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Review

The molecular basis of simple relationships between exposure concentration and toxic effects with time

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ABSTRACT

Understanding the toxicity of chemicals to organisms requires considering the molecular mechanisms involved as well as the relationships between exposure concentration and toxic effects with time. Our current knowledge about such relationships is mainly explained from a toxicodynamic and toxicokinetic perspective. This paper re-introduces an old approach that takes into account the biochemical mode of action and their resulting biological effects over time of exposure. Empirical evidence demonstrates that the Druckrey–Küpfmüller toxicity model, which was validated for chemical carcinogens in the early 1960s, is also applicable to a wide range of toxic compounds in ecotoxicology. According to this model, the character of a poison is primarily determined by the reversibility of critical receptor binding. Chemicals showing irreversible or slowly reversible binding to specific receptors will produce cumulative effects with time of exposure, and whenever the effects are also irreversible (e.g. death) they are reinforced over time; these chemicals have time-cumulative toxicity. Compounds having non-specific receptor binding, or involving slowly reversible binding to some receptors that do not contribute to toxicity, may also be time-dependent; however, their effects depend primarily on the exposure concentration, with time playing a minor role. Consequently, the mechanism of toxic action has important implications for risk assessment. Traditional risk approaches cannot predict the impacts of toxicants with time-cumulative toxicity in the environment. New assessment procedures are needed to evaluate the risk that the latter chemicals pose on humans and the environment. An example is shown to explain how the risk of time-dependent toxicants is underestimated when using current risk assessment protocols.

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1. Introduction

Understanding the toxicity of chemicals to organisms is the basis for a correct risk assessment. Given the enormous variety of chemicals that contaminate the environment, their different modes of action and mechanisms of toxicity (Escher and Hermens, 2002) in different species, quantitative studies on the relationship between exposure levels to toxicants and toxic effects are necessarily complex (Bradbury, 1995; Rubach et al., 2010) and represent a major challenge to ecotoxicologists. Relating an observed exposure concentration–effect relationship to the mechanism of toxicity of a compound, which is a prerequisite for meaningful risk assessment of chemicals, is only the first step for such an understanding. A second step involves the time-dependency of toxic effects (Baas et al., 2010), which is often forgotten in ecotoxicological research although time is considered in risk assessment protocols (e.g. chronic toxicity). Certainly, the inclusion of time is becoming more common in experimental studies (Legierse et al., 1999; Newman and McCloskey, 1996; Smit et al., 2008) and models (Lee and Landrum, 2006; Jager et al., 2011). However, the underlying mechanisms of time-dependency are best understood in the case of baseline toxicity (i.e. narcotics), but not so much in chemicals with specific modes of action (e.g. reactive electrophiles, enzyme inhibitors, etc. – for a review see Escher and Hermens, 2002).

The influence of time of exposure on toxicity was suggested a long time ago (Bliss, 1937), but it has taken decades for time-to-event analyses of ecotoxicity data to be developed (Newman and McCloskey, 1996) and applied in risk assessment (Crane et al., 2002). Unfortunately, implementation of time-dependent approaches on standard toxicity protocols and regulatory risk assessment is still lagging behind. Standard acute test protocols (e.g. OECD tests) require that toxic effects are recorded at intermediate time-points, but the derivation of LC50 and other toxicity metrics is only done at fixed times (e.g. 48 or 96 h). Consequently, most of the information obtained is not used even if it could be analyzed further using appropriate descriptive methods (Jager et al., 2006). Two different approaches can be used to analyze toxicity test data that includes time information: time-to-event procedures (Newman and McCloskey, 1996) and mechanistic models (Mackay et al., 1992; Kooijman and Bedaux, 1996; Ashauer and Escher, 2010). Time-to-event (TTE) analysis is an empirical method, which describes the time-dependent toxicity of a particular chemical to a particular species by fitting a mathematical curve to the experimental data. Often the parameters in those mathematical equations cannot be explained in biological terms, but the equations thus obtained can predict the toxicity of the chemical to a species with reasonable accuracy within the tested conditions (Zhao and Newman, 2004). Many mechanistic models have been proposed to analyze the time-dependent toxicity of chemicals, and their inclusion here is outside the scope of this paper (for a comprehensive review see Jager et al., 2011). All these models are useful tools to describe the toxic effects observed over time. For the case of survival endpoints, the current trend is to integrate their different assumptions under a general unified threshold model of survival (GUTS) based on toxicokinetics and toxicodynamics (Jager et al., 2011). However, for these mechanistic models to be realistic they need to be based on sound toxicological concepts.

The objective of this paper is three-fold: firstly, a short and critical review of current approaches to time-dependent toxicity is made in order to provide a background. Secondly, an old approach developed by Druckrey and Küpfmüller (1949) to study the toxicity of carcinogenic substances (Druckrey et al., 1963) will be introduced, as it is almost unknown among ecotoxicologists. Recent experimental evidence with aquatic and terrestrial organisms confirm that relatively simple exposure concentration–effect relationships are identical to those derived from the theoretical (mathematical) approaches of Druckrey and Küpfmüller (Tennekes, 2010). Thus, the observed exposure concentration–effect relationship can be related to the mechanism of action of a toxicant. The third objective is to show a number of case studies taken from the literature that confirm the validity of this old approach, followed by a brief discussion of the mechanisms involved in each case. Finally, some suggestions for new risk assessment procedures are made, using an example to explain how the risk of toxicants with time-cumulative toxicity, i.e. those for which toxic effects are greatly enhanced by exposure time, is underestimated in current risk assessment protocols.

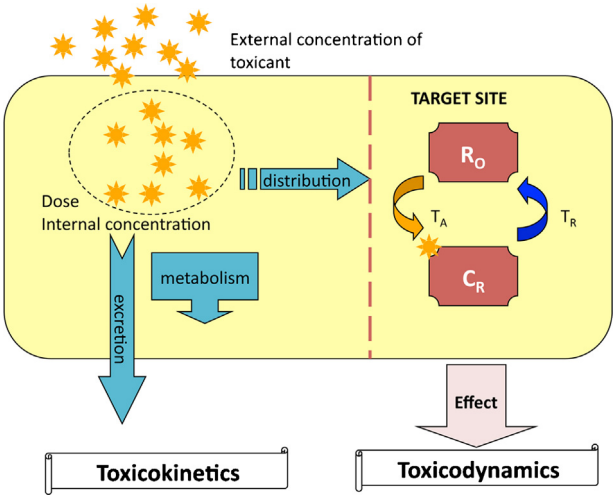

Fig. 1. Structure of the Druckrey–Küpfmüller model. The internal concentration or dose is determined by the toxicokinetic processes that take place inside the organisms. Only the toxicant molecules that reach the target receptors (R_0) can have a toxic effect. The toxicodynamics are based on binding of toxicant molecules to the target receptors (C_R), a process that takes place in time and depends on the time constants for association (T_A) and dissociation (T_R) to and from the receptor.

1.1. Current status on time-dependent ecotoxicity

Most of the research aimed at explaining the toxicity of chemicals in organisms is based on toxicokinetics, that is the processes of uptake, distribution within an organism, biotransformation (metabolism) and elimination (Fig. 1). Toxicokinetics determine the relationship between exposure concentration of a toxicant in the external media (or dose ingested in dietary exposures) and its concentration at the site of action, as well as its time course. Therefore, information on all aspects of the kinetics of toxicants is of particular relevance for understanding and predicting the toxicity of chemicals (Escher and Hermens, 2002). However, it is the concentration of the toxicant at the site of action that is of major interest, since this concentration determines critical receptor binding that may eventually elicit a toxic effect. A linear relationship between exposure levels to toxicants and their toxic effect, therefore, requires strict proportionality for each process.

More recently the concept of toxicodynamics, that is the interactions that link the internal concentration to an effect in an individual organism over time, has been incorporated as well (Ashauer and Brow, 2008; Voicu et al., 2010). Several interactions have been proposed, including damage-repair mechanisms (Lee et al., 2002), killing rates and recovery constants (Ashauer et al., 2007), which are appropriate for narcotics and some chemicals with specific mode of action. For the latter chemicals, the Druckrey–Küpfmüller model uses the time constants for association and dissociation of the toxicant to the target receptor, which

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determine the strength of the binding (Fig. 1). Toxicodynamics influence the effect outcomes (Billoir et al., 2012) and are important for risk assessment procedures, since organisms can be exposed to chemical pulses, constant exposures or intermittent and variable exposures with time (Rubach et al., 2010).

Common approaches to both toxicokinetics and toxicodynamics (TK/TD) use mathematical models that are based on one or several toxicological concepts, e.g. critical body residues (CBR (McCarty et al., 1992)); bioaccumulation (van Leeuwen et al., 1985); energy budgets and homeoestasis (DEBtox (Kooijman and Bedaux, 1996)); threshold hazards (THM (Ashauer et al., 2007)). Usually, first order-kinetics for one or two compartment models are used. These models have been quite successful in describing the observed toxic effects of narcotics and chemicals with a multiple mode of action such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and metallic elements in fish (Hoogen and Opperhuizen, 1988; Hoang et al., 2007; Baas et al., 2009), aquatic invertebrates (Meador, 1997; Pery et al., 2001; Landrum et al., 2003), soil arthropods (Crommentuijn et al., 1994; Widianarko and Straalen, 1996) and birds (Barron et al., 1995). The toxic effect of compounds with specific mode of action such as insecticides (e.g. acetylcholinesterase inhibitors, pyrethroids) can also be predicted with accuracy using DEBtox (Jager and Kooijman, 2005; Pieters et al., 2006), receptor mediated TK/TD (Jager et al., 2006) and critical target occupation (CTO) (Legierse et al., 1999) models.

Our understanding of toxicity processes has benefitted enormously from the above modelling. For example, CBR approaches have confirmed that systemic concentrations of toxicants (that lead to critical receptor interactions) are preferable to their environmental concentrations in order to explain toxicity (Landrum et al., 1992; Solomon et al., 2009). The most obvious consequence is that the toxicity of a mixture of chemicals with identical or similar mode of action can be determined by adding the molar concentrations of the individual components (Landrum et al., 2003); hence the concept of toxic equivalent concentrations (TEC), which is very useful in assessing the overall impact of mixtures of persistent chlorinated compounds such as polychlorinated biphenyls (PCBs) dibenzodioxins and dibenzofurans (Berg et al., 1998). Synergistic effects in mixture toxicity are rare, occurring in less than 10% of 200 cases reported (Deneer, 2000). The best well-known cases include estrogenic effects of some phenolic and organochlorine compounds (Escher et al., 2001) and the synergism between the herbicide atrazine and organophosphorus insecticides (Howe et al., 1998; Belden and Lydy, 2001). Toxicokinetic approaches have also been instrumental in establishing the relationship between bioconcentration factors (BCF), toxicity of PAHs and their octanol–water partitioning coefficients (K_{ow}) (Baas et al., 2009; Hattum and Montanes, 1999). And the DEBtox model has highlighted the importance of feeding conditions in modifying toxicokinetics (Pieters et al., 2006; Kooijman, 1991). However, the parameters used in all these models (e.g. rates of uptake and elimination, lipophilicity, etc.) can be related to the toxicity of narcotics, but not to the mechanism of action of other compounds, which is ultimately what causes their toxic effects (Escher and Hermens, 2002). Models that wish to explain the toxicity of toxicants with specific mode of action in relation to exposure must take into account the molecular mechanism of action of the compounds to their respective target sites. This may help explain the differences in sensitivity between species to a given compound. For example, the toxicity of neonicotinoid insecticides to *Daphnia magna* is two to three orders of magnitude lower than the toxicity of the same compounds to other planktonic and benthic crustaceans (Hayasaka et al., 2012a; Sánchez-Bayo and Goka, 2006a). The tolerance of *Daphnia* towards those neurotoxic insecticides must be found in the particular toxicodynamics of that species.

Current research aims at elucidating the toxic effects resulting from different patterns of exposure such as pulses and time-variable concentrations, which are more frequently found in the environment than constant exposures. For example, mortality in *D. magna* exposed to various metals is a function of concentration, duration, and recovery time between exposures (Hoang et al., 2007). While similar sensitivity of *D. magna* to cadmium was observed in flow-through conditions compared to static ones (Billoir et al., 2012), toxicokinetics determined by time-weighted-average (TWA) and time-variable water concentrations were not statistically different in *Diporeia* amphipods exposed to PAHs (Landrum et al., 2003), indicating that toxic effects are integrated over time.

In this regard, it has been known for some time that lethal median concentrations (LC50s) decrease exponentially with time of exposure (Santos and Cabral, 2004; Sánchez-Bayo, 2006). As time progresses, the median concentrations eliciting mortality reach a threshold (Widianarko and Straalen, 1996; Brown, 1978). For narcotics and chemicals with a reversible binding, this threshold is determined by bioaccumulation kinetics, i.e. it is inversely proportional to the BCF of the compound, which in turn is related to the partitioning coefficients between lipid and aqueous phases and the elimination rate (Escher and Hermens, 2002). However, when a toxicant is not eliminated, or binding to specific receptors is virtually irreversible the LC50 threshold can eventually be zero (Crommentuijn et al., 1994). These observations have led to the concept of no-effect concentration (NEC (Kooijman et al., 1996)), which appears to be constant for a given toxicant and organism and is independent of the time-variable exposure patterns (Pery et al., 2001). In ecotoxicology, NECs should replace outdated toxicity metrics such as the no-observed effect level or concentration (NOEL/NOEC) because the former are based on sound biological and toxicological facts whereas the latter are statistically flawed (Landis and Chapman, 2011). However, recent developments in ecotoxicology suggest that some toxicants can produce effects at any concentration provided their exposure time is sufficiently long (Tennekes, 2010). This means the concept of NEC may not apply for these toxicants when the life span of the organisms affected is longer than the theoretical maximum exposure time.

1.2. Conceptual bases of the Druckrey–Küpfmüller model

The toxicity approach developed by H. Druckrey and K. Küpfmüller some 60 years ago is obviously not based on the ecotoxicological concepts mentioned above but rather on the conceptual pharmacokinetics of their time. Because of this, some of the terminology they used has changed and needs to be translated to our current understanding of the TK/TD processes. Also, since the majority of ecotoxicologists are unaware of their model, which focuses on chemicals with specific mode of action and was applied to cancer research, not to survival of organisms in the environment, a brief explanation of its biological and mathematical concepts is required first. (*Note*: the original mathematical notations used by those authors are also used here.)

It is assumed that a toxicant molecule will react with a specific receptor in a bimolecular reaction, and that bound receptors determine the toxic effect. Denoting the initial concentration of critical receptors that a toxicant reacts with as R_0, the concentration of receptors that a toxicant is reacting with as C_R, and the toxicant concentration at the site of action as C (in today's terms, C is equivalent to the internal concentration C_i with dimensions of mole per volume or weight), the velocity of receptor binding (association) is:

$$KC(R_0 - C_R) \quad (1)$$

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where K is the reaction constant for association. The velocity of dissociation of bound receptors is:

$$\frac{C_R}{T_R} \tag{2}$$

where T_R is the time constant for dissociation. Therefore, the reaction kinetics of receptor binding in the case of a bimolecular reaction are

$$\frac{dC_R}{dt} = KC(R_0 - C_R) - \frac{C_R}{T_R} \tag{3}$$

Replacing the concentration of bound receptors C_R by the relative concentration of bound receptors C_R/R_0 and the reaction constant $K\,R_0$ by $1/T_A$ (where T_A is regarded as the time constant for association), we obtain

$$\frac{[dC_R/R_0]}{dt} = \frac{[C(1 - C_R/R_0)]}{[R_0 T_A]} - \frac{[C_R/R_0]}{T_R} \tag{4}$$

The relative concentration of bound receptors (C_R/R_0) determines the relative toxic effect, and Eq. (4) indicates that effects over time depend on the concentration of toxicant at the site of action C and the strength of the binding to the receptor, which is determined by T_A and T_R. The interaction of a toxicant with the critical receptors that lead up to an effect cannot be measured directly in a toxicity study, but has to be assessed indirectly by using the effect as an indicator of the extent of those interactions (Jager et al., 2010). Obviously C results from the equilibrium between the external exposure concentration (c) and internal concentration, driven by time-dependent toxicokinetics, and we have to assume proportionality between critical receptor interactions and effects, although this may not always be the case. Confounding influences and compensatory mechanisms attenuating effects may occur. Moreover, multiple processes determine critical receptor interactions that lead up to toxic effects, and modulations – such as bioactivation or enzyme induction – may be critical in many cases (Crommentuijn et al., 1994; Yurk and Barron, 1992).

Eq. (4) can be simplified to indicate the relative concentration of bound receptors C_R/R_0 in steady-state, i.e. when $[dC_R/R_0]/dt = 0$; then

$$\frac{C_R}{R_0} = \frac{[C/R_0] \cdot [T_R/T_A]}{1 + [C/R_0] \cdot [T_R/T_A]} \tag{5}$$

A plot of the relative concentration of bound receptors C_R/R_0 as a function of the relative toxicant concentration C/R_0 for defined values of T_R/T_A in steady-state is shown in Fig. 2A. It shows that when a substantial proportion of a specific receptor is used up by reaction with a toxicant, then saturation may occur (second-order kinetics).

Irrespective of the actual T_R/T_A value, the relationship between relative concentration of bound receptors C_R/R_0 and relative toxicant concentration C/R_0 is a hyperbole. Eq. (5) applies generally for a bimolecular reaction of a toxicant with a specific receptor in an individual organism. The value of the time constant for dissociation T_R relative to the time constant for association T_A determines the strength of the binding and, therefore, is crucial to the toxic effects: the higher the ratio, the higher the toxicity. Druckrey and Küpfmüller referred to the quotient T_R/R_0T_A as an *index of relative efficacy*. Thus, toxicity of substances with T_R/T_A ratios <1 requires high relative toxicant concentrations C/R_0. This is their theoretical explanation of *Dosis facit venenum* (Paracelsus).

If both T_R and T_A are low, i.e. when both association and dissociation are fast processes, the equilibrium between C and receptor binding (and effect) will be established quickly but the toxic effect will also regress quickly. The time course of the effect will be the same as the time course of the concentration at the site of action C, and the maximum effect will occur when the concentration at the site of action C is at its maximum. The effects will thus be strictly concentration-dependent.

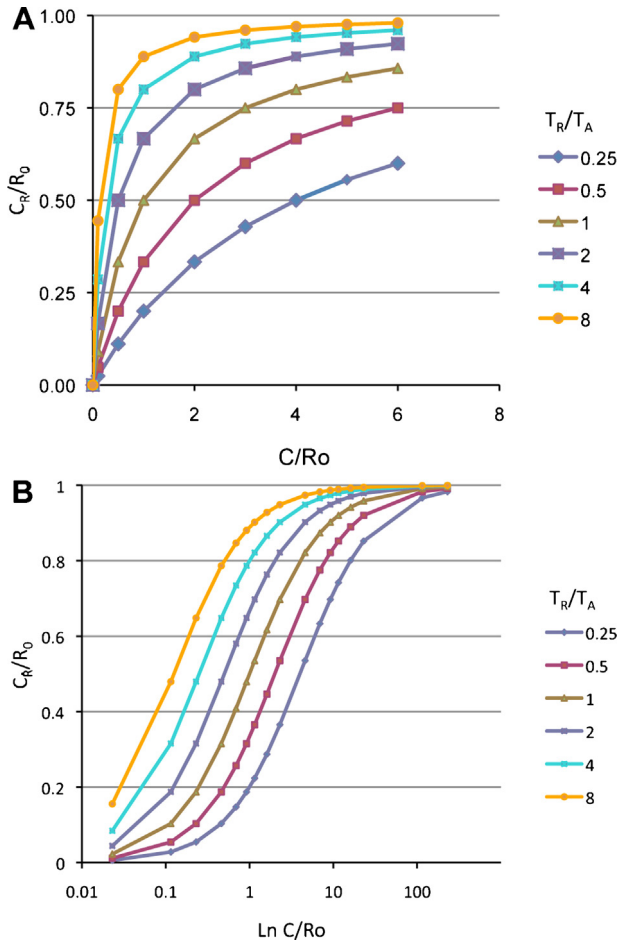

Fig. 2. (A) Relative concentration of bound receptors (C_R/R_0) as a function of the relative toxicant concentration (C/R_0) for several values of the ratio between the time constant for dissociation (T_R) and the time constant for association (T_A). (B) Same plot but using natural logarithms of the relative toxicant concentration ($\ln C/R_0$).

If the time constant for dissociation T_R is high, i.e. when receptor binding is only slowly reversible, the time to maximum effect will be delayed, and the toxic effect will also be slowly reversible. The higher T_R is, the longer is the time to maximum effect. Upon repeated exposure in quick succession there will be cumulative effects. Because equilibrium between C and receptor binding will be established very slowly, toxicity becomes a process that takes place in time. There will be a latency period (i.e. when small amounts of toxicant are bound to the receptors but no toxic effects are observed yet) up to a defined effect, which can be shortened, of course, by increasing the concentration of the toxicant at the site of action.

Taking the logarithm of the relative toxicant concentration at the site of action C/R_0 for several values of the ratio between the time constant for dissociation T_R and the time constant for association T_A, the typical sigmoid toxicity curve is obtained using Eq. (5) (Fig. 2B). This logarithmic plot is practically linear when the relative concentration of bound receptors C_R/R_0 is between 20% and 80%. Assuming proportionality between the exposure concentration c and the concentration at the site of action C, this would explain the typical relationship between effect and the logarithm of exposure concentration c observed for many toxicants in laboratory tests, which can be empirically described by a sigmoid curve of the type

$$\text{Proportional effect} = a + \left\{ \frac{1 - a}{1 + \exp(b - \gamma \cdot \ln c)} \right\} \tag{6}$$

where α is the background mortality, β is the middle point in the curve (i.e. EC_{50}) and γ is the slope (Sánchez-Bayo and Goka, 2007).

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While other mathematical models can also fit this toxicity pattern (e.g. logit, probit, Weibull models used to estimate LC50s), the mathematical approaches by Druckrey and Küpfmüller provide a theoretical explanation for such toxicity curves. Its essential feature is that the effect requires a high degree of receptor binding. It is interesting to note in this context that variation in the number of target receptors among the individuals of a population is presumed to follow a log-normal distribution, as it depends on size and other individual variables; consequently, toxicity bioassays use logarithms of the concentration to determine toxic effects on a group of organisms (Pieters et al., 2006).

1.3. Effects proportional to exposure concentration in time

Fig. 2A also shows that up to 25% receptor binding the hyperbole is practically linear. A linear concentration:effect relationship may only occur whenever receptor binding less than 25% leads to a toxic effect. Furthermore, if receptor binding happens to be virtually irreversible, i.e. when the time constant for dissociation approaches infinity ($T_R \to \infty$), Eq. (3) reduces to

$$\frac{dC_R}{dt} = KC(R_0 - C_R) \tag{7}$$

If the effect occurs in the linear section of the hyperbole and the concentration of the specific receptor remains virtually unchanged (first-order kinetics), i.e. when $C_R \ll R_0$, then

$$\frac{dC_R}{dt} = KR_0C \tag{8}$$

If, under such circumstances, an exposure concentration c is kept constant throughout a study, and, as a result, the toxicant concentration at the site of action C remains constant as well, integration yields

$$\frac{C_R}{R_0} = KCt \tag{9}$$

This is a theoretical explanation for Haber's rule, which states that the product of exposure concentration c and exposure duration t produces a constant toxic effect E (for a review, see Witschi, 1999). So, when Haber's rule applies, there would be proportionality between relative receptor binding C_R/R_0 and E, and effects would already begin to occur with the onset of receptor binding. However, in many other cases the toxic effects will only begin to occur as from a certain level of relative receptor binding C_R/R_0. In these cases, a 'threshold' of constant value would need to be introduced for both the administered concentration c and the time to effect t:

$$(c - c_m) \cdot (t - t_m) = \text{constant} \tag{10}$$

where c_m is a threshold concentration and t_m a minimum time of response. For toxicants that follow Haber's rule this would merely imply that the threshold concentration c_m and the minimum time of response t_m are so small as not to produce a measurable error.

From a theoretical point of view, a number of conditions have to be met if a toxicant is to follow Haber's rule. Firstly, proportionality is required between the exposure concentration c and the concentration at the site of action C, which must also increase over time in a strictly linear fashion

$$\frac{dC}{dt} = Kc \tag{11}$$

where K has the dimension of reciprocal time, i.e. of velocity. Secondly, the effect E has to be proportional to the concentration at the site of action C (and thus to exposure concentration c) as well, so that

$$\frac{dE}{dt} = Kc \tag{12}$$

and

$$E = K\int_0^t c\,dt \tag{13}$$

If, under such circumstances, the exposure concentration c is kept constant then

$$E = Kct \tag{14}$$

and the toxicant will follow Haber's rule, that is the velocity of the effect E/t will be linearly related to the exposure concentration c

$$\frac{E}{t} = Kc \tag{15}$$

Eqs. (9) and (14) assume proportionality between the concentration of occupied receptors and the effect, but this may not always be the case because the reversibility of an effect can have the same significance for dose–response characteristics as the reversibility of receptor binding. Denoting the time constant for the reversibility of the effect as T_r, three types of dose–response characteristics were identified by Druckrey and Küpfmüller when the time constants T_R and T_r approach either zero (reversible) or infinity (irreversible), as shown in Table 1. Thus, Haber's rule may be obtained when either receptor binding or the effects are irreversible.

1.4. Effects reinforced by exposure over time

Eq. (8) indicates that if the effect E indeed occurs in the linear section of the hyperbole when $C_R \ll R_0$ (first-order kinetics), and if receptor binding also happens to be virtually irreversible (i.e. when $T_R \to \infty$), the concentration of bound receptors C_R would be proportional to the integral of the concentration of the toxicant at the site of action C over time:

$$C_R \sim \int C\ dt \tag{16}$$

If the subsequent effect happens to be irreversible as well (e.g. death), the effect E would be proportional to the integral of the concentration of bound receptors C_R over time:

$$E \sim \int C_R\ dt \tag{17}$$

So, in cases of irreversible receptor binding and an irreversible effect, the effect E would be proportional to the double integral of the toxicant concentration at the site of action C over time, as the combination of Eqs. (16) and (17) shows

$$E \sim \int\int C\ dt \tag{18}$$

Integration yields E as the product of exposure concentration and exposure duration to a power (i.e. $c \cdot t^2$), with the implication that exposure time will enhance the effect (Fig. 3).

Druckrey demonstrated experimentally the validity of this equation using genotoxic carcinogens (Druckrey et al., 1963; Druckrey and Dischler, 1963), and found values of 2 or higher for the time exponent, indicating a stronger enhancement of effects with time (called 'reinforcement' henceforth) in the case of compounds like 4-dimethylaminostilbene. Consequently, such exposure concentration–effect relationships were described by the following Druckrey–Küpfmüller equation:

$$c \cdot t^n = \text{constant} \tag{19}$$

where the exponent n can be viewed as a exposure time reinforcement factor that may take a value higher than 1 when irreversible effects are greatly enhanced (i.e. reinforced) by time of exposure. For $n = 1$ the toxic effects would follow Haber's rule, which turns

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Table 1
Dose–response characteristics according to Druckrey and Küpfmüller.

Reversibility of receptor binding	Bound receptors in relation to toxicant concentration	Reversibility of the effect	Effect in relation to bound receptor concentration	Effect in relation to toxicant concentration	Dose–response characteristics
$T_R \to 0$	$C_R \sim C$	$T_r \to 0$	$E \sim C_R$	$E \sim C$	Dose-dependent
		$T_r \to \infty$	$E \sim \int C_R\,dt$	$E \sim \int C\,dt$	$C{\cdot}t$ = constant[a]
$T_R \to \infty$	$C_R \sim \int C dt$	$T_r \to 0$	$E \sim C_R$	$E \sim \int C\,dt$	$C{\cdot}t$ = constant[a]
		$T_r \to \infty$	$E \sim \int C_R\,dt$	$E \sim \int\int C\,dt$	Reinforced by time [$C{\cdot}t^n$ = constant, with $n > 1$]

[a] Assuming that an exposure concentration c is kept constant and that, as a result, the toxicant concentration at the site of action C remains constant as well.

out to be a particular case of the general Eq. (19). The essence of such relationships is that whenever $n > 1$ the product of exposure concentration c and exposure time t, which reflects the total dose required for an effect, decreases with decreasing exposure concentration c, even though the time-to-effect t increases with decreasing exposure concentration c. Sánchez-Bayo (2009) demonstrated empirically that the toxicity of neonicotinoid insecticides to aquatic arthropods followed a simple relationship between exposure concentration and time-to-effect which is identical to the Druckrey–Küpfmüller equation (19), and could be used, therefore, to validate their model.

An important consequence of this time-dependent toxicity is that what we observe at high dose levels is bound to happen at low dose levels as well, with the passage of time. Of course, all the above equations apply when the predominant toxic effects are caused by receptor-binding. Other sublethal and side effects that result from mechanisms not involving specific receptors (e.g. disturbed homeostasis) may or may not comply with these equations. The fact that sublethal effects "appear" to occur at low concentrations only is because if the toxicant levels are near or above the LD50 the high mortality would mask any other side effects.

1.5. Experimental validation of the Druckrey–Küpfmüller model

The above analysis shows that empirical relationships between exposure concentration and time-to-effect, such as Haber's rule or the Druckrey–Küpfmüller equation (19), can be explained by irreversible receptor binding. However, other toxicants follow the same relationship between exposure concentration and time-to-effect but produce a value of $n < 1$ (Tennekes and Sánchez-Bayo, 2012) (Fig. 3). Examples of these three types of toxicant behaviour are shown in Fig. 4 for the mortality of *D. magna* exposed to the neonicotinoid imidacloprid, the metalloid selenium and the essential trace element zinc. While data for the three toxicants fit Eq. (19), each one of them follows a different toxicity pattern: $n > 1$ for imidacloprid, $n = 1$ for selenium, and $n < 1$ for zinc.

The exponent n is calculated as the reciprocal of the absolute value of the slope in the regression line to the experimental data points

$$\mathrm{Ln}\, t50 = a + b\,\mathrm{Ln}\, c \tag{20}$$

where $t50$ is the median time-to-effect and c the concentration tested (or dose in the case of terrestrial organisms). Eq. (20) is identical to Eq. (19) but in a logarithmic form, with parameters estimated from least-squares regression analysis and statistics by ANOVA. Consequently, Eq. (20) can be used to validate experimentally the Druckrey–Küpfmüller model. These three patterns of time-dependent toxicity (see also Table 1) can be easily determined by looking at the product of c and $t50$ (Table 2), which reflects the actual dose that produces the effect:

(i) when $c \times t50$ decreases as c decreases, then the slope < 1 and the toxic effects are reinforced by time of exposure ($n > 1$);
(ii) when $c \times t50$ is constant for any combination of c and time-to-effect, then the slope (and n) takes the value 1 (Haber's rule); and
(iii) when $c \times t50$ decreases as c increases, then the slope > 1 and the toxic effects are more readily expressed at higher exposure concentrations, with time having a minor influence ($n < 1$).

The intersection of the regression line with the life span of the organism determines the lowest concentration for lethal effects of a particular chemical (LLC50), which can be considered close to the NEC even though sublethal effects may have taken place. In the case of *D. magna*, the estimated LLC50 for imidacloprid and

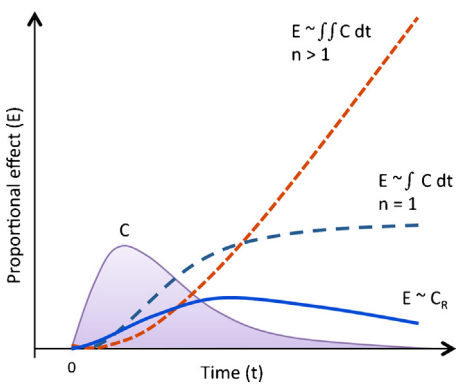

Fig. 3. Concentration–effects relationships as described by the Druckrey–Küpfmüller equation (see text). C = concentration at the site of action; C_R = concentration of bound receptors; E = effect.

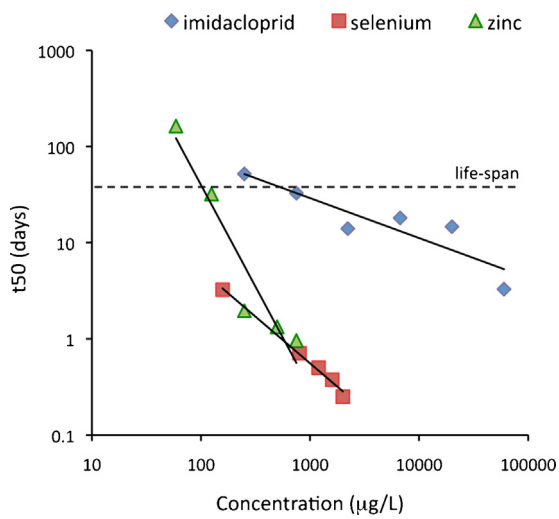

Fig. 4. Mortality of *Daphnia magna* exposed to several concentrations of the neonicotinoid imidacloprid, the metalloid selenium and the essential trace element zinc over time. $t50$ is the median time to death.

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Table 2
Toxicity patterns of imidacloprid, selenium and zinc on mortality of *Daphnia magna*. See also Fig. 4.

Chemical[b]	Concentration (c) ($\mu g\,L^{-1}$)	Median time to effect ($t50$) (days)	Dose = $c \times t50$ ($\mu g\,L^{-1}$ days)
Imidacloprid	250	52.0[a]	13,000
	750	32.8	24,594
	2220	14.0	31,080
	6700	18.1	121,158
	20,000	14.7	293,333
	60,000	3.3	197,500
Selenium	158	3.25	514
	800	0.71	567
	1200	0.50	600
	1600	0.38	600
	2000	0.25	500
Zinc	59	163.0[a]	9617
	125	32.0	4000
	250	2.0	490
	500	1.3	667
	750	1.0	719

[a] Estimated $t50$ longer than life span of *D. magna*.
[b] Data for imidacloprid from Sánchez-Bayo (2009); for selenium and zinc from Hoang et al. (2007).

zinc are 466 and 100 $mg\,L^{-1}$, respectively (Fig. 4); for selenium, an extrapolation would render a LLC50 of 12.3 $mg\,L^{-1}$. Evidently, for long-lived organisms the LLC50 can be zero, as other authors found with cadmium and certain soil arthropods (Crommentuijn et al., 1994).

2. Case studies

The examples of chemical toxicity with time shown in this section are taken from the published literature. Data were selected based on the availability of several test concentrations and results expressed for a number of time points. The original data sets were re-analyzed using Eq. (20), and results are shown in Tables 3–5: regression parameters, their statistical significance, and the value of the exponent n in the Druckrey–Küpfmüller equation were derived as indicated above. Lethality was the endpoint in all cases except with the carcinogenic substances, for which the time to tumour induction is usually recorded. A brief discussion of the data is included.

2.1. Chemicals that reinforce toxicity over time of exposure

Chemicals that follow the Druckrey–Küpfmüller equation (19) are shown in Table 3. The genotoxic carcinogens 4-dimethylaminostilbene (4-DAST) and diethylnitrosamine (DENA) were the first examples of toxicants that showed reinforcement of effects (i.e. tumour induction) over time of exposure (Druckrey et al., 1963, 1970; Druckrey and Dischler, 1963). The obvious critical target of these substances is DNA (Jager and Kooijman, 2005; Berg et al., 1998).

Neonicotinoid insecticides show reinforcement of lethal effects over time of exposure. Apart from *D. magna* (Fig. 4), exposure to imidacloprid renders values of $n > 1$ in freshwater ostracods (*Cypridopsis vidua*), in the hymenopteran parasitoid *Chelonus blackburni* and in honey bees (*Apis mellifera*). The same pattern applies to exposures of honey bees to thiamethoxam and dragonfly nymphs (*Sympetrum striolatum*) to thiacloprid (Table 3). The toxicity pattern of imidacloprid and thiacloprid suggests that these and other neonicotinoid compounds have irreversible binding to their nicotinic acetylcholine receptors (nAChRs) in arthropods, as indicated by several authors (Abbink, 1991; Buckingham et al., 1997; Zhang et al., 2000). Neonicotinoids are agonist of the nAChR,

Table 3
Examples of chemicals that show cumulative effects over time, i.e. follow the Druckrey–Küpfmüller equation $c \cdot t^n$ = constant, with $n > 1$. The value of the exponent n is determined as the reciprocal of the absolute value of the slope in the regression line $\ln t50 = a + b \ln c$, where $t50$ is the median time to effect and c the exposure concentration (or dose administered in the case of terrestrial organisms). Parameters of the regression are shown.

Chemical	Type[a]	Species	Taxa	n	Intercept (a)	Slope (b)	r^2	P	No. c tested	Exposure time	Reference
4-DAST	C	*Rattus* sp.	Mammal	2.89	6.01	−0.35	0.99	<0.001	7	900 days	Druckrey and Dischler (1963)
Cartap	I	*Danio rerio*	Fish (embryos)	1.66	8.24	−0.60	0.62	0.212	4	6 days	Zhou et al. (2009)
DENA	C	*Rattus* sp.	Mammal	2.30	5.60	−0.43	1.00	<0.001	8	840 days	Druckrey et al. (1963)
Diphacinone	R	*Falco sparverius*	Bird	1.60	6.86	−0.63	0.95	0.004	6	7 days	Rattner et al. (2011)
Imidacloprid	I	*Chelonus blackburni*	Insect	1.51	5.38	−0.66	0.99	0.049	3	24 h	Preetha et al. (2010)
Imidacloprid	I	*Daphnia magna*	Cladoceran	2.41	6.54	−0.41	0.89	0.043	5	10 days	Sánchez-Bayo (2009)
Imidacloprid	I	*Apis mellifera*	Insect	5.83	5.19	−0.17	0.95	0.012	5	240 h	Suchail et al. (2001)
Imidacloprid	I	*Cypridopsis vidua*	Ostracod	4.67	5.11	−0.21	0.88	0.006	6	96 h	Sánchez-Bayo (2009)
Methylmercury	OM	*Rattus rattus*	Mammal (cells)	1.69	7.82	−0.59	0.99	0.052	3	72 h	Fujimura and Usuki (2012)
Thiacloprid	I	*Sympetrum striolatum*	Aquatic insect (nymph)	1.53	7.43	−0.65	1.00	0.002	4	24 h[b]	Beketov and Liess (2008)
Thiamethoxam	I	*Apis mellifera*	Insect	2.21	4.04	−0.45	0.95	0.208	3	18 days	Oliveira et al. (2013)

[a] Codes: 4-DAST = 4-dimethylaminostilbene; C = carcinogen; DENA = diethylnitrosamine; I = insecticide; OM = organometallic compound; R = rodenticide.
[b] Exposure followed by three weeks in uncontaminated media (see text).

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Table 4
Examples of chemicals which follow Haber's rule, i.e. $c{\cdot}t^n$ = constant, where n approximates 1 (value of n determined as in Table 3). Parameters of the regression Ln $t50 = a + b$ Ln c are shown.

Chemical	Type[a]	Species	Taxa	n	Intercept (a)	Slope (b)	r^2	P	No. c tested	Exposure time	Reference
4-DAB	C	*Rattus* sp.	Mammal	1.01	6.89	−0.99	1.00	<0.001	5	350 days	Druckrey (1943)
Azinphos-methyl	I	*Poecilia reticulata*	Fish	1.21	1.30	−0.83	0.93	<0.001	6	14 days	Legierse et al. (1999)
Benzaldehyde	CR	*Lepomis macrochirus*	Fish	1.23	4.61	−0.81	1.00	<0.001	4	96 h	Phipps and Holcombe (1985)
Carbaryl	I	*Aedes aegypti*	Aquatic insect (larvae)	1.02	8.69	−0.98	0.94	<0.001	6	24 h	Parsons and Surgeoner (1991)
Carbofuran	I	*Aedes aegypti*	Aquatic insect (larvae)	1.30	6.46	−0.77	0.97	<0.001	6	24 h	Parsons and Surgeoner (1991)
Fenitrothion	I	*Aedes aegypti*	Aquatic insect (larvae)	1.12	4.19	−0.89	1.00	<0.001	7	24 h	Parsons and Surgeoner (1991)
Fipronil	I	*Apis mellifera*	Insect	0.97	2.15	−1.03	1.00	–	2	264 h	Aliouane et al. (2009)
Fipronil sulfide	Im	*Folsomia candida*	Collembola	1.01	9.79	−0.99	0.99	0.085	3	96 h	San Miguel et al. (2008)
Methidathion	I	*Poecilia reticulata*	Fish	1.13	0.71	−0.89	0.96	<0.001	6	14 days	Legierse et al. (1999)
Permethrin	I	*Aedes aegypti*	Aquatic insect (larvae)	0.99	1.77	−1.01	0.96	<0.001	7	24 h	Parsons and Surgeoner (1991)
Phenthoate	I	*Poecilia reticulata*	Fish	1.30	0.78	−0.77	0.96	<0.001	6	14 days	Legierse et al. (1999)
Phosmet	I	*Poecilia reticulata*	Fish	1.04	2.24	−0.96	0.96	<0.001	6	14 days	Legierse et al. (1999)
Se	M	*Daphnia magna*	Cladoceran	1.03	6.12	−0.97	0.99	<0.001	5	21 days	Hoang et al. (2007)
Thiacloprid	I	*Gammarus pulex*	Amphipod	1.11	11.40	−0.90	0.99	<0.001	5	24 h[b]	Beketov and Liess (2008)

[a] Codes: 4-DAB = 4-dimethylaminobenzene; C = carcinogen; CR = chemical reagent; I = insecticide; Im = insecticide metabolite; M = metal/metalloid.
[b] Exposure followed by three weeks in uncontaminated media (see text).

Table 5
Examples of chemicals that follow the equation $c{\cdot}t^n$ = constant, where $n < 1$ (value of n determined as in Table 3). Parameters of the regression Ln $t50 = a + b$ Ln c are shown.

Chemical	Type[a]	Species	Taxa	n	Intercept (a)	Slope (b)	r^2	P	No. c tested	Exposure time	Reference
1,3-Dichloro-4,6-dinitrobenzene	CR	*Lepomis macrochirus*	Fish	0.11	−18.81	−8.92	0.96	0.019	4	96 h	Phipps and Holcombe (1985)
2-Chloroethane	CR	*Lepomis macrochirus*	Fish	0.55	10.01	−1.81	0.97	0.015	4	96 h	Phipps and Holcombe (1985)
2,4-Pentanedione	CR	*Lepomis macrochirus*	Fish	0.58	11.88	−1.74	0.95	0.027	4	96 h	Phipps and Holcombe (1985)
Carbaryl	I	*Lepomis macrochirus*	Fish	0.30	11.18	−3.35	0.87	0.069	4	96 h	Phipps and Holcombe (1985)
Cd	M	*Lepomis macrochirus*	Fish	0.28	11.21	−3.54	0.91	0.190	3	96 h	Phipps and Holcombe (1985)
$CdCl_2$	CR	*Daphnia magna*	Cladoceran	0.60	7.33	−1.66	0.98	<0.001	6	21 days	Kooijman (1981)
Chlorthion	I	*Poecilia reticulata*	Fish	0.81	4.26	−1.24	0.97	<0.001	6	14 days	Legierse et al. (1999)
Cu	M	*Daphnia magna*	Cladoceran	0.30	14.17	−3.36	0.89	0.015	5	21 days	Hoang et al. (2007)
Fenobucarb	I	*Hyale barbicornis*	Amphipod	0.48	7.39	−2.10	0.80	0.107	4	96 h	Añasco et al. (2010)
Hexachloroethane	CR	*Lepomis macrochirus*	Fish	0.51	4.54	−1.95	0.91	0.047	4	96 h	Phipps and Holcombe (1985)
Malathion	I	*Poecilia reticulata*	Fish	0.54	4.10	−1.85	0.90	<0.001	6	14 days	Legierse et al. (1999)
Malathion oxon	I	*Rana boylii*	Amphibian	0.58	9.80	−1.72	0.79	0.111	4	96 h	Sparling and Fellers (2007)
Pentachlorophenol	I	*Lepomis macrochirus*	Fish	0.25	−3.36	−3.95	0.97	0.014	4	96 h	Phipps and Holcombe (1985)
Zn	M	*Daphnia magna*	Cladoceran	0.47	13.43	−2.12	0.92	0.009	5	21 days	Hoang et al. (2007)
Zn	M	*Poecilia reticulata*	Fish	0.36	34.37	−2.74	0.80	0.107	4	32 h	Widianarko et al. (2001)
α-Br-2′,5′-dimethoxyacetophenone	CR	*Lepomis macrochirus*	Fish	0.39	−1.19	−2.57	0.86	0.071	4	96 h	Phipps and Holcombe (1985)

[a] Codes: C = carcinogen; CR = chemical reagent; I = insecticide; M = metal/metalloid.

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which mediate fast cholinergic synaptic transmission and play roles in many sensory and cognitive processes in invertebrates (Armengaud et al., 2002; Jones et al., 2006). The lower affinity of neonicotinoids for vertebrate's nAChRs has been attributed to the different ionic structure of the vertebrate subtypes (Tomizawa et al., 2000). Given that nAChRs are embedded in the membrane at the neuronal synapses, their replacement seems unlikely because neurons do not grow. Irreversible binding of the receptors should not be confused with lack of recovery; some neuronal or motor functions are recoverable after an initial shock even though the affected neurons remain damaged. Note that the time reinforcement factor (n) for lethality of imidacloprid varies in the range 1.5–5.8, whereas the factor for thiacloprid is only 1.5. A possible explanation is that the exposure pattern was different: imidacloprid exposures were constant during the time period, whereas thiacloprid data was obtained with *Sympetrum* nymphs exposed to the insecticide for 24 h, after which time the nymphs were placed in uncontaminated media for three weeks (Beketov and Liess, 2008).

American kestrels (*Falco sparverius*) orally dosed with diphacinone in gelatin capsules resulted in reinforced mortality over time, and produced values of $n = 1.6$ (Table 3). It should be noted that the kestrels received diphacinone as a divided dose administered 4 times in a 24-h period (considered an acute dose). A dead chick hatching was provided as food between administration of each of the capsules (Rattner et al., 2011). Like other anticoagulant rodenticides, diphacinone binds irreversibly to vitamin K epoxide reductase, impairing the carboxylation of the serine protease coagulation factors that result in haemorrhages and ultimately death (Wallin and Martin, 1987). Full recovery of the individuals that survive is dependent upon metabolism and excretion of the toxicant, and synthesis of new enzyme and clotting factors (Watt et al., 2005).

The developmental toxicity of the new dithiol insecticide cartap to the zebrafish (*Danio rerio*) appears to be reinforced by exposure time as well. The mortality of fish embryos exposed to increasing concentrations of cartap followed the Druckrey–Küpfmüller equation with a value of $n = 1.66$ (Table 3). Note the regression line does not have a good fit, as the lowest concentration (50 mg L^{-1}) resulted in mortality below 10% after 6 days of exposure. This only shows that more concentrations should have been tried to obtain an accurate value of n, but does not invalidate the observed reinforcement of mortality over time. Cartap is bioactivated by oxidation to the natural toxicant nereistoxin [4-(dimethylamino)-1,2-dithiolane]. Both cartap and nereistoxin are ion channel blockers of the nAChR, stopping cholinergic neuronal transmission (Lee et al., 2003).

Finally, the viability of rat cerebrocortical neurons exposed to methylmercury (MeHg) follows a pattern of toxicity that is reinforced with time of exposure ($n = 1.69$, Table 3). Formed by anaerobic microorganisms in aquatic sediments, MeHg is biomagnified through the food chain. As with inorganic mercury, MeHg also forms covalent bonds with sulfide groups in proteins, but it is more toxic than mercury because it penetrates the tissues and reaches the central nervous system (Walker, 2001). Its neurotoxicity symptoms include motor difficulties, sensory problems and mental retardation, an irreversible condition known as Minamata disease (Takeuchi, 1982).

2.2. Chemicals that follow Haber's rule

4-dimethylaminobenzene (4-DAB) was the first carcinogenic compound reported by Druckrey to comply with Haber's rule (Druckrey, 1943). The appearance of liver cancer in rats treated with 4-DAB was inversely proportional to the daily doses of this compound, and the product of the daily dosage and the median tumour induction time, i.e. the total dose, was found to be practically constant. This is indicated by a value of n close to 1 in Eq. (19) (Table 4).

Mortality of bluegill fish (*Lepomis macrochirus*) after exposure to benzaldehyde is slightly reinforced by time of exposure, but a value of $n = 1.2$ rather suggests this compound follows Haber's rule (Table 4). Benzaldehyde irreversibly inactivates the antioxidant enzyme glutathione peroxidase but has no effect on other antioxidant enzymes. Since this is the main enzyme responsible for the removal of hydrogen peroxide and organic hydroperoxides in brain, its inactivation by benzaldehyde produces neurotoxic effects (Tabatabaie and Floyd, 1996). It is likely that a fast regeneration of glutathione peroxidase counteracts to some extent those effects, which tend to approximate Haber's rule.

Toxicity of the carbamate insecticides carbaryl and carbofuran, the organophosphorus (OP) fenitrothion and the synthetic pyrethroid permethrin to mosquito larvae (*Aedes aegypti*) also appears to follow Haber's rule, at least during 24-h exposures. The OPs azinphos-methyl, methidathion, phenthoate and phosmet tested on guppies (*Poecilia reticulata*) during 14 days exposures follow the same rule (Table 4). While both carbamates and OPs are cholinesterase (AChE) inhibitors, the binding of carbamates to the enzyme is temporary whereas that of alkyl OPs is irreversible (Matsumura, 1985). In the case of irreversible receptor binding, recovery is possible only by novel synthesis of free AChE once the toxicant has been eliminated from the body. For carbofuran, a value of $n = 1.3$ may indicate that either carbofuran inhibition lasts longer than that of other carbamates or its systemic properties resulted in an uptake rate that prevented regeneration of the inhibited cholinesterase in the 24-h laboratory test (Parsons and Surgeoner, 1991). Whether this toxicity pattern applies for longer exposures is unknown. Permethrin is a type II synthetic pyrethroid that irreversibly destabilizes the voltage-dependent sodium channel, but can also enhance norepinephrine release at presynaptic nerve terminals (Clark and Brooks, 1989). The regeneration of the enzymes involved may result in eventual recovery of the individuals that survive a given dose (Coats et al., 1989).

The same toxicity pattern applies to the phenylpyrazole insecticide fipronil when honey bees ingest contaminated nectar, and to its sulfide metabolite when exposed to collembola. Both parent and metabolite are antagonists of the γ-aminobutyric acid (GABA)-gated chloride channel, binding irreversibly to this receptor (Cole et al., 1993).

Toxicity of the neonicotinoid insecticide thiacloprid to the freshwater amphipod *Gammarus pulex* appears to follow also Haber's rule ($n = 1.1$, Table 4), whilst the same compound reinforced lethality in dragonfly larvae under the same exposure regime (Table 3). In either case, the experimental evidence is not contrary to the irreversible nature of the nAChR binding.

Selenium's toxicity also follows Haber's rule (Table 4) even though selenium is known to be a component of the enzyme glutathione peroxidase and few other proteins (Hogberg and Alexander, 1986). This is probably a case where threshold concentration c_m and the minimum time of response t_m are so small as not to produce a measurable error, as inferred earlier (see Eq. (9)). Virtually the entire administered concentration will probably relate to selenium's toxicity in the test system, since the fraction which reacts with inert receptors that make no contribution to toxicity would be negligible by comparison.

2.3. Chemicals with toxicity predominantly dependent on concentrations

A number of chemicals follow the Druckrey–Küpfmüller equation (19) but with the exponent $n < 1$ (Table 5). In these cases, the toxicity is more dependent on a concentration gradient than on time. Plausible explanations could be that when the concentration of the toxicant at the site of action C is proportional to the exposure concentration c, a substantial fraction of the toxicant may react

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with inert receptors that make no contribution to toxicity, or the threshold concentration c_m and the minimum time of response t_m influence the toxicity profile. Also, some compounds may have non-specific receptor binding, only a fraction of which may be slowly reversible or even irreversible. Consequently, their effects depend primarily on the toxicant concentration and appear to diminish with exposure time. Narcotics may not fit here, as the Druckrey–Küpfmüller model requires slowly reversible or irreversible binding; further research is needed to elucidate this point.

This may, for example, be the case for mortality of *D. magna* induced by metals that are also essential trace elements, such as zinc or copper (Fig. 4 and Table 5). Copper proteins have diverse roles in biological electron transport and oxygen transportation, such as cytochrome-C oxidase. Copper is also found in many superoxide dismutases, proteins that detoxify superoxides (Mertz, 1981). This could explain why low copper concentrations are less poisonous than higher copper concentrations, because small amounts of the former would be either used to make biomolecules or would be involved in reactions with inert receptors; only the excess amounts would become poisonous. Apart from excretion of that excess, some animals have also developed biochemical pathways to eliminate it, such as sequestration by metallothionein (Steinebach and Wolterbeek, 1994; Fritsch et al., 2010). Similar reasoning may apply to the toxicity of zinc. Zinc is essential for the normal growth and reproduction in animals and plants. It is vital for the functionality of more than 300 enzymes, for the stabilization of DNA and gene expression, and plays an immune function role (Frassinetti et al., 2006).

Similarly, the toxicity of cadmium to bluegill fish (*L. macrochirus*) may be influenced by intracellular glutathione functions which protect against Cd^{2+} toxicity. This tripeptide provides a first line of defense against Cd^{2+} before induction of metallothionein synthesis occurs, which may quickly neutralize the potential impact of low intracellular Cd^{2+} concentrations (Singhal et al., 1987). Through these mechanisms, some mammals (e.g. shrews, voles) have developed great tolerance against cadmium (Marques et al., 2007), whereas some soil invertebrates are able to store metals such as Cd in special hepatopancreatic cells as inert granules (Morgan et al., 2002).

Bluegill fish exposed to the carbamate insecticide carbaryl for 4 days show a toxicity pattern where $n < 1$, and the same occurs with tadpoles of *Rana boylii* exposed 4 days to malathion oxon and guppies exposed to malathion or chlorthion for 14 days (Table 5). Although carbaryl and other OPs generally follow Haber's rule (see Table 4), it is conceivable that different TK/TD processes may determine toxicity in these organisms. Apart from the specific inhibition of AChE, a carbaryl metabolite is formed in liver microsomes by cytochrome P-450 mixed function oxidases, which has been shown to bind covalently to amino acid residues of microsomal proteins in vertebrates (Miller et al., 1979). The latter mechanism is likely to be influenced by intracellular defence mechanisms. The toxicity of fenobucarb, another carbamate with contact action, on the amphipod *Hyale barbicornis*, follows the same pattern over a 4-day exposure period (Table 5).

Unlike insecticides with a specific mode of action, pentachlorophenol is an all-purpose biocide that undergoes bioactivation to generate benzoquinone electrophiles that react covalently with biopolymers, not all of which may contribute to toxicity. These processes are also influenced by intracellular defence mechanisms that may be more effective at lower toxicant concentrations (van Ommen et al., 1986). This would explain why toxicity of pentachlorophenol to bluegill fish follows the Druckrey–Küpfmüller equation with $n = 0.25$ (Table 5).

In fact, all the chemicals listed in Table 5, which follow the Druckrey–Küpfmüller equation with values of $n < 1$, interact with several molecules in the organisms, not all of which contribute to toxicity or may only contribute to toxicity after a certain level of receptor binding. For example, 2,4-pentanedione binds iron in peroxidase and thus prevents the oxidation of human serum proteins, but also inactivates several enzymes through reactions involving arginine and lysine (Bingham et al., 2001). Hexachloroethane can be genotoxic by binding to nucleic acids in various organs (US-DHHS, 1997). It is metabolized by the mixed function oxidase system involving cytochrome P-450 and the resulting metabolites have free radicals that interact with many proteins in the cell (Lattanzi et al., 1988).

2.4. Risk assessment of time-dependent toxicants

The traditional approach to toxicity testing is to consider dose (concentration):effect relationships at arbitrarily fixed exposure durations, which are supposed to reflect 'acute' or 'chronic' time scales. This approach measures the proportion of all exposed individuals responding by the end of such exposure times. This is valid when toxicity is mainly dependent on exposure concentrations, but it is insufficient when toxic effects are reinforced by exposure time, because the impact of low exposure concentrations may be underestimated if the duration of the experiment is shorter than the latent period for toxicity. Toxicological databases established in this way are collections of endpoint values obtained at fixed times of exposure. As such these values cannot be linked to make predictions for the wide range of exposures encountered by humans or in the environment. By contrast, TTE approaches (Newman and McCloskey, 1996) provide more information on the exposure concentrations and times needed to produce toxic effects on tested organisms. Indeed, TTE bioassays differ from standard chronic toxicity tests in that TTE approaches record effects at consecutive times during the exposure, so the data form a matrix that can be analyzed to extract information about the effective concentrations (e.g. NEC, EC10, LC50, etc.) or about the time to effect for a given endpoint (e.g. $t50$). This is an essential requirement for risk assessment of chemicals showing time-dependent toxicity, particularly for those that have time-cumulative toxicity, as it allows prediction of toxic effects for any combination of concentration and time found in the environment.

An improper understanding of the mechanisms of toxicity with time can be found in the current regulatory framework for honey bees (*A. mellifera*). The European Food Safety Authority (EFSA) has recommended the inclusion of chronic toxicity tests in pesticide risk assessment of honey bees, thereby expanding current oral and contact acute toxicity data for 24 and 48 h (EPPO guidelines 170 and OECD 213 and 214). Mortality is recorded daily during at least 48 h and compared with control values. If the mortality rate is increasing between 24 and 48 h whilst control mortality remains at an accepted level (i.e. $\leq$10%) the duration of the test is extended to a maximum of 96 h. The results are used to calculate the LD50 at 24 h and 48 h and, in case the study is prolonged, at 72 h and 96 h as well. EFSA proposes to use mortality data and a mathematical model based on Haber's rule to detect repeated dose-effects. This approach is bound to fail because it does not take into account that toxic effects may be reinforced by exposure time, as indicated for imidacloprid in Table 3. EFSA data are of little use for prediction of toxic effects for any combination of concentration and time because it would contain no more than four $t50$ values – one for each day.

What would be required is information on the exposure concentrations and exposure times needed to kill bees. Mortality should be determined under continuous exposure conditions to a range of concentrations monitored at defined time intervals (say after 1, 2, 3, 7 and 14 days of exposure), especially in the case of oral exposure. In that way, not only LC50 values can be established for each of these time points but also the $t50$s can be estimated by regression

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Table 6
Risk assessment for the neonicotinoid insecticide imidacloprid to honey bees (*Apis mellifera*). Predicted times to 50% mortality ($t50$) of workers by ingesting nectar or pollen contaminated with imidacloprid, after taking into account that 11% of plants are contaminated (Chauzat et al., 2011). By contrast, standard hazard quotients (HQ) for dietary NOEL of 20 $\mu g\,L^{-1}$ (Blacquière et al., 2012) suggest that imidacloprid poses no danger to honey bees.

Residues	Imidacloprid (PEC) ($\mu g\,L^{-1}$ or kg^{-1})	c = PEC × frequency ($\mu g\,L^{-1}$ or kg^{-1})	Predicted $t50$[a]		HQ = PEC/NOEL
			(h)	(days)	
Nectar	1	0.11	263	11.0	0.05
	3	0.33	218	9.1	0.15
Pollen	0.7	0.08	280	11.7	0.04
	10	1.1	177	7.4	0.50

[a] Based on Ln $t50$ (h) = 5.19–0.17 Ln c ($\mu g\,L^{-1}$ or kg^{-1}) from Table 3.

analysis. Once this information is obtained, the risk assessment should consider the pesticides residues found in pollen, and the frequency of such residues in the environment, in order to estimate the time to 50% mortality ($t50$) using equation 19. An example for the case of imidacloprid is given in Table 6.

Since imidacloprid and other neonicotinoid insecticides have time-cumulative effects on arthropods (Tennekes and Sánchez-Bayo, 2012), the risk of foraging worker bees feeding on tiny levels of residues becomes an issue that cannot and should not be ignored. In the example shown here, 50% of worker bees would die within 7–12 days if feeding on a field where 11% of plants have residues of imidacloprid in the specified range (Table 6). By contrast, standard hazard quotients (HQ) for dietary NOEL of 20 $\mu g\,L^{-1}$ (Blacquière et al., 2012) are misleading because they suggest that imidacloprid poses no danger to honey bees. Given that honey bee workers can live up to a few months in winter time the NEC for imidacloprid is close to zero, which means that any residue concentration found in pollen will have a lethal effect provided there is sufficient time of exposure. Recommendations of this kind have been suggested before (Halm et al., 2006; Mommaerts et al., 2010; Alix and Vergnet, 2007), but their implementation has not happened yet.

The same type of assessment should be applied to estimate the risk of neonicotinoids and fipronil to all other terrestrial and aquatic arthropods. Apart from having time-cumulative effects over time, these compounds are persistent in the environment (Gunasekara et al., 2007) (Footprint database http://sitem.herts.ac.uk/aeru/iupac/). Cumulative effects of fipronil on rice mesocosms during two consecutive years have been observed (Hayasaka et al., 2012b), while aquatic arthropods as diverse as ostracods, mayflies, dragonflies and aquatic beetles are eliminated during the rice growing season after a single application of imidacloprid (Hayasaka et al., 2012c; Sánchez-Bayo and Goka, 2006b). This suggest the integrity and functionality of the aquatic ecosystems affected by these insecticides would be lost if these systemic insecticides are applied year after year.

3. Conclusions

The interaction of a toxicant with the specific receptors that lead up to an effect is essential to understand the mechanisms of toxicity. Toxicokinetic and toxicodynamic models must be based on a molecular approach that considers the mechanisms of action of chemicals. Only then they will be able to explain the time-dependent effects observed in toxicity testing, and predict environmental impacts with reasonable accuracy.

The model of Druckrey and Küpfmüller explained in this paper complies with that requirement. In fact, their concentration–effect relationship with time (Eq. (19)) has been validated by a diverse array of empirical data, using chemicals with very different modes of action and organisms, both aquatic and terrestrial (Tables 3–5). This universal model also serves as a screening tool to identify toxicants that show time-dependent and time-cumulative toxicity. In addition to the laboratory evidence, field observations on the impacts that neonicotinoid and phenylpyrazole insecticides have on arthropods also confirm the validity of the time-cumulative mechanisms explained here.

Finally, the implications for risk assessment are obvious: while most toxicants with a generic mode of action can be evaluated by the traditional concentration–effect approaches, a certain number of chemicals, including carcinogens, methylmercury, rodenticides, neonicotinoids and cartap insecticides have toxic effects that are reinforced with time of exposure, i.e. time-cumulative effects. Therefore, the traditional risk approach cannot predict the impacts of the latter chemicals in the environment. A new risk assessment, as proposed here, is needed to evaluate the effects that such time-dependent chemicals have on humans and the environment.

Conflict of interests

The authors declare no conflict of interests.

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