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Time-Dependent Toxicity of Neonicotinoids and Other Toxicants: Implications for a New Approach to Risk Assessment

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Introduction

A fundamental goal of toxicology is to determine safe levels of exposure to potentially poisonous substances for humans and the environment [1]. Traditionally, safe levels have been estimated in laboratory toxicity bioassays by calculating the non-observable effect level (NOEL) of a chemical to a variety of organisms which are representative of certain taxa, i.e. mammals, birds, fish, crustaceans, algae, etc. There are, however, fundamental problems with the validity of this approach, both conceptual and statistical in nature, as indicated by Landis and Chapman [2] and other authors [3]. Thus, the outdated NOEL concept is being replaced by the no-effect concentration (NEC) level [4], which assumes that toxic chemicals do not have any effect on a population of organisms at very low concentrations.

Druckrey and Küpfmüller [5] reasoned that the following processes determine the complex relationship between exposure levels to poisons and a toxic effect:

1. **Absorption, distribution, metabolism and excretion**, which determine the relationship between dose and the concentration of the poison at the site of action and its time course in various parts of the body, commonly referred to as pharmacokinetics. A linear relationship between dose and effect would require proportionality between dose (or concentration in a medium) and the concentration of the poison at the site of action. This may be the case for many poisons, for as long as the poison does not influence pharmacokinetics within the considered dose range;
2. **The interaction of the poison with critical receptors,** which determines the relationship between the concentration of the poison at the site of action and receptor binding. A linear relationship between dose and effect would require proportionality between the concentration of the poison at the site of action and the concentration of bound receptors. This is only the case, as will be shown in section 2, when the proportion of bound receptors sufficient for an effect is relatively small compared to the initial level of the receptor; and
3. **Receptor binding**, which determines the relationship between the concentration of bound receptors and the actual biological effect. A linear relationship between dose and effect would require proportionality between relative receptor binding and the actual biological effect.

The pharmacokinetics of most pesticides is well known after having been the subject of intense research in recent decades. For example, the kinetics of acetylcholinesterase inhibitors such as carbamate and organophosphorus insecticides was described long ago, and can be found in most toxicology textbooks (e.g. [6]). The biochemical interactions between these insecticides and their target enzyme (acetylcholinesterase), together with the kinetics of uptake, form the basis of mechanistic models of toxicity such as DEBtox [7]. In a similar way, the kinetics of narcotic chemicals has been described by models which consider the time course in their uptake and bioconcentration by fish [8].

In this review we will focus on the relationship between the concentration of the poison at the site of action and the actual biological effect, which Druckrey has referred to as *ergokinetics* (Druckrey, personal communication with Tennekes, 1985). The reason for this is that recent developments in ecotoxicology suggest that some toxicants can produce effects at any concentration level provided their exposure time is sufficiently long [9]. This means that 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. Consequently, risk assessment of these chemicals, which includes neonicotinoid insecticides and certain metallic compounds, may require entirely new approaches.

Conceptual model for toxicant effects with time

In their book *Dosis und Wirkung* (dose and response) [5], Druckrey and Küpfmüller analyzed ergokinetics in mathematical terms, and their reasoning leads to fundamentally important conclusions on the effects caused by interaction of poisons with critical receptors, which are highly relevant for toxicological risk assessment.

Denoting the initial concentration of critical receptors that a poison reacts with as R_0, the concentration of receptors that a poison has reacted with as C_R, and the poison concentration at the site of action as C, the velocity of receptor binding (association) is:

$$K\,C\,(R_0 - C_R) \quad (1)$$

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

$$C_R / T_R \quad (2)$$

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

$$dC_R/dt = K\,C\,(R_0 - C_R) - C_R / T_R \quad (3)$$

If the effect occurs under circumstances where $C_R \ll R_0$, i.e., with first order kinetics, then R remains practically constant, in which case

$$K\,(R_0 - C_R) = 1 / T_A \quad (4)$$

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where T_A can be regarded as the time constant for association. Equation (3) then simplifies to

$$dC_R/dt = C / T_A - C_R / T_R \quad (5)$$

In equilibrium, where $dC_R/dt = 0$, Equation (5) simplifies to

$$C_R = C (T_R / T_A) \quad (6)$$

Replacing the concentration of bound receptors C_R by the relative concentration of bound receptors C_R/R_0, we obtain

$$C_R / R_0 = (C / R_0) . (T_R / T_A) \quad (7)$$

This expression indicates that, with first order kinetics, i.e. when $C_R \ll R_0$, the proportion of bound receptors is dependent on the concentration of poison in the body of the organism (exposure dose) and the relative time for association and dissociation (exposure duration). Based on this reasoning, Druckrey and Küpfmüller drew the following conclusions for all poisons that interact with specific receptors in a first order bimolecular reaction where the toxic effect is determined by the relative concentration of bound receptors C_R/R_0:

- The effect is proportional to the concentration of the poison at the site of action C (Paracelsus).
- The effect is inversely proportional to R_0. If the concentration of specific receptors R_0 is low, the poison may induce pronounced toxicity at very low concentrations at the site of action C.
- The effect is proportional to T_R/T_A, i.e., to the quotient of the time constant for dissociation T_R and the time constant for association T_A.
- If T_R/T_A is high, the poison may induce pronounced toxicity at very low concentrations at the site of action C.
- If $T_R/T_A < 1$, a compound is innocuous and can only induce toxicity at relatively high concentrations at the site of action C.
- If both time constants (T_R and T_A) are low, i.e. when both association and dissociation are fast processes, equilibrium between C and receptor binding (and effect) will be established quickly but the toxic effect will also regress quickly.
- The character of a poison is primarily determined by T_R, i.e., by the time constant for dissociation of bound receptors.
- If T_R is low, i.e. when receptor binding is quickly reversible, the time course of the effect will be the same as the time course of C and the maximum effect will occur when C is at its maximum. Under these circumstances, there will be a linear relationship between effect and the actual concentration of the poison at the site of action C, and between dose and effect, provided C is proportional to dose (or concentration in a medium). Such poisons can therefore be termed "*concentration poisons*". The value of T_A will only determine the fraction of the poison that reacts with the specific receptors R.
- 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 is T_R is, the longer is the time to maximum effect. Upon repeated exposure in quick succession there will be cumulative effects. Such poisons can therefore be termed "*cumulative poisons*". With cumulative poisons, a quantitative or even linear relationship between effect and the actual concentration of the poison at the site of action C , as seen with concentration poisons, will not exist. Because equilibrium between C and receptor binding (and effect) will be established very slowly, toxicity becomes a process that takes place in time. There will be a latency period up to a defined effect, which can be shortened, of course, by increasing the concentration of the poison at the site of action C.

Thus, according to Druckrey and Küpfmüller, depending on the time constant for dissociation T_R, the toxicity of a compound may become a process that takes place in time. 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 specified exposure times. 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. Thus, current toxicological risk assessment can be compromised by this approach to toxicity testing, as will be demonstrated in this paper, leading to serious underestimates of actual risk.

Time effects of toxicants

In order to overcome this handicap, an increasing number of researchers are using a variant of the traditional toxicity testing protocol which includes time to event (TTE) methods. This TTE approach measures the times to respond for all individuals, and provides information on the acquired doses as well as the exposure times needed for a toxic compound to produce any level of effect on the organisms tested [10]. Consequently, extrapolations and predictions of toxic effects for any combination of concentration and time are now made possible [11]. We will demonstrate that this approach is superior to current toxicological testing procedures, and has important implications for risk assessment of chemicals.

Following the conceptual model by Druckrey and Küpfmüller, if receptor binding is virtually irreversible, then $T_R \rightarrow \infty$ and Eq. (3) reduces to

$$dC_R/dt = K C (R_0 - C_R) \quad (8)$$

If the toxic effect occurs when $C_R \ll R_0$, then

$$dC_R / dt = K R_0 C \quad (9)$$

If a dose level is kept constant throughout a study, and, as a result, C remains constant as well, integration yields

$$C_R = K R_0 C t \quad (10)$$

This reasoning by Druckrey and Küpfmüller provided a theoretical explanation for Haber's rule [12]. Haber's rule (for a review, see [13]) states that the product of exposure concentration and exposure duration produces a constant toxic effect. Haber had noted that exposure to a low concentration of a poisonous gas for a long time often had the same effect (death) as exposure to a high concentration for a short time. The results of Druckrey's ground-breaking study on the carcinogenicity of 4-dimethylaminoazobenzene (4-DAB) in BDIII rats [14] were consistent with Haber's rule: doubling the daily 4-DAB dose, and thereby presumably doubling the concentration of the carcinogen at the site of action, halved the time up to the appearance of liver cancer.

In the 1960s the molecular biology of this carcinogenic effect was elucidated and confirmed the theorem of Druckrey and Küpfmüller. DNA was recognized by Brookes and Lawley [15] as the target for chemical carcinogens, as recently inferred by Wunderlich [16], and

Warwick and Roberts confirmed irreversible receptor binding by demonstrating covalent binding of a 4-DAB metabolite to DNA [17].

It is now apparent that Haber's rule is highly relevant for ecotoxicological risk assessment as well. It was recently shown to describe the toxicity of the neonicotinoid insecticide imidacloprid to midges *Chironomus tentans* [18]. The product of exposure concentration and exposure duration to 50% mortality (t50) for *C. tentans* was very similar under acute and chronic exposure conditions. These observations have also confirmed the theorem of Druckrey and Küpfmüller and Haber's rule. Abbink certified in 1991, when the compound was first introduced, that "imidacloprid is the first highly effective insecticide whose mode of action has been found to derive from almost complete and virtually irreversible blockage of postsynaptic nicotinic acetylcholine receptors (nAChRs) in the central nervous system of insects" [19]. Imidacloprid mimics the action of acetylcholine, but unlike acetylcholine, imidacloprid is not deactivated by acetylcholinesterase and thus persistently activates nAChRs [20]. Chronic exposure of insects to imidacloprid therefore leads to cumulative and virtually irreversible blockage of nAChRs in their central nervous system, which play roles in many cognitive processes.

Exposure time reinforcing the toxic effects

Although equation (10) provided a theoretical explanation for Haber's rule, it assumed proportionality between the concentration of bound receptors C_R and the *effect*. This may not always be the case. As mentioned earlier, Druckrey and Küpfmüller had pointed out that the relationship between the poison concentration at the site of action C and the effect E involves at least 2 steps: the first is the interaction of the poison with critical receptors leading to receptor binding, the second is the subsequent biological effect resulting from receptor binding. As we have seen earlier, if receptor binding is virtually irreversible (i.e. $T_R \rightarrow \infty$), the concentration of bound receptors C_R is proportional to the integral of C over time:

$$C_R \sim \int C\, dt \quad (11)$$

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

$$E \sim \int C_R\, dt \quad (12)$$

In cases of irreversible receptor binding and an irreversible effect, the effect E is thus proportional to the double integral of the poison concentration at the site of action C over time, as the combination of equation (11) and (12) shows:

$$E \sim \int \int C\, dt \quad (13)$$

The implication is that exposure time will reinforce the effect. Reinforcement of an effect by exposure time was subsequently demonstrated by Druckrey and co-workers with benchmark studies of the production of ear duct and liver carcinomas by 4-dimethylaminostilbene (4-DAST) [21] and diethylnitrosamine (DENA) [22], respectively, in BDII rats (Table 1).

The total carcinogenic dose *decreased* with decreasing daily 4-DAST or DENA dose levels, even though the median tumor induction times *increased* with decreasing daily dose levels. In a logarithmic system of coordinates, there was a linear relationship between the median tumor induction time (t50) and the daily dosage (d):

$$\ln d = \ln k - n \ln t50 \quad (14)$$

or

$$d\, t50^{\,n} = \text{constant} \quad (15)$$

where the time exponent *n* was 3.0 and 2.3 for 4-DAST and DENA, respectively.

The essence of equation (15) is that the total dose required to produce the same effect decreases with decreasing exposure levels, even though the exposure times required to produce the same effect increase with decreasing exposure levels. So, it should be possible, in principle, to induce cancer with a single dose of a chemical carcinogen. In fact, this was achieved by Druckrey *et al* [23] in single dose experiments with the direct-acting ethylating nitrosamide N-nitroso-N-ethylurea (ENU) in BD IX rats.This carcinogen is rapidly lost from the blood after intravenous injection; it has an *in vivo* half-life of 5-6 minutes.

Upon single dose treatment in early life (1, 10 or 30 days after birth), the overwhelming majority of animals died from malignancies of the nervous system [23]. The quantitative relationship (Figure 1) between (post-conception) age at treatment (a), dose (d), and the median induction period of neurogenic malignancies (t50) can be described as follows [24]:

$$a / t50 = K\, d^{\,r} \quad (16)$$

Chemical	Daily dose, d (mg kg^{-1})	Median tumor induction time, t (days)	Total dose, D (mg kg^{-1})	n
4-DAST	3.4	250	850	3.0
	2.0	340	680	
	1.0	407	407	
	0.5	550	275	
	0.28	607	170	
	0.2	700	140	
	0.1	900	90	
DENA	9.6	101	963	2.3
	4.8	137	660	
	2.4	192	460	
	1.2	238	285	
	0.6	355	213	
	0.3	457	137	
	0.15	609	91	
	0.075	840	64	

Table 1: Induction of ear-duct carcinomas in BDII rats by 4-DAST and of liver carcinomas by DENA, after Druckrey and Dischler [21], and Druckrey et al. [22].

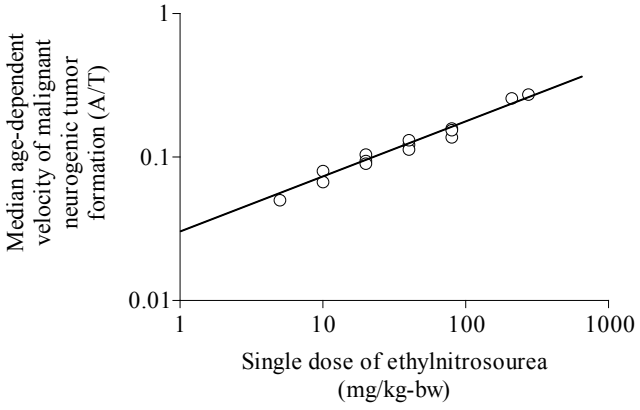

Figure 1: Median age-dependent velocity of malignant neurogenic tumor formation (A/T) in BD IX rats versus single dose levels of ethylnitrosourea, on logarithmic coordinates. Linearity leads to equation (17).

with K = constant , where r = 0.426

or

$$d\,(t50 / a)^{n} = \text{constant} \quad (17)$$

where n = 1 / r = 2.35.

Equation (17) indicates that, in this case, the *velocity* of carcinogenesis is determined by *the initiating dose* and *the state of the relevant targets at treatment*. This is reminiscent of Wilder's law of initial value, which states that the direction of response of a body function to any agent depends to a large degree on the initial level of that function. Target cells for ENU in early life are subependymal cells in the brain and subpial cells in the spinal cord, which are destined to differentiate into glial cells, i.e., astrocytes or oligodendrocytes. The evidence suggests that the critical genetic changes induced by the carcinogen in these target cells lead to inhibited normal cell differentiation processes, as has been observed in leukemogenesis [25,26]. Yuspa and Morgan reported that cells resistant to terminal differentiation can be readily isolated from skin of BALB/c mice exposed to an initiating dose of carcinogen *in vivo* but not from control mouse skin [27].

Applications of the Druckrey-Küpfmüller model

Genotoxic carcinogens

The Druckrey-Küpfmüller equation (15) can serve as a basis for risk assessment of carcinogens. Carlborg pointed out that this equation is implied by a Weibull model for dose-response functions in carcinogenesis [28]. The simple form of the Weibull model is a sigmoid curve defined by four parameters:

$$P = 1 - e^{\exp}, \text{ with the exponent} = -(\alpha + \beta\, x^{m}) \quad (18)$$

where x is the dose, P is the tumor rate and m, α, β are parameters to be estimated from the data. The parameter α is determined by the background tumor probability, β is a scale parameter related to the units measuring the dose, and m is the important shape parameter. At very low doses the excess risk over background is approximately $\beta\, x^{m}$. The virtual safe dose (VSD) corresponding to a one-in-a-million risk over background is then given by

$$VSD = (10^{-6} / \beta)^{1/m} \quad (19)$$

The extended form of the Weibull model includes the time to a tumor (t):

$$P = 1 - e^{\exp}, \text{ with the exponent} = -(\alpha + \beta\, x^{m})\, t^{k} \quad (20)$$

where k is a new parameter. Now suppose that t measures the time to a tumor. Also, suppose that the background tumor rate is zero (α = 0). For a test group at some dose x, consider the median time to tumor t50 – that is, the value of t such that P = 0.50. The extended Weibull model for this dose and time is

$$0.50 = 1 - e^{\exp}, \text{ with the exponent} = -\beta\, x^{m}\, t50^{k} \quad (21)$$

This reduces to

$$[-\ln 0.50 / \beta]^{1/m} = x\, t50^{k/m} = x\, t50^{n} \quad (22)$$

where n = k / m and the left side of the equation is a constant. This is the Druckrey-Küpfmüller equation (15). The exponent n is given by the slope of the regression between the logarithms of the dose and that of the median time to tumor.

Neonicotinoid insecticides

Similar relationships have been demonstrated for the toxicity of neonicotinoid insecticides to aquatic invertebrates, in particular imidacloprid to the freshwater ostracod *Cypridopsis vidua* and to *Daphnia magna* and thiacloprid to *Gammarus* and *Sympetrum*. Sánchez-Bayo [29] demonstrated that the relationship between the concentration of the neonicotinoid insecticides imidacloprid and thiacloprid in a medium (C) and the time to 50% mortality (t50) of several species of exposed arthropods followed a hyperbolic curve described by the equation

$$t50 = a^{*}C^{-b} \quad (23)$$

Accordingly, there was a linear relationship when the logarithms of the variables C and t were used

$$\ln t50 = a' - b \ln C \quad (24)$$

where a' is the intercept and b is the slope of the regression. Equation (24) can be transformed to

$$C^{b}\, t50 = \text{constant} \quad (25)$$

or

$$C\, t50^{1/b} = \text{constant} \quad (26)$$

Equation (26) is very similar to the Druckrey-Küpfmüller equation (15) for the action of chemical carcinogens such as DENA or 4-DAST.

Similar to the dose-response characteristics of DENA, exposure time was found to reinforce the toxicity of imidacloprid and thiacloprid to the tested arthropod species – Beketov and Liess [30] have referred to this feature of the neonicotinoids as having "delayed effects". The C t50 product, which reflects the total dose required for a lethal effect, decreased with decreasing toxicant concentration C (Table 2), even though the times to 50% mortality t50 *increased* with decreasing toxicant concentration C.

This toxic behaviour of neonicotinoid insecticides has also been pointed out by other researchers. For example, Suchail et al. [31] noted that at concentrations of 0.1, 1, and 10 μg of imidacloprid per liter, the total cumulate dose ingested by honeybees in chronic intoxication was about 60 to 6,000 times lower than the doses needed to produce the same effect in acute intoxication tests. The same effect was also apparent with two of the metabolites of imidacloprid (5-hydroxyimidacloprid and olefin). Although the authors could not explain the huge discrepancies between the results from acute toxicity tests of imidacloprid and its feeding effects in honeybees, their observations are consistent with the time-dose model described here. Consequently, a correct understanding of the ergokinetics of imidacloprid is essential for its risk assessment in relation to bees. In addition, at sub-lethal doses imidacloprid can alter honey bee foraging and learning [32-35]. Imidacloprid has been detected at levels of 5.7 μg kg^{-1} in pollen from French hives [36] and foraging honey bees reduced their visits to a syrup feeder when it was contaminated with 3 μg kg^{-1} of imidacloprid [37]. Foraging as well as hive worker bees and brood are likely to be continuously exposed to imidacloprid when contaminated food is collected and stored inside the hive [38]. A honey bee during a foraging flight must learn and recall many complex visual patterns [39,40]. These cognitive functions may be perturbed when nAChRs, necessary for the formation of long-term memory and involved in acquisition and retrieval processes, are persistently blocked [41]. These observations are consistent with the theorem of Druckrey and Küpfmüller [5]. Both receptor binding and the effect of receptor binding are virtually irreversible, and exposure time will therefore reinforce the effect, which may in the course of time be detrimental to the bee colony, and ultimately cause colony collapse.

Species	Chemical	Concentration (C) in µg L^{-1}	Time to 50% mortality (T50) in days	C x T50 product in µg L^{-1}.days	n
Cypridopsis vidua	Imidacloprid	4	5.2	20.8	4.67
		16	3.0	48	
		64	3.3	211.2	
		250	2.3	575	
		1,000	2.0	2,000	
		4,000	0.9	3,600	
Daphnia magna	Imidacloprid	250	384.7	96,175	1.35
		750	69.7	52,275	
		2,220	18.6	41,292	
		6,700	15.0	100,500	
		20,000	18.4	368,000	
		60,000	3.0	180,000	
Gammarus pulex	Thiacloprid*	99	63.6	6,296.4	1.11
		364	16.7	6,078.8	
		988	6.5	6,422	
		3,100	3.2	9,920	
		9,520	0.9	8,568	
Sympetrum striolatum	Thiacloprid*	7.2	20.6	148.3	1.53
		8.0	17.2	137.6	
		12.7	13.0	165.1	
		113.3	3.2	362.6	

*Original data from Beketov and Liess [30]

Table 2: Mortality of Arthropods Induced by Neonicotinoid Insecticides (After Sánchez -Bayo [29]). Data fit the equation C $T50^n$ = constant.

Thus, low environmental concentrations of these insecticides (that may not be acutely toxic) could be detrimental to many invertebrate species in the medium to long term, in particular to aquatic invertebrates because these compounds are quite soluble in water and their toxicity may be reinforced by exposure time. The results of recent studies are consistent with this concept. Thus, imidacloprid applied to rice mesocosms eliminated all zooplankton species for at least two months when concentrations were above 1 µg L^{-1} [42], which is 500 to 75,000 times lower than its acute LC50s for ostracods and cladocerans. By contrast, the insecticide fipronil applied to the same mesocosms produced only small, non-significant adverse effects on zooplankton, even if this insecticide is 100–1000 times more toxic to those organisms than imidacloprid and more persistent in soil [43]. Mayflies of the genera *Baetis* and *Epeorus* showed a reduction in reproductive success when exposed to concentrations of imidacloprid as low as 100 ng L^{-1} [44]; whether this is the result of a time-exposure effect or a sublethal effect is not clear. A single pulse contamination of mesocosms with the neonicotinoid insecticide thiacloprid resulted in long-term alteration of the overall invertebrate community structure because some species did not recover [45]. One species, the stonefly *Nemoura cinerea*, was affected at the lowest tested concentration, 70 times below the lowest known LC50. Imidacloprid can be applied as a systemic insecticide to trees by direct stem injections or by soil injections and drenches, in which case it may be indirectly introduced to aquatic systems via leaf fall or leaching: at realistic concentrations in leaves (18-30 µg g^{-1} fresh weight) imidacloprid can inhibit leaf litter breakdown through adverse effects on decomposer invertebrates [46], but it has no effect on microbial decomposers.

Terrestrial ecosystems have also been reported as negatively affected by imidacloprid. A study conducted over 3 years on an experimental home lawn [47] revealed that three consecutive years of imidacloprid applications to the same field plots suppressed the numbers of total hexapods, *Collembola*, *Thysanoptera* and *Coleoptera* adults by 54-62%. However, applications of imidacloprid to an experimental vegetable patch had only a temporary effect on the arthropod communities of the eggplant crop [48], perhaps because immigration of insects from nearby areas compensated the losses caused by the insecticide. When imidacloprid is applied as a systemic insecticide to the soil around trees it may cause sublethal effects on earthworms if concentrations in the litter reach or exceed 3 mg kg^{-1} [49,50]. A recent study indicates high toxicity of imidacloprid to the non-target terrestrial arthropod *Porcellio scaber*, at similar levels as the organophophorus diazinon [51]. However, because the blocking of AChRs by diazinon is temporary (it may last only a few hours), its toxic effect would last as long as it is present in soil at sufficient concentrations, whereas imidacloprid effects could last much longer if the nAChRs are permanently blocked.

Other chemicals

Time-dependent effects have also been observed with toxic metals. Sánchez-Bayo [29] analysed the data provided by other authors and concluded that the toxicity of copper, zinc, selenium and cadmium (as $CdCl_2$) to *Daphnia magna* and that of zinc to the guppy (*Poecilia reticulata*) followed the same pattern described by equation (26). The median times to 50% mortality (t50) decreased with increasing concentrations of metals, as seen with carcinogens and neonicotinoids, suggesting that for all metals the time constant for dissociation from the critical receptor $\mathbf{T_R}$ is high. However, with the exception of selenium, and in complete contrast to the action of carcinogens and neonicotinoids, the total metal dose taken up by and lethal to these aquatic organisms also *decreased* with increasing concentrations of metals, as shown here for *Daphnia magna* (Table 3). Selenium's toxicity followed Haber's Rule, with n=1, and can safely be assumed to bind irreversibly to the critical receptor, but the n-values characterizing the toxicity of copper, zinc, and cadmium were 0.3, 0.47, and 0.60, respectively. So, whereas with carcinogens and neonicotinoids, lower concentrations are more effectively poisonous than higher concentrations, it turns out that lower metal concentrations are less effectively poisonous than higher metal concentrations. This could reflect some elimination process which minimizes metal toxicity with time of exposure.

Chemical	Concentration (C) in µg L^{-1}	Time to 50% mortality (T50) in days	C x T50 product in µg L^{-1}.days	n
Copper*	28	32.6	912	0.30
	32	6.5	208	
	48	3.2	154	
	56	2.0	112	
	64	1.3	80	
$CdCl_2$**	3.2	292	935	0.60
	5.6	58	325	
	10	38	375	
	18	11	203	
	32	6	181	
	56	2	105	
Selenium*	158	3.3	514	1.03
	800	0.7	567	
	1200	0.5	600	
	1600	0.4	600	
	2000	0.3	500	
Zinc*	59	163	9617	0.47
	125	32	4000	
	250	2	490	
	500	1.3	667	

*Original data from Hoang et al. [64]
**Original data from Kooijman [65]

Table 3: Mortality of *Daphnia magna* induced by metallic elements or compounds (After Sánchez -Bayo [29]). Data also fit the equation C $T50^n$ = constant.

Metal toxicity is particularly insidious, as it affects not only aquatic but also most terrestrial organisms through the food chain. While more information is necessary to prove that other metallic and metalloid elements (e.g arsenic) produce time-dependent toxic effects, the evidence so far obtained from toxicity experiments carried out with fish (e.g. [52-54]) illustrates a toxicity pattern similar to that observed with neonicotinoid insecticides. This is likely to result from metal accumulation in the body, which increases with time until the organisms reach a sufficient dose to cause a toxic effect (see review in [55]). At least we know this is the case with mercury contamination in humans, which leads to the condition known as Minamata disease [56].

It is apparent that any chemical that permanently binds to a receptor in the body to produce a toxic effect may have time-dependent effects whenever the bound receptors remain in the body of the organism. The examples shown here for carcinogenic substances that bind to DNA, the neonicotinoid insecticides that deactivate the nAChRs and the metallic elements or compounds, like $CdCl_2$, that accumulate in the organisms' tissues, suggest this is probably the case. This suggests that many other substances may behave in the same way, even though we may be unaware of their ergokinetics.

So far we have discussed only toxicants that affect animals, but in principle time-dependent toxicity could also apply to the mode of action of herbicides in plants and algae. For example, if recalcitrant herbicidal compounds (e.g. PSII inhibitors) can block permanently the photosynthetic pathway in the chloroplasts, it is likely they may also show a time-dependent toxic effect in algae and higher plants. This would have serious implications for the risk assessment of herbicides in agricultural crops and coral reefs, among others. Research in this area of ecotoxicology is still lacking, and therefore we can only speculate about it.

Implications for risk assessment

Although time-to-event models have been considered in recent years for inclusion in risk assessments of environmental contaminants in areas as diverse as agriculture [57], occupational health [58], engineering [59] and ecology [60], the implications of the time-dependent toxicity of some chemicals have not been realised yet. First of all we need to know what kind of toxicants behave in a time-dependent manner, which is the same as asking which chemicals bind irreversibly to specific receptors. So far, the evidence pointed out here indicates that neonicotinoids, some carcinogens and metal/metalloids fit such description but other chemical groups may also follow this pattern.

Secondly, if a toxicant has time-dependent effects, the standard risk assessment procedures would not be valid in situations where there is exposure to sublethal concentrations of the toxicant for long periods of time. Traditionally this type of exposure has been considered as chronic toxicity, and its relationship with standard acute toxicity endpoints (e.g. LC50) has been studied in many aquatic species [61] ever since Kenaga introduced a ratio to describe it [62]. However, this traditional approach ignores the underlying mechanism of toxicity with time that has been described in this review.

There is no doubt that once ecotoxicologists realise the full potential and advantages of time-to-event approaches, they will become the standard tool "for analysis of toxicity from pulse exposures, and the latent toxic effects emerging after exposure has ceased, because both of these phenomena are time related" [63]. We already pointed here the experimental work by Beketov and Liess [30] in this regard, and only hope that more researchers may follow the same pathway.

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