Sektion 3: Herbizid-Management
Session 3: Herbicide management

Predicting hormesis in mixtures of herbicidal compounds – where are we and how far can we go?

Vorhersage hormetischer Mischwirkungen von herbiziden Wirkstoffen – wo stehen wir und wie weit können wir kommen?

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Abstract

Predicting the occurrence and expression of stimulatory effects of subtoxic doses of phytotoxins or herbicides (hormesis) in mixtures is a challenging and needed task, considering that herbicide exposures in practice often occur in mixtures at low doses due to drift deposition, errors in application, protection by mulch, herbicide resistance, small-scale dose heterogeneity, and other causes. While joint effects in toxin mixtures can be straightforwardly modelled and predicted at toxic doses, the evaluation at stimulatory doses lacks a common statistical approach. Prediction of effective hormetic doses can be adequately facilitated by adopting joint-action models that have been developed for monotonic responses. In contrast, prediction of the magnitude of hormesis as one of the key quantitative features of hormesis is not so easy. Currently, there are no mechanistic models available that could be adopted to predict the hormetic magnitude in mixtures nor is there a generally accepted model available. Nevertheless, some promising attempts were made to predict the hormetic magnitude in herbicidal mixtures demonstrating the fundamental possibility of modelling hormesis in mixtures and providing valuable insights into the phenomenon. The success of these attempts is summarized and future research needs and limits are discussed.

Keywords: Biphasic, dose-response, growth stimulation, joint action, maximum stimulatory response

Zusammenfassung

Die Vorhersage des Auftretens und des Ausmaßes von stimulierenden Wirkungen subtoxischer Dosierungen (Hormesis) in Herbizidmischungen ist eine anspruchsvolle und notwendige Aufgabe, da Herbizid-Expositionen in der Praxis häufig in Mischungen und bei niedrigen Dosierungen erfolgen können, z.B. bei Abdrift, Anwendungsfehlern, Schutz durch Mulchauflagen, Herbizidresistenz oder kleinräumiger Heterogenität der Applikationsmenge. Während Mischungswirkungen im toxischen Dosisbereich zuverlässig modelliert und vorhergesagt werden können, fehlt für die Auswertung im hormetischen Dosisbereich bisher ein einfacher statistischer Ansatz. Es zeigte sich, dass eine Vorhersage von Hormesis-induzierenden Dosierungen durch Modelle, die für monotone Dosis-Wirkungszusammenhänge entwickelt wurden, hinreichend möglich ist. Im Gegensatz dazu gestaltet sich die Vorhersage der Amplitude der Stimulation, als eines der Hauptmerkmale der Hormesis, als schwierig. Derzeit stehen keine mechanistischen Modelle zur Verfügung um die Amplitude in Mischungen vorherzusagen, noch gibt es ein allgemein akzeptiertes statistisches Modell. Dennoch wurden einige vielversprechende Versuche unternommen, die hormetische Amplitude in Mischungen von herbiziden Wirkstoffen vorherzusagen. Diese Versuche zeigen, dass es grundsätzlich möglich ist, eine hormetische Stimulation in Mischungen zu modellieren und dadurch wertvolle Einblicke in das Phänomen der Herbizid-Hormesis zu gewinnen. Die Errungenschaften dieser Versuche werden zusammengefasst und zukünftiger Forschungsbedarf und Grenzen werden diskutiert.

Stichwörter: Dosis-Wirkungsbeziehung, maximale stimulierende Wirkung, Mischwirkung, Wachstumsstimulation, zweiphasisch

Introduction

Soon after the phenomenon of stimulatory effects of low, subtoxic doses of a toxin or stressor was termed "*hormesis*" in 1943 (SOUTHAM and ERLICH, 1943), hormetic effects of herbicides on plant

growth and/or physiology were first noticed and described in the mid-twentieth century (BELZ and DUKE, 2017; BRITO et al., 2018). Herbicide hormesis has been recorded with almost all herbicide classes and modes of action (BELZ and DUKE, 2017). The phenomenon is believed to be relevant for regular herbicide applications whenever the active dose reaching a plant turns into a subtoxic dose as for example in case of drift deposition, run-off, errors in application, small-scale dose heterogeneity, herbicide resistance, leaf contact of treated and untreated plants, protection by taller plants or mulch, absorption of low doses from soil, *etc.* (VELINI et al., 2010, 2017; BELZ et al., 2011; BRITO et al., 2018). Since herbicides often act in mixtures, the question if and how herbicide hormesis is affected in mixtures has been studied for about a decade. As chemical exposures in general mostly occur in mixtures and at low doses, the issue is highly relevant for many other toxicological disciplines as well (OHLSSON et al., 2010). Our knowledge on the prediction of mixed chemical exposures is well advanced for the high-dose response zone leading to toxic effects, and joint effects between mixture partners can be straightforwardly statistically modelled (SØRENSEN et al., 2007; RITZ and STREIBIG, 2014). Toxic mixture effects are traditionally evaluated on the dose *x,* such that an interaction between mixture partners occurs if the dose needed for a certain level of inhibition lowers in mixture (synergism) or increases (antagonism). Our knowledge on the prediction of hormesis in mixtures is instead marginal, and only a few studies have addressed this issue (CALABRESE, 2008b, 2010; BELZ and PIEPHO, 2017). In contrast to a traditional toxicological focus on the dose, mixture effects on hormesis need to address two perspectives: the doses inducing hormesis and the magnitude of hormesis represented by the maximum stimulatory response y_{max}. The dose levels inducing hormesis, primarily represented by the dose *M* causing maximum stimulation, are assumed to follow our traditional understanding of mixture effects in the toxic response zone (CALABRESE, 2008a, b). Synergism between mixture partners is thus expected to lower the *M* dose needed to achieve a maximum stimulation, while antagonism will lead to enhanced *M* doses. When it comes to the question how an interaction between mixture partners affects y_{max}, our traditional toxicological perceptions are instead believed to fail. Hence, a different concept of interaction on the magnitude of hormesis is assumed (CALABRESE, 2008a, b).

The hormetic concept of chemical interaction

Hormesis is believed to be constrained in magnitude by limitations in biological plasticity of the boosted organism. The maximum stimulation y_{max} in a plant trait will generally not differ from a typical boost of 30-60% above the control, independent of an interaction between mixture partners (FLOOD et al., 1985; CALABRESE, 2008a, b). Hence, the changes in y_{max} observed during an incidence of chemical interaction will not fall short of a 30% stimulation response nor exceed 60% (CALABRESE, 2008b, 2010). Consequently, a chemical interaction in the low-dose range will be most perceivable by changes in doses inducing hormesis rather than in magnitude (CALABRESE, 2008a, 2010). However, this concept of no marked hormetic type of synergism/antagonism on y_{max} remains, for the most part, a hypothesis that needs empirical support. A prerequisite to do so will be a sound statistical approach allowing for a prediction of the hormetic key features in mixtures.

Statistical modelling of hormesis in mixtures

The modelling of mono- or biphasic (*i.e.* hormetic) dose-response relationships as the basis for comprehensive joint action analyses, and the estimation of several quantitative key features characterizing a hormetic or toxic response (*e.g.* dose *M*, y_{max} or ED_K values leading to K% inhibition) is statistically feasible whether a single compound or multiple compounds are in action (BELZ and PIEPHO, 2012). Most mixture studies consider binary mixtures of two herbicidal compounds. Based on the estimates for the single compounds separately, predefined reference models can be adapted to the data. A few such reference models have been developed and applied for monotonic mixtures (STREIBIG and JENSEN, 2000). Deviations of mixture data from a reference model can be statistically assessed and interpreted in terms of synergism, additivity, or antagonism between mixture partners. On the basis of an adapted reference model or the modelling of a synergis-

tic/antagonistic deviation pattern, the biological performance of any mixture ratio can be predicted.

Predicting interactions on a hormetic dose

Since joint effects are expected to change hormetic doses similar to toxic doses, available statistical mixture models developed for monotonic mixtures are used. The most frequently used reference models for monotonic mixtures are *concentration addition* (CA) and *independent action* (IA) assuming both additivity of doses (SØRENSEN et al., 2007). The IA model or *multiplicative survival model* (MSM) or better known as Colby's method in weed science, assumes a dissimilar mode of action of the mixture partners and multiplicativity of effects up to a maximum response of 100% (STREIBIG and JENSEN, 2000). The IA model is thus inapt to model hormetic doses leading to a response of >100% (BELZ et al., 2008), but has been used for ED_K predictions in the presence of hormesis (OHLSSON et al., 2010; ZOU et al., 2013). The CA model assumes similarity of action, but proved suitable modeling hormesis for mixtures violating its underlying assumption of similar mode(s) of action (OHLSSON et al., 2010). The mechanisms behind individual stimulatory responses are rarely known, and the stimulatory mode of action at lower doses may differ from the inhibitory mode of action at higher doses (CEDERGREEN, 2010; BELZ and DUKE, 2017). Therefore, selection of a reference model like CA that can accurately describe mixtures of dissimilarly and similarly acting compounds seems most appropriate for the prediction of hormetic doses. If the observed mixture data deviates synergistically or antagonistically from a reference model, the predefined curved isobole models of *Hewlett* or *Vølund* are available to model observed deviation patterns (SØRENSEN et al., 2007). All these models predict mixture effects based on the effective doses of the single compounds tested separately and, hence, a prediction of changes in hormetic doses can only be done if both single compounds induce hormesis (BELZ et al., 2008; BELZ and PIEPHO, 2017). Nearly all previous studies trying to evaluate hormetic doses in mixtures, tried to apply these traditional toxicological models.

Predicting interactions on the hormetic magnitude y_{max}

At the moment, there is no reference model available that could be adopted, nor is there a generally accepted model for y_{max} predictions (OHLSSON et al., 2010). In accordance with the hormetic mixture concept and based on empirical observations, BELZ et al. (2008) proposed a *linearity model* for y_{max} predictions, assuming a linear change of y_{max} with the mixture ratio. Of the few studies trying to evaluate y_{max} in mixtures, most tried to apply this linear model, however, there are also other modelling attempts (ZOU et al., 2013, 2017).

Where are we?

The present state-of-the-art in predicting hormesis in mixtures is demonstrated below on the basis of two examples evaluating binary mixture effects of two herbicidal compounds on root elongation of the model plant *Lactuca sativa*.

The first example refers to a mixture of two allelochemicals jointly acting in allelopathy of the invasive weed *Parthenium hysterophorus* L., namely, the sesquiterpene lactones parthenin and tetraneurin-A (BELZ et al., 2008). Both allelochemicals induced pronounced and reproducible hormesis in root elongation of *L. sativa* as single compounds, separately and in mixture (Fig. 1 up left). Mixture effects of these sesquiterpene lactones on the dose could be adequately assessed using the CA model as a reference independent of the dose level. Mixtures were always additive and significantly followed the CA model or the *Hewlett* isobole model, but with insignificant curvature parameter. Figure 1 (up middle) demonstrates this additivity for the *M* dose level in the form of a fraction plot based on the mixture ratio of parthenin (*x*) versus the associated *M* values (*y*) (BELZ and PIEPHO, 2017). This shows that mixture effects on hormetic doses can be equally predicted with available statistical models developed for monotonic mixtures. Studies with other toxins and/or test systems widely confirmed an adequate predictive power of the CA model and the curved

isobole models for hormesis evaluations (BELZ et al., 2008; OHLSSON et al., 2010; GE et al., 2011). Furthermore, ignoring hormesis by using monotonic instead of biphasic dose-response relationships for ED_K estimations did not notably influence the prediction of joint effects on inhibitory doses (BELZ et al., 2008). Changes in y_{max} in mixtures of parthenin versus tetraneurin-A widely followed the linearity model or remained within the limits of a typical boost. Figure 1 (up right) shows the fraction plot for the mixture ratio of parthenin (x) versus the associated y_{max} values. This finding showed that the linearity model can act as a reference model for y_{max} predictions and supported the hormetic mixture concept in the absence of an interaction on the dose. A subsequent study also revealed the validity of the linearity model in case of additivity of doses (OHLSSON et al., 2010). The question if the linearity model may still hold true in case of synergism/antagonism between mixture partners is addressed in the second example.

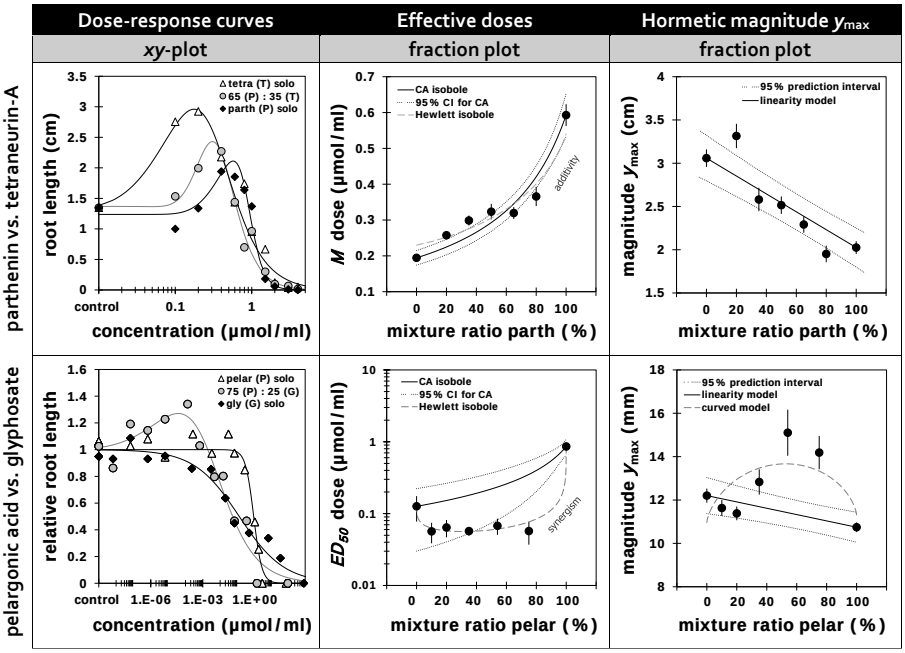

Fig. 1 Selected dose-response relationships for the effect of herbicidal compounds and their mixtures on root length of *Lactuca sativa* and deduced fraction plots for mixture effects on effective doses (M, ED_{50}) and the maximum stimulatory response y_{max}. (Upper) Mixture of parthenin (parth) versus tetraneurin-A (tetra); (lower) Mixture of pelargonic acid (pelar) versus glyphosate (gly). Error bars represent the standard error. (Data are from BELZ et al. (2008) and BELZ and PIEPHO (2017)).

Fig. 1 *Ausgewählte Dosis-Wirkungsbeziehungen für die Wirkung herbizider Stoffe und ihrer Mischungen auf das Wurzelwachstum von* Lactuca sativa *und abgeleitete ‚Fraction Plots' für Mischwirkungen auf effektive Dosierungen* (M, ED_{50}) *und die maximale stimulierende Wirkung* y_{max}. *(Oben) Mischung von Parthenin (parth) gegenüber Tetraneurin-A (Tetra); (Unten) Mischung aus Pelargonsäure (Pelar) gegenüber Glyphosat (Gly). Fehlerbalken zeigen den Standardfehler. (Daten aus BELZ et al. (2008) und BELZ und PIEPHO (2017)).*

The second example refers to a mixture of two herbicides jointly acting in commercial products, namely pelargonic acid and glyphosate (BELZ and PIEPHO, 2017). This mixture is believed to act synergistically, and glyphosate is known for its growth stimulation at low doses in plants (BRITO et al., 2018). Within five independent experiments, both herbicides showed inconsistent hormesis as single compounds separately and in mixture. Figure 1 (lower left) shows an experiment where the single compounds lacked hormesis in contrast to a mixture of 75% pelargonic acid and 25% glyphosate. As a consequence, mixture effects on hormetic doses could not be predicted. Evaluat-

ing mixture effects on ED_K doses against CA consistently revealed additivity at the ED_{20} level, changing to strong synergism at ED_{90}. Figure 1 (lower middle) shows one of two experiments where strong synergism appeared already at the ED_{50} level. Despite this synergism, changes in y_{max} in mixtures roughly followed the linearity model in three out of the five experiments. Observed atypical y_{max} deviations from linearity proved significant, however, changes always remained within the limits of a typical hormetic boost. Figure 1 (lower right) shows one experiment with y_{max} values deviating atypically from a linear trend and rather following a one-sided, curved trend of higher than expected values. Noticeably, both experiments with curved y_{max} trends were those with strong synergism already at the ED_{50} level. This indicated that the linearity model may only apply for mixtures showing no/minor interactions at ED_{50} level, while y_{max} predictions seem more critical for strongly interacting mixtures. Despite this, the hormetic mixture concept did not seem to be violated even in case of strong synergism. Judging on this discrepancy, between a statistically significant deviation pattern for y_{max} from linearity and a fulfillment of the hormetic concept of chemical interaction, will be one of the future challenges in this area.

How far can we go?

Despite the progress made in recent years, it is evident that there are still difficulties associated with the prediction of hormesis in mixtures. A major limit is the fact that the occurrence and expression of hormesis is the result of a complex, dynamic interplay of a low-dose exposure with several influencing factors (*e.g.*, growth conditions, time of exposure, plant age, *etc.*) so that a herbicide is not always and everywhere consistently hormetic (CEDERGEEN et al., 2007; BELZ and DUKE, 2014). If hormesis is missing with the single compounds tested separately, traditional reference models for dose predictions cannot be defined. Therefore, it is yet impossible to predict the mixture hormetic effect when the hormetic dose features of single mixture partners are missing (ZOU et al., 2013). Moreover, when hormesis does not occur in certain mixtures, a putative deviation pattern for hormetic doses cannot be evaluated.

Assessing joint effects on y_{max} is independent of the occurrence of hormesis, as in case of a lack of hormesis, the y_{max} equals the upper level of the monotonic dose-response relationship (BELZ and PIEPHO, 2017). Despite this, absence of hormesis in any of the mixture ratios may impact y_{max} evaluations as well by making atypical deviations from the linearity model more likely. An absence of hormesis results in a maximum response of 100%, while under hormesis the maximum response is expected to range typically between 130-160% of control. A response difference of up to 60% is more likely to be statistically significant and to represent an atypical deviation that violates the linearity model. This was, for example, the case in the pelargonic acid versus glyphosate study where atypical y_{max} deviations from linearity were observed if hormesis was lacking with just both single compounds or with almost all mixtures (BELZ and PIEPHO, 2017). Therefore, an absence of hormesis can play a great role for conclusions drawn from hormetic mixture studies (BELZ and PIEPHO, 2017). In addition, even if hormesis occurs consistently over mixture ratios and as a typical boost, a moderate change in y_{max} of between 30-60% may be statistically significant against the linearity model (BELZ and PIEPHO, 2017). This discrepancy between a significant, non-linear change indicating a real mixture effect on y_{max} and biological performance still in line with the hormetic mixture concept should be tackled in the future, *e.g.* by specifying model deviation ratios that are still in line with the hormetic mixture concept (BELZ and PIEPHO, 2017). BELZ et al. (2008) further supposed that atypical deviations may be the result of experimental variance, especially, in case of a low magnitude of hormesis that is more susceptible to variance. Hence, for now it can be noted that mixtures with lower magnitudes of hormesis and strongly interacting mixtures seem more prone to significantly deviate from the linearity model, but may still follow the hormetic concept of chemical interaction (BELZ and PIEPHO, 2017). Or vice versa, the linearity model seems promising to model mixture effects if hormesis is large and reproducible and if no/minor chemical interactions between mixture partners occur (BELZ et al., 2008; BELZ and PIEPHO, 2017).

Studies recording a clear one-sided deviation trend for y_{max} indicated an interrelation to a strong interaction on the dose, especially at the ED_{50} level (BELZ et al., 2008; BELZ and PIEPHO, 2017). If such one-sided deviation trends can be confirmed for additional mixtures showing strong interactions on the dose, a real mixture effect on y_{max} may have to be taken into account. Based on this, it should also be considered if synergism/antagonism on the dose leads to a characteristic higher or lower than expected change in y_{max} (BELZ and PIEPHO, 2017). However, the prediction of any putative atypical trend from the linearity model would first require development of a curved model equation similar to the *Hewlett* or the *Vølund* model for synergism/antagonism on the dose.

Future research should also provide further empirical support for determining if the linearity model can be applied to multiple compound mixtures, not only binary mixtures, and if it can be extrapolated to various biological systems (CEDERGREEN, 2010). A simplification and further refinement of the modelling approach is also needed, since modelling several biphasic dose-response relationships at once as necessary for adapting predefined mixture models can be a rather exhausting task.

Conclusions

The experience we currently have with the prediction of hormesis in mixtures is still limited. At the moment it seems that hormetic doses can be reliably predicted with available statistical models provided that all mixture partners and mixture ratios induce hormesis. Predictions of the magnitude of hormesis are independent of the occurrence of hormesis and seem to follow a linear trend according to the hormetic concept of chemical interaction provided that no/minor interactions occur in mixtures. Least predictable are changes in magnitude of hormesis if a strong interaction occurs in mixtures.

Against the background of the high relevancy and great concern of mixed low-dose exposures for many toxicological disciplines, including herbicide toxicity, an incorporation of hormesis into mixture toxicity evaluations should be more often done in future. There are still some open challenges and obvious limitations in predicting hormesis in mixtures, but the phenomenon deserves more study to understand its full impact on herbicidal joint actions.

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References

BELZ, R.G., N. CEDERGREEN and H. SØRENSEN, 2008: Hormesis in mixtures – Can it be predicted? Science of the Total Environment **404**, 77–87.

BELZ, R.G., N. CEDERGREEN and S.O. DUKE, 2011: Herbicide hormesis – can it be useful in crop production? Weed Research **51**, 321–332.

BELZ, R.G. and H.P. PIEPHO, 2012: Modelling effective dosages in hormetic dose-response studies. PLoS ONE **7(3)**: e33432. doi:10.1371/journal.pone.0033432.

BELZ, R.G. and S.O. DUKE, 2014: Herbicides and plant hormesis. Pest Management Science **70**, 698–707.

BELZ, R.G. and H.P. PIEPHO, 2017: Predicting biphasic responses in binary mixtures: pelargonic acid versus glyphosate. Chemosphere **178**, 88–98.

BELZ, R.G. and S.O. DUKE, 2017: Herbicide-mediated hormesis. ACS Symposium Series **1249**, 135–148.

BRITO, I.P.F.S., L. TROPALDI, C.A. CARBONARI and E.D. VELINI, 2018: Hormetic effects of glyphosate on plants. Pest Management Science (doi:10.1002/ps.4523).

CALABRESE, E.J., 2008a: Hormesis: why it is important to toxicology and toxicologists. Environmental Toxicology and Chemistry **27**, 1451–1474.

CALABRESE, E.J., 2008b: Hormesis and mixtures. Toxicology and Applied Pharmacology **229**, 262–263.

CALABRESE, E.J., 2010: Hormesis is central to toxicology, pharmacology and risk assessment. Human and Experimental Toxicology **29**, 249–261.

CEDERGREEN, N., J.C. STREIBIG, P. KUDSK, S.K. MATHIASSEN and S.O. DUKE, 2007: The occurrence of hormesis in plants and algae. Dose Response **5**, 150–162.

CEDERGREEN, N., 2010: Predicting hormesis in mixtures. Integrated Environmental Assessment and Management **6**, 310–311.

FLOOD, J.F., G.E. SMITH and A. CHERKIN, 1985: Memory enhancement – supraadditive effect of subcutaneous drug-combinations in mice. Psychopharmacology **86**, 61–67.

GE, H.L., S.S. LIU, X.W. ZHU, H.L. LIU and L.J. WANG, 2011: Predicting hormetic effects of ionic liquid mixtures on luciferase activity using concentration addition model. Environmental Science and Technology **45**, 1623–1629.

OHLSSON, Å., N. CEDERGREEN, A. OSKARSSON and E. ULLERÅS, 2010: Mixture effects of imidazole fungicides on cortisol and aldosterone secretion in human adrenocortical H295R cells. Toxicology **275**, 21–28.

RITZ, C. and J.C. STREIBIG, 2014. From additivity to synergism – A modelling perspective. Synergy **1**, 22–29.

SØRENSEN, H., N. CEDERGREEN, I.M. SKOVGAARD and J.C. STREIBIG, 2007: An isobole-based statistical model and test for synergism/antagonism in binary mixture toxicity experiments. Environmental and Ecological Statistics **14**, 383–397.

SOUTHAM, C.M. and J. ERLICH, 1943: Effects of extract of western red-cedar heartwood on certain wood-decaying fungi in culture. Phytopathology **33**, 517–524.

STREIBIG, J.C. and J.E. JENSEN, 2000: Actions of herbicides in mixtures. *In*: COBB, A.H. and R.C. KIRKWOOD (eds.): Herbicides and their mechanisms of action. Sheffield Academic Press, Sheffield, UK, pp. 153-180.

VELINI, E.D., M.L.B. TRINDADE, L.R.M. BARBERIS and S.O. DUKE, 2010: Growth regulation and other secondary effects of herbicides. Weed Science **58**, 351–354.

VELINI, E.D., C.A. CARBONARI, M.L.B. TRINDADE, G.L.G.C. GOMES and U.R. ANTUNIASSI, 2017: Variations in pesticide doses under field conditions. ACS Symposium Series **1249**, 47–60.

ZOU, X., Z. LIN, Z. DENG and D. YIN, 2013: Novel approch to predicting hormetic effects of antibiotic mixtures on *Vibrio fischeri*. Chemosphere **90**, 2070–2076.

ZOU, X., X. XIAO, Y. HE, L. HU, C. HU and X. HUANG, 2017: Hormetic effects of metal ions upon *V. fischeri* and the application of a new parameter for the quantitative assessment of hormesis. Journal of Hazardous Materials **322**, 454–460.