

Response to comments from the associate editor

Comment

Dear Authors,

The pre-print of your manuscript led to many constructive exchanges on the way of modelling the short-term temperature response of enzyme activities. There were also discussions of great relevance to the biogeochemistry community on the level of complexity of the models we need, for which applications and how to evaluate the model performances by integrating the number of parameters (model complexity).

I invite you to submit a revised version of your manuscript integrating the fruit of these exchanges. Special attention should be paid to the way you compare and evaluate the different models. As pointed out by the referees, this would require a more complete and balanced presentation of existing models, and to consider in your evaluation the number of parameters (level of complexity) and not only the “realism” of the models and explained variability (R₂).

Response: We thank you for handling our paper. In our revision, we have carefully addressed the reviewer’s concerns. For the comparison and evaluation of “different models”, we expanded our literature review of the most relevant models, and explained their conceptual differences. We highlight that our chemical kinetics theory not only is an extension of the Ratkowsky model that has been demonstrated to be effective for hundreds of datasets, but it also leads to new insights that cannot be captured by MMRT and other existing models. Particularly, by linking this study with other published studies, we show that the chemical kinetics theory can logically upscale from single-enzyme-single-substrate chemical reactions to individual organisms, and even populations. Therefore, we believe the paper will be a very valuable addition to “Biogeosciences”.

Response to reviewer 1.

We appreciate Prof. Louis Schipper’s careful assessment of our manuscript, which has helped us greatly improve its readability and scientific quality. We respond to his comments point by point below. It is noted that while addressing his comments, we also have taken the other reviewer’s suggestions into account, so that a balanced decision is made on the requested changes to the manuscript. We italicize the corresponding changes in the manuscript for clarity.

Comment 1: This paper comprises two major elements (i) the paper starts with a critique of macromolecular rate theory (one of several models of the temperature response of enzyme activity) and (ii) development of chemical kinetics theory to predict temperature dependence of

enzymatic reactions which includes the assumption of an equilibrium between active and reversibly denatured enzymes. It is important that we acknowledge that no ‘simple’ model will fully capture the complexity of individual enzymes and their coupled activity in biochemical pathways. There are hundreds/thousands of interacting atoms that play a role in enzyme catalysis and so we are attempting to develop much simpler models that capture the general behaviour of enzymes and biogeochemical processes in useful and predictive ways.

Response: We fully agree with the statement that no simple model can fully capture the complexity of individual enzymes and their coupled activity in biochemical pathways. Our attempt here is to address features missed in the macromolecular rate theory by considering the alternative chemical kinetics theory. In particular, we believe, as the developers of MMRT did, that a good theory to provide mechanistic insights should be able to incorporate as many observed major phenomena as possible, while being sufficiently simple and interpretable.

Comment 2: The authors present a model for the temperature dependence of enzyme rates that postulates an equilibrium between native and non-native conformations of the free enzyme (Figure 1b). This is a variation on the “equilibrium model” of (Daniel and Danson, 2013) that postulates an equilibrium between active and inactive conformations of the enzyme-substrate complex. This equilibrium model gave rise to macromolecular rate theory (MMRT) which captures the equilibrium in a heat capacity term. These three models are part of a large family of models that date back at least 70 years which try to rationalise the deviations from “Arrhenius behaviour” seen for enzymes, organisms and ecosystems and specifically capture the observed temperature optima. Thus, the current manuscript continues that tradition and testing this new model is a useful endeavour – in particular characterising the equilibrium via experiment and/or simulation.

Response: We agree that our chemical kinetics theory-based model is one kind of equilibrium model that tries to explain the deviation from “Arrhenius behaviour” observed for enzymes, organisms, etc. It is also our purpose to review the fundamental mechanisms responsible for observed temperature sensitivities and to compare and contrast how previous efforts have included these processes. We hope our manuscript will help readers to better understand existing models that parameterize these deviations from “Arrhenius behaviour” and how they may be applied in their analyses.

Comment 3: The model presented in this manuscript is motivated (in the title, abstract and introduction) by two criticisms/assertions (ln 36 onwards) about MMRT which are incorrect: Firstly, MMRT does not assert that the Michealis-Menten equilibrium constant, K_M , is independent of temperature. This would be to ignore the basic thermodynamic relationship between equilibrium and temperature: $\ln K = (-\Delta G/RT)$. Secondly, MMRT does not assume that the enzyme is saturated at all temperatures. MMRT models the temperature dependence of the

rate constant, k , for rates at different scales (enzymes, organisms, ecosystems). For example, at high concentrations of substrate, the Michealis-Menten equation simplifies to rate = $k_{cat}[E]$ and MMRT provides a model for the temperature dependence of k_{cat} . At low substrate concentrations the Michealis-Menten equation simplifies to rate = $(k_{cat}/K_M)[E][S] = k_2[E][S]$ and MMRT provides a model for k_2 (which includes K_M). This is discussed in some detail by Arcus and Mulholland (2020). When modelling respiration rates in soil, for example, we expect that substrate concentrations are low and thus the latter simplification applies. We can nonetheless rule out K_M as the source of deviations from Arrhenius behaviour for the following reason: If the non-Arrhenius behaviour for soil respiration were a consequence of K_M then we would expect this behaviour to disappear at high substrate concentrations and this is not the case when glucose is added to soil. Indeed, the deviations from Arrhenius kinetics become more pronounced when glucose is added rather than less (e.g., Numa *et al.*, 2021). Thus, the deviations from Arrhenius behaviour, as modelled by MMRT, are not a function of the temperature-dependence of K_M .

Response: We partially agree with this comment. Yes, it is true that MMRT does not assert that the Michealis-Menten equilibrium constant, K_M , is independent of temperature. Nor does MMRT assume that the enzyme is saturated at all temperatures. However, we noted that the two experimental studies used by Hobbs *et al.* (2013) to derive the parameters of MMRT and therefore the overall parametric fitting, did not consider the temperature dependence of K_M or various levels of substrate concentrations. Specifically, Hobbs *et al.* (2013), in their description of “Enzyme assays”, wrote that “Temperature profiles were determined by measuring activity at 2–3 °C intervals using substrate at either $2 \times K_M$ (barnase) or $10 \times K_M$ (Mall). Barnase and A43C/S80C data were corrected for V_{max} based on K_M determinations at two temperatures.” From this, we infer that the temperature dependence of K_M is convolved with the estimated temperature dependence of V_{max} , and therefore may have led to uncertainties. Based on our new theory, we found that the temperature dependence of K_M will lead to a substrate modulation of the emergent temperature dependence of catalysis rate (Figure 3), where at a given temperature for a given substrate, higher substrate concentration will increase the catalysis rate temperature sensitivity. In particular, we clarify that the chemical kinetics theory does not suggest that the K_M temperature dependence will lead to the catalysis rate falloff as temperature increases over an optimal, but it does modulate the value of optimal temperature for a given substrate through its relationship with substrate abundance (Figure 3).

Comment 4: While at least half of the manuscript is devoted to development of a chemical kinetics theory, this is not reflected in the title and only gets a single line in the abstract. To me, this paper is about the new model and both the title and abstract should reflect this approach. It is more than reasonable to debate the relative value of MMRT in the discussion when taking into account the general comment above.

Response: We agree and have revised the title to be “A reanalysis of the foundations of the macromolecular rate theory and an alternative based on chemical kinetics theory”. We also revised the abstract accordingly:

“The macromolecular rate theory (MMRT) has been proposed as a mechanistic scheme to describe the temperature dependence of enzymatic reactions, and has enjoyed quite some popularity recently. MMRT was motivated by assuming that enzyme denaturation is not sufficient to explain the decline of enzyme activity above an optimal temperature, and was derived with two experimental assumptions: (1) the half saturation parameter is independent of temperature; and (2) when the substrate concentration is kept at 10 times of the half saturation parameter at reference temperature, the enzyme assays are substrate saturated under all experimental temperatures. We show that thermally reversible enzyme denaturation could be essential to consistently interpret the temperature dependence of enzymatic reactions, and due to the temperature-dependence of the half saturation parameter, neither of the experimental assumptions of MMRT held. Consequently, the MMRT estimated temperature sensitivity of the maximum catalysis rate is inaccurate. It can mischaracterize temperature-related biochemical behaviors, such as inferring the existence of a unique optimal temperature where biochemical rate peaks, and the shift of this optimal temperature as an indicator of thermal acclimation or adaptation. We proposed a chemical kinetics theory that explicitly incorporates the observed thermally reversible enzyme denaturation, von Smoluchowski’s diffusion-limited chemical reaction theory, and Eyring’s transition state theory to interpret the temperature dependence of enzymatic reactions. Since the chemical kinetics theory performed equally successful in fitting the enzyme assay data used in deriving MMRT, and has incorporated more relevant empirical observations and well-established theories than MMRT, we recommend it as a better candidate for mechanistic modeling of the temperature dependence of biogeochemical rates. However, MMRT is still a better model than the conventional Q_{10} and Arrhenius functions for describing the emergent temperature dependence of biochemical rates.”

Comment 5: In the introduction, not only should MMRT be described and critiqued as a temperature dependence model but the array of other equilibrium models, and (e.g. the DAMM model) (Davidson *et al.*, 2012), square root model etc...

Response: We now expanded the introduction per this suggestion and a similar suggestion raised by the other reviewer, so that a more comprehensive landscape is painted for the progress on modeling the deviation from “Arrhenius equation”. Particularly, we added a paragraph to discuss some important historical developments of the equilibrium model, and highlighted the commonality of thermally reversible enzyme denaturation.

“Besides MMRT, a few other models with mechanistically interpretable parameters are also capable of equally well interpreting the non-monotonic temperature dependence of enzyme modulated reactions, including growth rates. Notably, Sharpe and Demichele (1977) proposed a model that incorporates the empirical observation of thermally reversible enzyme denaturation

and the transition state theory (Eyring, 1935). Specifically, they considered that enzymes are in reversible transition between three states, one cold-induced inactive state, one heat-induced inactive state, and one active state which is able to carry out the catalysis. By assuming reactions to be substrate unlimited, they obtained a model with five thermodynamic parameters that is able to almost perfectly fit published temperature dependent growth rates of eight poikilothermic organisms (see their Figures 5 and 6). (The applicability of the Sharpe-Demichale model to growth rates of an organism is based on the assumed existence of control or master enzymes (Johnson and Lewin, 1946).) Motivated by the success of Sharpe and Demichale (1977) and the work on thermally reversible protein denaturation by Murphy et al. (1990), Ratkowsky et al. (2005) grouped the two inactive states into one, and, again assuming no-substrate limitation, derived a model with two thermodynamic parameters and two enzyme informatic parameters, which was able to very accurately fit 35 sets of observed temperature dependent bacterial growth rates. The model by Ratkowsky et al. (2005) was later used by Corkrey et al. (2012) and Corkrey et al. (2014) to successfully interpret the temperature dependent growth rates of many more poikilothermic organisms. Ghosh et al. (2016) extended the model by Ratkowsky et al. (2005) to include the thermally reversible denaturation of many enzymes and proteins informed by proteomics, and were able to satisfactorily interpret the measured temperature-dependent growth rates of mesophiles and thermophiles.

The thermally-reversible enzyme denaturation occurs due to the thermal motion of molecules and ions in the solution of enzyme proteins (Finkelstein and Ptitsyn, 2016). As thermal motion is ceaseless, according to Boltzmann's law in statistical mechanics (Feynman et al., 2011), enzyme molecules will be distributed among different configurations that can be quantified by their respective energy status. Therefore, under any biologically feasible temperature, some of the enzyme molecules will not be in their biologically active native states. That is, at any life amenable temperature, only a fraction of enzymes is able to catalyze the corresponding biochemical reaction. Consequently, by not explicitly taking into account the thermally-reversible enzyme denaturation (or by assuming all enzyme denaturation are irreversible), we believe MMRT may have missed some important mechanistic insights on the temperature control of enzymatic reactions.”

Comment 6: The developed theory is only tested on data from single enzymes (Hobbs et al 2013) and not biogeochemical processes so how this work translates to a journal that focuses on biogeochemical modelling is not clear. Would this be better submitted to a biochemistry journal that focusses on enzymes? Also, see line 149 the authors state a more complex model is needed for soil, so it is not clear how the authors suggest a developed model fits in biogeochemistry.

Response: Since our purpose is to reanalyze MMRT and propose an alternative, and MMRT is built upon the data that are used in this manuscript, we believe that it is reasonable to leave further development in future studies. Additionally, because MMRT is now popularly used for biogeochemical modeling or analysis, we believe that publishing our manuscript with biogeoscience can help the research community to improve the understanding and modeling of various relevant biochemical processes.

We noted that Ghosh et al. (2016) have successfully applied the Ratkowsky et al. (2005) model to include many lethal proteins sampled from proteomes of some mesophilic and

thermophilic bacteria. Since the Ratkowsky model is a special case of the chemical kinetics theory, these previous studies by Ghosh et al. (2016), Ratkowsky et al. (2005), and Corkrey et al. (2012) together thus show that the chemical kinetics theory here is scalable from single enzyme to unicellular, and multicellular organisms.

Finally, in order to further show the validity of the chemical kinetics theory, we show that the observed reduction in optimal temperature upon addition of plant litter or glucose by (Robinson *et al.*, 2020; Numa *et al.*, 2021) can potentially be explained as a shift in substrate type. Specifically, in the newly added Figure 4, which is also shown below (Figure S1), we argue that since organo-mineral interaction tends to increase the activation energy of existing SOM substrates, our theory predicts that the lower activation energy of newly added plant litter or glucose will lower the emergent temperature optimal. Thus, we believe a strong case to publish this manuscript with biogeosciences is presented.

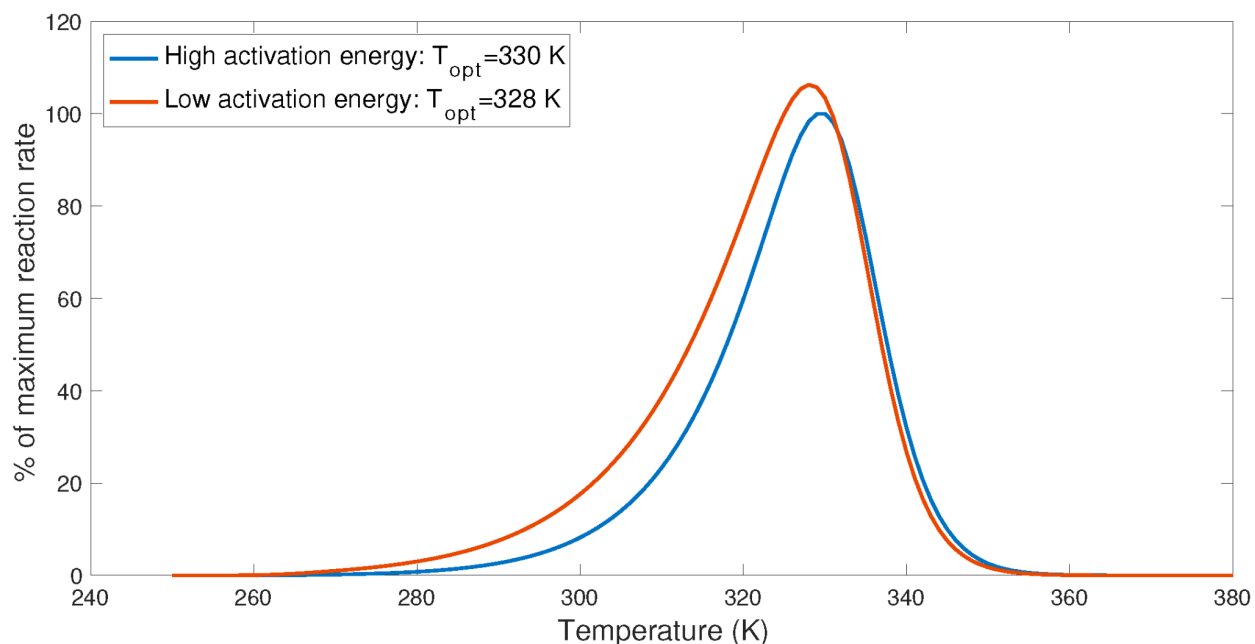


Figure S1. Lowering the activation energy will shift the optimal temperature towards lower values. This is shown as Figure 4 in the revised manuscript.

Comment 7: Ln 42 the authors should define what reversible denaturation is in their framework. As large molecules, enzymes constantly undergo large fluctuations resulting in an array of conformations that bind the substrate to varying degrees. Are the authors assuming only 2 states (active and inactive) rather than a continuum of binding/catalytic states with varying K_m s?

Response: We simply define reversible denaturation as the transition between native states (that are able to bind the substrates and catalyze the reaction) and non-native states (that are not able to bind the substrate and catalyze the reaction). This is simpler than considering more than two states, but is sufficient to account for the observed (1) existence of thermally reversible enzyme

denaturation, and (2) the non-monotonic temperature dependence of enzymatic reactions. In the revised manuscript, we now defined the reversible denaturation as “the dynamic transition between its native folded state and the unfolded state as a function of temperature and solution conditions (e.g., Oliveberg et al., 1995; Anfinsen, 1973)”, and made a more explicit description about how thermally reversible enzyme denaturation was used in several existing models by adding a new paragraph in the introduction. We have also consulted Prof. Ken A. Dill, an expert in protein physics, to make sure this definition is simple but sufficient. (Also see response to **comment 5**.)

Comment 8: Ln 90 Schipper and Liang and others primarily suggested using MMRT rather than Q10 or Arrhenius functions because neither latter function resulted in a measure of a T_{opt} (and therefore T_{inf}).

Response: We agree with this point, and acknowledge that MMRT improved on the Q10 and Arrhenius functions in this regard. However, we also acknowledge that there are alternative models that can equally well capture the non-monotonic relationship between biochemical rates and temperature. Also, per the suggestion from the other reviewer, we expanded the introduction to make it more inclusive (also see response to **comment 5**).

Comment 9: Ln 205 and elsewhere (Ln 79). There needs to be a clear description of the differences between the dC_p for the different models. In the current paper, dC_p is the change in heat capacity between the reversibly denatured state and the active state. In MMRT, the dC_p is argued to be the heat capacity difference between the enzyme-substrate complex and the enzyme transition state. There is no reason that these should be the same size of sign as these are not the same processes.

Response: We agree that this issue is important to clarify. In particular, we dedicated the following paragraph to address these differences:

“One outstanding difference between MMRT and the chemical kinetics theory is that MMRT generally infers a negative heat capacity (associated with the catalysis process), while the chemical kinetics theory infers a positive heat capacity of protein unfolding (associated with thermally reversible enzyme denaturation). Interestingly, if the heat capacity of protein refolding is substituted for the heat capacity of unfolding in the description of thermally reversible enzyme denaturation, its sign becomes negative (Oliveberg et al., 1995). Some other studies that have used a similar framework of MMRT have associated the heat capacity with the enzyme-substrate binding process and have also found its value as being negative (e.g., Wang et al., 2009; Buczek and Horvath, 2006; Dullweber et al., 2001). However, using molecular dynamics simulations, Aqvist and Van der Ent (2022) inferred the heat capacity to be zero for both catalysis and binding processes for a designer enzyme 1A53-2.5. Moreover, Aqvist and Van der Ent (2022) and Aqvist (2022) suggested that the non-monotonic relationship between temperature and

catalysis rate can be explained by the existence of an equilibrium between active enzyme substrate complex and inactive enzyme substrate complex. To some extent, the conceptual model by Aqvist and Van der Ent (2022) is equivalent to the chemical kinetics theory, if the latter allows the inactive enzymes to form inactive enzyme-substrate complexes. We acknowledge that the finding of zero heat capacity for both catalysis and binding processes has been debated in Lear et al. (2023) and Aqvist (2023), but it is concluded that different kinetic models can fit the measured temperature dependent catalysis rates equally well. In particular, Aqvist (2023) noted that a kinetic model considering thermally reversible enzyme denaturation fits the observations equally well.”

Comment 10: Ln 133 enzyme denaturation was shown experimentally to have T_{opt} without enzyme denaturation in Hobbs et al 2013. Are the authors invoking reversible denaturation here? See also Ln 143 are the authors arguing for including irreversible denaturation or reversible denaturation? These are very different concepts.

Response: Hobbs et al. (2013) stated that enzyme denaturation cannot satisfactorily explain the observed T_{opt} , so they propose MMRT which does not require enzyme denaturation. By attributing the observed T_{opt} to changes in heat capacity associated with enzyme catalysis, MMRT effectively assumes that all enzymes are in their native states and are capable of forming enzyme-substrate complexes. In contrast, our new approach is based on well documented observations of thermally-reversible enzyme denaturation, and that models incorporating this reversible denaturation are able to describe the observed nonmonotonic temperature dependence of enzymatic reactions (e.g. the model by Ratkowsky et al.(2005)). We now revised this part of the text as:

“Equation (16) or (17) can be used to analyze the motivating assumption and the two basic experimental assumptions that underlie MMRT. First, Hobbs et al. (2013) suggested that MMRT was motivated by noting that enzyme denaturation cannot satisfactorily explain the temperature dependence of catalysis rates. They then assumed that all enzymes are effectively in their active state to do catalysis, and attributed the decline in enzyme catalysis rate above an optimum temperature to the change of heat capacity associated with the enzyme catalysis. However, thermally reversible enzyme denaturation, as one type of enzyme denaturation, has been observed by many studies (Sizer, 1943; Alexandrov, 1964; Huang and Cabib, 1973; Maier et al., 1955; Weis, 1981), as well as by molecular dynamics simulations (McCully et al., 2008), and is ensured to occur by the ceaseless thermal motion of molecules and ions in the enzyme solution. Nonetheless, irreversible denaturation driven by heat does occur (Perdana et al., 2012), as it is necessary for the cooking of eggs or meat. Second, equations (16) and (17) clearly show that the enzyme assay-derived $k(T)$ is affected by substrate abundance, through the ratio (S/K_0) of substrate concentration S to K_0 . Third, because K is temperature dependent, taking (S/K_0) to be a relatively large value (e.g. 10 as adopted by Hobbs et al. (2013) and Peterson et al. (2004), hoping to “minimize the effect of any possible increase in K_M with temperature”) does not

guarantee that the term $(1+S/K_0)/(f_K(T)+S/K_0)$ reduces to a value of one (see section 3.1 for more details). Therefore, not only is the validity of their motivating presumption compromised, but also that of their experimental assumptions for deriving MMRT. Instead, as we will show later, a proper incorporation of thermally reversible enzyme denaturation is sufficient to explain the non-monotonic temperature dependence of enzyme catalysis rate, and substrate availability and the temperature dependence of K can modulate the optimal temperature (T_{opt}) where biochemical reaction rate is maximized.”

Comment 11: Ln 155 why can the uncertainty ranges of the fitted parameters not be determined? This should be about the fit of the data and not the variation in data extraction?

Response: Because that the parameter fitting problem is close to singular, we were not able to derive the uncertainty from the Hessian matrix using finite difference approximation. An alternative approach is to use Monte Carlo method with perturbed observations, but we were not able to meaningfully extract the uncertainty of observations from the log plots in Hobbs et al. (2013) and Peterson et al. (2004). However, we believe this shortcoming will not change the conclusion of our manuscript.

Comment 12: Ln 180, Figure 3 Is this modelled data? The authors argue that T_{opt} increases with decreasing substrate concentration but do not provide error bounds on T_{opt} for substrate concentrations ranging between K_0 and infinite substrate. Is this a real change in T_{opt} , that is, do the authors believe that a modelled increase in T_{opt} of 1C (figure 3d) or 3C (Figure 3a) is real, based on a 4-parameter fitted model? This is something that could be experimentally tested. In contrast, measurement of respiration when glucose or litter was added to soil shows decreases the temperature optima (Robinson *et al.*, 2020; Numa *et al.*, 2021)

Response: Yes, Figure 3 presents model predictions using some of the parameters derived in Figure 2 (so there is no uncertainty). For measurements of temperature dependence from real soils, the situation is more complicated due to the involvement of many microbes, enzymes, substrates and mineral particles, as the reviewer mentioned previously. We note that our predictions should be confronted with measurements based on single enzyme catalyzed reactions in solution, and have indicated this point in the revised text. For real soil systems, our previous work (Tang and Riley (2013, 2015)) shows that soil minerals will effectively increase the affinity parameter. Therefore, adding labile substrates can trigger a respiratory reaction that has lower affinity parameter and activation energy compared to the stabilized substrate already in soil. These interactions will cause a shift of the temperature optimal towards smaller values, as observed by the two studies quoted above. We acknowledge these complexities for real soils in the revised text, and note that the chemical kinetics theory needs to be implemented in a more comprehensive model to explain the observations by Robinson et al. (2020) and Numa et al. (2022). We also explained these issues in our response to **comment 5**.

Comment 13: Figure 3b why is the maximal rate of c3 (substrate 10x K_o) so much lower than when substrate is limiting c1. Would you not expect higher rates when substrate was not limiting?

Response: Thanks for catching this, we double checked the matlab script and identified a bug when plotting the results. Now the figures are corrected, but the derived numbers and conclusions remained the same.

Comment 14: Also figure 3d seems to have bulged in the curve at around 300 K, which is not normally seen in experimental data. Is this considered as realistic or a function of the model?

Response: The bulge was derived from the pattern in Figure 2k for Aryl-acylamidase, whose data are derived from Peterson et al. (2004). Their original data were also not very smooth. However, it was also caused by the bug in the plotting script. The corrected Figure 3d is smoother.

Comment 15: Ln 187 what is meant by the physiological optimum - what concentration of substrate is this referring to?

Response: We clarified that it refers to the optimal temperature at unlimited substrate concentrations as computed by equation (15).

Comment 16: Ln 197 MMRT has been used to capture differences due to changes in substrate availability on T_{opt} in soil (Numa *et al.*, 2021).

Response: As we explained in the response to comments 6 and 12. We hypothesize that Numa et al. (2021) may have observed a competition effect on T_{opt} associated with the change in substrate type. Our chemical kinetics theory when integrated with a complete model for SOM dynamics will be able to resolve this pattern, as we suggested in a prototype model presented in Tang and Riley (2015)).

Comment 17: Ln 201 Is the assertion that higher T_{opt} occurs with high substrate availability is a consequence of the developed model but not observations? Should be clearly stated. Is this extrapolating beyond the parameters used to model the enzyme in the first place?

Response: It is a prediction based on the chemical kinetics theory. We clarified this point in the revision by adding a discussion on observations by Numa et al. (2021) and Robinson et al. (2020). Please also see responses to comments 6 and 12.

Comment 18: Ln 206 a comparison between dCp between the model developed by the authors and MMRT is not reasonable these are different phenomena as pointed out above. In the current paper, dCp is the change in heat capacity between the reversibly denatured state and the active state. In MMRT, the dCp reported is argued to be the heat capacity difference between the enzyme-substrate complex and the enzyme transition state. The dCp for transition state binding (that is MMRT dCp) for MTAP has been measured by Firestone et al (2017) and is same as inferred by MMRT fits within 5%.

Response: We now clarified the conceptual difference between these two heat capacities, to ensure readers are aware of the different definitions. Also see response to comment 9.

Comment 19: Ln 215 Can the authors formalise what was considered wrong with the La Mer study rather than broad statement that was considered flawed. This paper is not referenced in the early MMRT papers (Hobbs *et al.*, 2013) (Arcus *et al.*, 2016)

Response: One reviewer (who decided to be anonymous but may have been involved in the original author list of MMRT papers) of the earlier draft of this manuscript suggested that MMRT was partially motivated by the study La Mer (1933). In that paper, La Mer suggested that the rate constant should be $k_f = Z_f \cdot \exp(-\Delta G_f/RT)$ for forward reaction, and $k_b = Z_b \cdot \exp(-\Delta G_b/RT)$ for backward reaction, so that the equilibrium constant is $K_e = k_f/k_b = Z_f/Z_b \cdot \exp(-\Delta G^o/RT)$, with $\Delta G^o = \Delta G_f - \Delta G_b$ (being a linear function of temperature). Because La Mer (1933) assumed a non-zero heat capacity for the free energy of activation, one will find $Z_f \neq Z_b$. However, $Z_f = Z_b$ is needed to make $K_e = \exp(-\Delta G^o/RT)$, so that the theory is consistent with the transition state theory and thermodynamics. However, since the original MMRT papers did not cite La Mer (1933), and because that paper has subsequently been shown to be flawed, we removed discussion of it in our revised manuscript.

Comment 20: Ln 221. How can the authors argue that the developed model is a “better mechanistic representation of temperature dependent biochemical rates than MMRT”? This inference is based on a better fit to data using a model with 4 parameters (1 more than MMRT, as noted Ln 140, so not surprising that the fit is better). There is no experimental testing of the underlying mechanism proposed in their model. There is also no accounting for the addition of another parameter using, for example, AIC, which probably should be done.

Response: Our argument for the better mechanistic representation of our new model is that it is built upon its equally good parametric fitting (as compared to MMRT), inclusion of more observed phenomena (e.g., the existence of thermally reversible enzyme denaturation), consistent incorporation of the transition state theory and von Smoluchowski’s chemical reaction theory, and indirect support from the molecular dynamics simulations. These make it worthwhile to introduce one more physically meaningful parameter. In particular, we are not proposing an

alternative model based on statistical regression, rather than the value of our alternative approach is that it is based on a solid theoretical foundation of chemical kinetics and thermodynamics, which themselves are built upon a wide range of empirical observations. To address the reviewer comment and clarify these points, we have added text to the revised manuscript:

“In summary, we contend that the chemical kinetics theory, by incorporating the observed thermally reversible transitions of enzymes between their native and non-native states (which occurs even in the absence of substrate molecules due to the ceaseless thermal motion of molecules and ions in the enzyme solution) (Anfinsen, 1973; Finkelstein and Ptitsyn, 2016; Sizer, 1943; Oliveberg et al., 1995), the diffusion-limited chemical reaction theory by (von Smoluchowski, 1917), and the transition state theory by (Eyring, 1935), can satisfactorily explain the non-monotonic relationship between temperature and catalysis rates, and is a better mechanistic representation of the temperature dependence of enzyme-catalyzed biochemical rates than MMRT.”

Comment 21: Ln 229. Again the authors argue their model is “better” than the Peterson model simply because the Peterson model has an extra parameter and no AIC calculated between comparative models. It is unclear how the authors are considering the relative merits of different models. Is it better to consider the number of fitted parameters to describe the response or mechanistic representation?

Response: We noted that the advantage of the Peterson model also involves an inconsistency between their theory and support from empirical experiments, and have used a Gibbs free energy of unfolding that contradicts what is widely known in protein physics. In the revised manuscript, we have clearly spelled out the issues associated with the Peterson model:

“Combining the transition state theory and the protein denaturation model by Lumry and Eyring (1954), Peterson et al. (2004) proposed an equilibrium model that includes both reversible and irreversible enzyme denaturation to explain their observed non-monotonic relationship between temperature and catalysis rates. However, because they assumed a constant enthalpy for the reversible enzyme denaturation, their Gibbs free energy of enzyme unfolding became a linear function of temperature. This linear function contrasts with the nonlinear function (i.e. equation (11)) and the existence of multiple native protein states that are usually observed in protein physics (Ghosh and Dill, 2009; Silverstein, 2020; Sheng and Pan, 2002; Finkelstein and Ptitsyn, 2016). Further, their model involves an explicit temporal dependence in the formulated catalysis rates, which introduces one more parameter (i.e., time) than the chemical kinetics model. Moreover, (Peterson et al., 2004) also assumed their enzyme assays are substrate saturated, while we show above that such an assumption could very well be invalidated by the temperature dependence of substrate affinity parameter.”

In addition, we now stated more clearly how we evaluated the merits of a model:

“In summary, we contend that the chemical kinetics theory, by incorporating the observed thermally reversible transitions of enzymes between their native and non-native states (even in the absence of substrate molecules) (Anfinsen, 1973; Finkelstein and Ptitsyn, 2016; Sizer, 1943; Oliveberg et al., 1995), the diffusion-limited chemical reaction theory by (von Smoluchowski, 1917), and the transition state theory by (Eyring, 1935), can satisfactorily explain the non-monotonic relationship between temperature and catalysis rates, and is a better mechanistic representation of the temperature dependence of enzyme-catalyzed biochemical rates than MMRT. ”

Comment 22: Overall, the current manuscript presents an interesting approach on the temperature dependence of enzyme-driven rates and investigation of the phenomena contained in the model warrants investigation. Our view is that this is sufficient in itself, and does not need to be prefaced by incorrect assertions about MMRT. In biogeosciences, we are seeking tradeoffs between fully mechanistic models of temperature response and those that are useful. As the saying goes: All models are wrong, some are useful.

Response: We thank the reviewer for his constructive comments. We fully agree with the statistician George E.P. Box’s sentiment that all models are wrong, some are useful. With that understanding and the reviewer’s comments, we have rewritten the overall tone and goal of our manuscript to be a discussion of how our new approach can integrate well-established theory (e.g., law of mass action, thermodynamics, chemical kinetics theory), previous efforts (e.g., MMRT, Peterson, ...), and observed phenomena (e.g., the widely observed thermally reversible enzyme denaturation).

References

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Response to Reviewer 2

We appreciate the reviewer's careful assessment of our manuscript. We respond to the comments point by point below. It is noted that while addressing his comments, we also had taken the other review's suggestions into account, so that a balanced decision is made on the requested changes to the manuscript. We highlight the corresponding changes in the manuscript by quoting them in italic font.

Comment 1: The paper provides a critique of the MMRT model, concentrating on its underlying assumptions as stated by the authors. This makes for a useful contribution since MMRT has

proved popular in several fields, such as soil science. Should the model be flawed or incomplete then these applications may have reduced value, particularly in the journal's subject area. The paper does not make a direct comparison with alternative models, though it refers to a number of them, collectively termed 'chemical kinetics theory'. Certainly, empirical models would not be of interest here, but in any case, I do not consider such comparisons are needed since they can needlessly burden a paper with material that the interested reader can seek out.

Response: We appreciate the reviewer's sentiment on our paper, which fully captured our intent for the design and content of this manuscript. We followed the reviewer's suggestions as much as we can, and believe that the revised manuscript is now much more helpful to readers of biogeosciences.

Comment 2: In the introduction there is a brief coverage of publications that refer to MMRT. I found this could have been more comprehensive. It could have dealt with a number of alternative models and, as noted earlier, that would burden the paper.

Response: We now expand the introduction of MMRT, and also the alternative mechanistic models. We now included the comprehensive review by (Grimaud et al., 2017) and (Noll et al., 2020), and added the following paragraph

“Besides MMRT, a few other models with mechanistically interpretable parameters are also capable of equally well interpreting the non-monotonic temperature dependence of enzyme modulated reactions, including growth rates. Notably, Sharpe and Demichele (1977) proposed a model that incorporates the empirical observation of thermally reversible enzyme denaturation and the transition state theory (Eyring, 1935). Specifically, they considered that enzymes are in reversible transition between three states, one cold-induced inactive state, one heat-induced inactive state, and one active state which is able to carry out the catalysis. By assuming reactions to be substrate unlimited, they obtained a model with five thermodynamic parameters that is able to almost perfectly fit published temperature dependent growth rates of eight poikilothermic organisms (see their Figures 5 and 6). (The applicability of the Sharpe-Demichale model to growth rates of an organism is based on the assumed existence of control or master enzymes (Johnson and Lewin, 1946).) Motivated by the success of Sharpe and Demichele (1977) and the work on thermally reversible protein denaturation by Murphy et al. (1990), Ratkowsky et al. (2005) grouped the two inactive states into one, and, again assuming no-substrate limitation, derived a model with two thermodynamic parameters and two enzyme informatic parameters, which was able to very accurately fit 35 sets of observed temperature dependent bacterial growth rates. The model by Ratkowsky et al. (2005) was later used by Corkrey et al. (2012) and Corkrey et al. (2014) to successfully interpret the temperature dependent growth rates of many more poikilothermic organisms. Ghosh et al. (2016) extended the model by Ratkowsky et al. (2005) to include the thermally reversible denaturation of many enzymes and proteins informed by proteomics, and were able to satisfactorily interpret the measured temperature-dependent growth rates of mesophiles and thermophiles.”

Comment 3: L20-25. The authors note that Hobbs et al. (2013), Schipper et al. (2014) claim MMRT is able to better than the Arrhenius-like functions for various ecological properties. I

suggest that it may be worth adding here that while Schipper et al. (2014) compares MMRT to an Arrhenius model it does not do any more than this, in that it does not, for example, choose to compare to the Ratkowsky 2005, Corkrey 2012 models.

Response: We now acknowledge that models, e.g. by Sharpe and DeMichele (1977), Ratkowsky et al. (2005), Corkrey et al. (2012) can be equally well in terms fitting the pattern. We also acknowledged how the MMRT papers mis-criticized the Ratkowsky model. In particular, the important study by Oliveberg et al. (1995) who reported negative heat capacity of protein refolding was actually supporting the Ratkowsky model and the chemical kinetics theory here.

Comment 4: L25-30. Similarly, I would add that Alster et al (1016) does the same thing, but in addition, I would add that those authors unnecessarily dismiss other models as 'empirical' when, on examination, they do not appear to be (e.g. Corkrey et al (2012), Peterson et al (2004), Daniel & Danson (2013)). This is a point of irritation since such claims are too easily repeated.

Response: We added this more balanced view throughout the manuscript. Also see response to comment 2.

Comment 5: L30-35. Examples (e.g., Ratkowsky et al (1983)) of papers as having parameters that are not biologically interpretable. This seems a little unfair since that particular model is explicitly empirical. I suggest adding in references to other models that do have interpretable parameters and/or are based on thermodynamic principles such as those listed above.

Response: We added references of other models, and annotated them fairly.

Comment 6: Section 2.3. I found this derivation somewhat hard to follow (though interesting). Perhaps a little more could be added to assist non-specialists?

Response: We added more verbal descriptions to mathematical derivations, so that they are easier to follow.

Comment 7: L130-135. I stumbled over the wording 'The motivating assumption ... many observations'. Perhaps tweak this to make it clearer.

Response: We rewrote this sentence:

“Equation (16) or (17) can be used to analyze the motivating assumption and the two basic experimental assumptions that underlie MMRT. First, Hobbs et al. (2013) suggested that MMRT was motivated by noting that enzyme denaturation cannot satisfactorily explain the temperature dependence of catalysis rates. They then assumed that all enzymes are effectively in their active state to do catalysis, and attributed the decline in enzyme catalysis rate above an optimum temperature to the change of heat capacity associated with the enzyme catalysis. However,

thermally reversible enzyme denaturation, as one type of enzyme denaturation, has been observed by many studies (Sizer, 1943; Alexandrov, 1964; Huang and Cabib, 1973; Maier et al., 1955; Weis, 1981), as well as by molecular dynamics simulations (McCully et al., 2008), and is ensured to occur by the ceaseless thermal motion of molecules and ions in the enzyme solution.”

Comment 8: Figure 2. Given the scale used it is difficult to closely examine the fits. But the majority of the fits appear excellent (except Barnase?). I was expecting comparisons to MMRT fitted lines since that is the point of the paper. For that matter, I speculate that the Ratkowsky 2005, Corkrey et al (2012) models and others would do as well and could be fitted. The fits are summarized as r2 values. The authors comment (L155-160) that uncertainties were not available, and that they were hindered by an 'ill-conditioned Hessian matrix', but I don't see why resampling could not be used to obtain 99% CIs bands (although they would be very narrow).

Response: We note that there were not many data points to extract from the original papers. Particularly, most of the data in those papers were plotted in log space, so we were not able to extract data uncertainty meaningfully. We thought about resampling, but with the few data points, it is problematic. Nonetheless, based on the excellent fitting we obtained, and the fact that Ratkowsky et al (2005) model is a special case of the chemical kinetics theory here, we think not presenting the uncertainty should not affect the conclusion of our analysis. In the past, we have also used the Ratkowsky model to fit similar data, and it worked well just as the reviewer pointed out.

Comment 9: L210-220. I found this enlightening. Please elaborate on what was flawed in La Mer (1933).

Response: We explain this below. Specifically, in that paper, La Mer suggested that the rate constant should be $k_f = Z_f \cdot \exp(-\Delta G_f/RT)$ for forward reaction, and $k_b = Z_b \cdot \exp(-\Delta G_b/RT)$ for backward reaction, so that the equilibrium constant is $K_e = k_f/k_b = Z_f/Z_b \cdot \exp(-\Delta G^o/RT)$, with $\Delta G^o = \Delta G_f - \Delta G_b$ (being a linear function of temperature). Because (La Mer, 1933) assumed a non-zero heat capacity for the free energy of activation, one will find $Z_f \neq Z_b$, whereas $Z_f = Z_b$ is needed to make $K_e = \exp(-\Delta G^o/RT)$, so that it is consistent with the transition state theory and thermodynamics. However, per suggestion from the other reviewer, we removed this paragraph from the revised text.

Comment 10: L230-235. The point made about Ohm's law is well made. This an important point since several of the references made (e.g. Corkrey et al 2012) refer to organismal growth rates and not enzymic data. While it might be argued that the complexity of processes involved at the

organism precludes such models being successful, this has been found to be incorrect, such in the above reference. Note that Corkrey et al (2014; 10.1371/journal.pone.0096100) may be relevant here since it also refers to multicellular strains. It is such extensions from the enzymic to large scale processes that makes the paper relevant to the journal.

Response: We are glad this point resonated with the reviewer's thoughts. We now related to more references, including Corkrey et al. (2012, 2014). We also highlighted that the success of Ghosh et al. (2016) using proteomic data to explain the temperature dependent growth rate of mesophilic and thermophilic bacteria suggests that the chemical kinetics theory is scalable.

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