we therefore may use model predicted values also to

estimate values at other incubation times, especially at $t = 0$ (start of incubation) where soil enzyme reaction is highest. The mathematical model predicts a DHA calculated according the "tangent method" (Małachowska-Jutysz and Matyja, 2019) [13] tangent activity) of 9.27 $\mu\text{g TPF}/(\text{g soil})/\text{h}$ for the T₅ at 28 °C incubation temperature. At 40 °C the tangent model-predicted DHA shows a value of 6.37 $\mu\text{g TPF}/(\text{g soil})/\text{h}$, which is a drop to ~69% and 1.83 $\mu\text{g TPF}/(\text{g soil})/\text{h}$ at 15 °C incubation temperature, at drop to nearly 20% of the value at 28 °C incubation temperature. Comparing the two fertilizer treatments (T₁, T₅) we see an increase of nearly 1.6-fold in the tangent DHA results at 28 °C incubation for the T₅ treatment with bio fertilizer applied (see Table 1).

Our findings confirm that an exponential function as mathematical model, as proposed by several authors, fits well to the measured data with resulting high values for the correlation quality indicator (R^2_{adj}) between 0.952 and 0.993. Both variable model parameters converge for each series towards a unique combination, leading to a maximum (optimum) R^2 for each investigated treatment (data series), as seen in the related contour plots; the exponential function hence defines a suitable model for this purpose.

The achieved model parameters clearly confirm a temperature dependency of the TPF formation. Results from soil treatment T₅ reveal a tendency towards faster reaction (smaller value of the time constant parameter) with rising incubation temperature for the TPF formation, which is in line with many reports on faster chemical reactions with rising temperatures, within a certain temperature range. We also observed a temperature dependency for the TPF saturation level (TPF_{max}) achieved. In the range of the investigated temperature levels between 15 °C to 40 °C we identified a peak value for the TPF_{max} parameter, in our case at an incubation temperature of 28 °C, for the soil treatment T₅.

Also, this observation is backed-up by other authors (Gu *et al.*, 2009) [4], where a rise in the level of the reaction product (*i.e.*, TPF level) has been observed when increasing the reaction temperature with a following drop of this level when temperature is exceeding a certain optimum level.

Results from treatment T₁ were gathered only at 28 °C and confirm that maximum TPF level as well as reaction rate (time constant) are not only temperature depending but change also with the soil treatment/type.

Conclusion

Soil dehydrogenase enzyme activity mainly depends on the environmental factors and associate methods used to determine it, even when estimated from the same rhizospheric soil. Measuring the enzyme activity is influenced not only by the soil itself but also by the measurement method. The widely used Casida method relies

on incubation of the soil sample with a TTC suspension and the subsequent formation of TPF. We observed (among others) that this process is strongly influenced by the temperature during the incubation time and the TPF formation rate changes over the incubation duration. From our findings we conclude that the exponential model is a suitable mathematical model for the TPF formation process in the course of soil DHA analysis.

The effort for model parameter fitting (to the measured data) is modest and straight forward and can be carried-out with any mathematics software, capable of basic functions. The numbers of additional samples required are also modest; where we recommend to skip some sampling times (incubation times) used and plan instead for 6, 12, 24, 48, 96 hrs as incubation times for the purpose of soil DHA analysis. Applying the dispersion parameters (e.g. standard deviation) from the measured replicates to the model parameters is not a straight-forward procedure, as the selected model is not linear in the measured data and the exponential factor (time-constant) has a different unit. We therefore applied the optimization procedure additionally onto the data given by the 95% confidence range derived from the replicates of the individual samples. Even though these resulting model parameters do not denote the 95% confidence range for the best-fit model parameters, they indicate a range for the sensitivity of the model parameters with respect to the variations seen in the measured data.

Having a suitable mathematical model for a bio-chemical reaction provides benefits, but comes mostly at the cost of additional measurements required and the modeling work to be done. In case of the selected TPF dynamics model with the two parameters, the minimum required measured values are two, compared to the Casida method, where one data sample after 24h incubation time is mandated. Using the bare minimum of measured data bears the risk of low model quality as any (not avoidable) measurement uncertainty becomes fully transferred into model uncertainty. The advantage of a valid mathematical model is the gain of insight into the bio-chemistry process, especially for time-evolving processes, with unknown reaction velocity. In our case we found a clear trend in reaction dynamics (faster reaction at higher temperature) and a peak in reaction intensity (i.e., the created TPF amount) with rising temperature during the incubation. Choosing the right amount of measured data at suitable sample points (sample time) can optimize the cost/benefit ratio between smaller cost for data collection at constant high model quality.

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