Dose: Time-to-effect analyses can identify hazardous chemicals at an early stage of product development.

Henk A. Tennekes*

Experimental Toxicology Services (ETS) Nederland BV, Frankensteeg 4, 7201KN Zutphen, The Netherlands

Abstract

Expenditure for discovery and development of a new crop protection product is now approaching the $ 300 million mark, while at the same time underpinning information gaps in environmental safety assessment. Large information gaps also exist for the safety of a vast number of existing chemicals in commerce. Addressing safety information gaps of new and existing chemicals with increased conventional toxicity testing would skyrocket costs and animal usage and contravene the 3Rs principles to refine, reduce, and replace animal testing. There is a great need to improve predictive safety assessment with minimum animal use. Recent progress in the understanding of the molecular biology of chemical dose response relationships, which has confirmed general applicability of the Druckrey-Küpfmüller theorem for receptor-mediated toxicity, can now be employed at an early stage of product development to identify (and eliminate) hazardous chemicals using short-term chronic dose response tests in invertebrates. Time-to-effect (TTE) approaches can identify hazardous chemicals with time-cumulative toxicity. This approach would shift research and development programs to chemicals with ascertained strictly dose-dependent toxicity, for which no-observable-adverse-effect levels (NOAELs) can be reliably established with short-term tests, which could make a myriad of resource-intensive animal studies obsolete.

Keywords: Toxicity, Chronic, Predictive Safety Assessment, Hazardous Chemicals, 3Rs principles.

Accepted on February 26, 2017

Introduction

A recent survey of five leading crop protection companies [1] revealed that the expenditure over the last 20 years for discovery and development of a new crop protection product has nearly doubled to $ 289 million, mostly due to markedly increased expenditure for environmental chemistry studies, field trials and regulatory affairs (Table 1), which reflects a tremendous rise in environmental safety data required by regulatory bodies. The average lead time from first synthesis to commercial introduction is now more than 11 years, and the biology of an average 160,000 newly synthesized molecules is screened for the registration of one new crop protection product [1].

Within the same time period, new products such as the neonicotinoid insecticides, which were developed according to latest regulatory standards, have become highly controversial because risks to beneficial non-target organisms were underestimated [2-4], underpinning information gaps in environmental safety assessment. There are additional large information gaps on the safety of a vast number of existing chemicals in commerce [5], and removing hazardous chemicals from the market and replacing them with safer alternatives has become a key objective of EU REACH chemical management regulation [6] and reform of the Toxic Substances Control Act in the United States [7]. Addressing safety information gaps of new and existing chemicals with increased conventional toxicity testing would skyrocket costs and animal usage and contravene the 3Rs principles to refine, reduce, and replace animal testing [8]. The chemical industry has a vital interest in predictive safety assessment rather than extensive and resource intensive toxicology programs and there has been a steady increase in development and use of in vitro alternatives to animal testing by industry over the last 30 years [9]. A paradigm shift is emerging in which computational approaches, systems biology, high-throughput in vitro toxicity assays, and high-throughput exposure assessments are beginning to be applied to mechanism-based risk assessments in a time- and resource-efficient fashion [10]. Structure-based (quantitative structure–activity relationship [QSAR]) prediction approaches were among the first developed and most commonly used cheminformatics tools for safety assessment [11], although there are concerns about the reliability of their predictions [12].

It seems possible to further improve predictive safety assessment with minimum animal use following recent progress in the understanding of the molecular biology of chemical dose response relationships [13], which can be employed at an early stage of product development to identify and eliminate hazardous chemicals with short-term dose response tests. This approach would shift research and development programs to chemicals with ascertained strictly dose-dependent toxicity, for which no-observable-adverse-effect levels (NOAELs) can be reliably established with short-term tests, which could make a myriad of resource-intensive animal studies obsolete.

The Paracelsus Paradigm and the Threshold Model for Risk Assessment

The Renaissance physician Paracelsus (1493–1541), often regarded as the 'Father of Toxicology', laid the groundwork for chemical risk assessment when he coined his dictum, *'What is*

Table 1. *Discovery and development costs (in million us dollars) of a new crop protection product, according to a survey of 5 leading crop protection companies [1].*

Activity	Activity Segment	1995	2010-14	Ratio 2010-14/1995
Research	Biology	30	51	1.70
	Chemistry	32	49	1.53
	Toxicology & Environmental Chemistry	10	7	0.70
	Total Activities	72	107	1.49
Development	Environmental Chemistry	13	35	2.69
	Toxicology	18	29	1.61
	Field trials	18	47	2.61
	Chemistry	18	35	1.94
	Total Activities	67	146	2.18
Registration	Total Activities	13	33	2.54
R&D	Total Activities	152	286	1.88

there that is not poison? All things are poison and nothing is without poison. Solely the dose determines that a thing is not a poison'. Paracelsus inferred that lower doses – below a threshold – could cause otherwise poisonous substances to become harmless [14]. Paracelsus paved the way for the threshold concept and the no-adverse effect level [15] for risk assessment. The notable exception is the linear non-threshold (LNT) dose-response model widely adopted for carcinogenic agents. Carcinogenic risk assessment originates from the assertion of Hermann J. Muller (in his Nobel Prize Lecture of December 12, 1946) that the dose-response relationship for radiation-induced mutations is linear [16] ruling out a threshold dose. Muller's statement received first recognition by the Genetics Panel of the U.S. National Academy of Sciences' Committee on Biological Effects of Atomic Radiation (BEAR) in 1956 [17], and was subsequently endorsed by leading regulatory authorities for estimates of the cancer risk from radioactive fallout [18] and genotoxic carcinogens [19,20]. However, the LNT dose-response model has been challenged by several authors who hypothesized potential thresholds and protective mechanisms throughout the process from initial DNA damage induction to tumor formation [21-26]. Chemical risk assessment was compounded even further by recent observations that some non-genotoxic chemicals show dose-response relationships identical to that of nitrosamines, arguably the most potent carcinogens known [27-31]. The dose response is described in quantitative terms by what is known as the Druckrey-Küpfmüller equation:

$$d\,t^{n}=\text{constant} \quad (1)$$

where d=daily dose and t=exposure Time-to-effect, and $n \geq 1$.

Adverse effects of genotoxic carcinogens and several hazardous non-carcinogens are dependent not only on exposure levels but also on exposure duration. The essence of equation (1) is that the total dose required to produce an adverse effect is much lower at low exposure levels even tough exposure times needed to produce the effect are much higher. From a mechanistic point of view, the common denominator of the dose-response relationship is irreversibility of receptor binding and irreversibility of the associated effect [13].

Shortly after the Second World War, two reputable German scientists, the pharmacologist and cancer researcher Hermann Druckrey, and Karl Küpfmuller, a mathematically adept electrical and communications engineer, while detained in an allied internment camp, developed groundbreaking theoretical knowledge about the relationship between drug dosage and tissue response in pharmacology and toxicology in general, and the action of carcinogenic substances in particular [32]. They hypothesized, with theoretical approaches to reaction kinetics, that irreversible receptor binding with an associated irreversible effect would lead to reinforcement of the effect by exposure time (Table 2) [33]. Many years later, the carcinogenicity of nitrosamines [30,31] was indeed shown to be a result of irreversible receptor binding (alkylation of DNA) associated with irreversible effects (gene mutations) [34,35]. Essentially similar receptor-mediated mechanisms of toxic action leading to time-cumulative toxicity have now been validated for a considerable number of non-carcinogens [13], indicating that the Druckrey-Küpfmüller theorem is generally applicable for chemical toxicity.

This evidence has major implications for chemical risk assessment. The Paracelsus paradigm and threshold model cannot be upheld for chemicals with time-dependent toxicity, and is valid only for reversible effects resulting from reversible receptor binding (Table 2), when effects are indeed entirely dose-dependent. An additional implication is that chemical risk assessment requires quantitative analysis of the dose-response relationship to discriminate between strictly dose-dependent toxicity and dose- and time-dependent toxicity.

Shift of research and development to chemicals with dose-dependent toxicity

The linear non-threshold (LNT) dose-response model and the ALARA principle ("as low as reasonably achievable"), currently used in risk assessment and management of nitrosamines, may have to be extended to *all chemicals with time-cumulative toxicity* [36], because risks of long-term exposure to low concentrations of such chemicals have been seriously underestimated. In fact, equation (1) suggests a threshold dose may not exist for chemicals with time-cumulative toxicity. The neonicotinoids are a case in point. Bayer scientists demonstrated that the neonicotinoid insecticide imidacloprid blocks nicotinic acetylcholine (nACh) receptors in the central nervous system of insects [37], leading to irreversible neuronal damage [38], underpinning the Druckrey-Küpfmüller theorem [32,33]. Unlike the normal neurotransmitter acetylcholine, acetylcholinesterase cannot remove imidacloprid from the nACh receptor. Although irreversible receptor binding has recently been retracted by Bayer experts [39] in response to

Table 2: *Dose-response characteristics according to Druckrey and Küpfmüller [33].*

Reversibility of receptor binding	Receptor binding in relation to compound concentration	Reversibility of the effect	Effect in relation to receptor binding	Effect in relation to compound concentration	Dose-response characteristics
$T_R \rightarrow 0$	$C_R \sim C$	$T_r \rightarrow 0$ $T_r \rightarrow \infty$	$E \sim C_R$ $E \sim \int C_R\, dt$	$E \sim C$ $E \sim \int C\, dt$	Dose-dependent Ct = constant*
$T_R \rightarrow \infty$	$C_R \sim \int C dt$	$T_r \rightarrow 0$ $T_r \rightarrow \infty$	$E \sim C_R$ $E \sim \int C_R\, dt$	$E \sim \int C\, dt$ $E \sim \int \int C\, dt$	Ct = constant Reinforced by time

T_R is the time constant for the reversibility of receptor binding
T_r is the time constant for the reversibility of the effect
C_R is the concentration of bound receptors
C is the concentration of the poison at the site of interaction
E=Effect
*known as Haber's Rule (the product of concentration and time produces a constant effect). These dose response relationships may occur with irreversible receptor binding or irreversible effect when C is practically constant over time

the discovery of time-cumulative toxicity of imidacloprid and thiacloprid to arthropods [27], it can hardly be disputed that dissociation, if it occurs at all, is bound to be very slow, and that cumulative nACh receptor binding leading to irreversible neuronal toxicity can be easily envisaged [40]. Time-cumulative toxicity to invertebrates has been demonstrated for the neonicotinoids imidacloprid, thiacloprid, thiamethoxam and clothianidin [13,27,28,41-43], which, due to their environmental properties, threatens the survival of invertebrates. They are prone to leach from soils [44], and have been demonstrated to contaminate surface water in Europe and North America [3,44]. In the Netherlands, surface water contamination with imidacloprid has been demonstrated to correlate with decline of macro-invertebrates [45] and insectivorous birds [44,46], and entomological surveys in Dutch and German nature reserves have revealed a staggering decline of ground beetles and flying insects since the introduction of imidacloprid in agriculture in the 1990s [44,47]. The assumption of thresholds of toxicity for neonicotinoids was a serious error of judgement and has resulted in disastrous insect decline with knock on effects on all insectivores, which could have been prevented if the ALARA principle would have been applied to time-cumulative toxicity.

Dose: Time-to-effect analyses to identify hazardous chemicals

The corollary of described dose response relationships is that repeated dose toxicity testing must first and foremost focus on establishing dose: Time-to-effect relationships and eliminate hazardous chemicals with time-cumulative toxicity at an early stage of development. Traditional repeated dose toxicity tests usually involve the administration of 3 dose levels of test chemicals to animals, which then are observed for adverse effects. Adverse effects are expected to be observed at high dose, while mid-dose and low-dose level are expected to provide the lowest-observed-adverse-effect level (LOAEL) and the NOAEL (no-observed adverse effect level).

This 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, and measures the proportion of all exposed individuals responding with adverse effects by the end of such exposure times. This is valid when toxicity is mainly dependent on exposure concentrations (Paracelsus), but it is insufficient when toxic effects are influenced 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, Time-to-effect (TTE) approaches 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 of at least 5 dose levels at consecutive times during the exposure, so the data form a matrix that can be analysed 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 to detect chemicals showing time-dependent toxicity, and it allows prediction of toxic effects for any combination of concentration and time found in the environment.

Short-Term Tests for Predictive Safety Assessment

The OECD is committed to the implementation of the 3R-principles, Replacement, Reduction and Refinement, as first laid down by Russel & Burch in 1959 [8], in their "The Principles of Humane Experimental Technique". Since the adoption in 1981 of the first set of Test Guidelines, many of the short-, and long-term toxicity tests have been developed or revised to introduce aspects of the 3R-principles [48]. A considerable number of guidelines for *in vitro* assays have been developed to predict diverse toxicological endpoints (Table 3) which not only provide in vitro alternatives to the notorious Lethal Dose-50% (LD50) and Draize tests that inflict severe suffering on animals [49], but may also identify carcinogens, mutagens, and endocrine disruptors. The industry's interest in *in vitro* assays has arisen from the need to support the early identification of promising new molecules but also through legislation requiring adherence to the 3Rs [50].

Carcinogenesis and mutagenesis are of special concern in product development, and *in vitro* genotoxicity studies (Table 3) are usually conducted at a very early stage. Now that the Druckrey-Küpfmüller theorem has been shown to be generally applicable, dose: Time-to-effect analyses can identify similarly hazardous non-carcinogens in early development, and improve hazard identification. Time-dependent toxicity can be conveniently investigated with short-term chronic toxicity tests in invertebrates [48,51]. Daphnia magna appears to be an excellent model because its sensitivity to lethal toxicity is very similar to mammals and humans [52,53]. Moreover, if short-term assays demonstrate absence of mutagenic potential and time-dependent toxicity, the Druckrey-Küpfmüller theorem (Table

Table 3*. Suggested screening tests for predictive safety assessment [48,51].*

Toxicity endpoints	Assay	OECD or EPA Guideline(s)
Eye corrosion or irritation	Bovine corneal opacity and permeability test	OECD 437
	Isolated chicken eye test	OECD 438
	Fluorescein leakage test	OECD 460
	Reconstructed human Cornea-like Epithelium (RhCE)	OECD 492
Skin corrosion or irritation	In vitro membrane barrier test	OECD 435
	Reconstructed human epidermis (RHE) test	OECD 431, 439
	Transcutaneous electrical resistance test	OECD 430
Acute toxicity to fish	Zebrafish embryo acute toxicity test	OECD 236
Skin sensitisation	Human Cell Line Activation Test (h-CLAT)	OECD 442E
Endocrine disruption	BG1Luc estrogen receptor transactivation test	OECD 457
	H295R steroidogenesis assay	OECD 456
	Amphibian metamorphosis assay	OECD 231
	Stably Transfected Transactivation to detect Androgen Receptor Agonists and Antagonists	OECD 458
Genetic toxicity	Bacterial reverse mutation test	OECD 471
	In vitro mammalian chromosomal aberration test	OECD 473
	In vitro mammalian cell gene mutation test	OECD 476
	In vitro mammalian cell micronucleus test	OECD 487
Skin absorption	Skin absorption *in vitro*	OECD 428
Phototoxicity	3T3 neutral red uptake phototoxicity test	OECD 432
Chronic toxicity	Sediment-water chironomid toxicity (spiked sediment)	OECD 218
	Sediment-water chironomid toxicity (spiked water)	OECD 219
	Sediment-water chironomid life-cycle toxicity test	OECD 233
	Daphnid chronic toxicity test	OPPTS 850.1300
NOAEL determination	Combined Repeated Dose Toxicity Study with the Reproduction/ Developmental Toxicity Screening Test	OECD 422

2) indicates that biological effects of a candidate product are bound to be strictly dose-dependent, which would also rule out carcinogenic potential (invariably a dose- and time-dependent process). Safe exposure levels can be extracted from short-term repeated dose experiments, such as the Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test adopted by the OECD on 22 March 1996 (Table 3) [48]. A myriad of resource-intensive animal studies would become obsolete, simplifying product development programs.

References

1. Anonymous. The Cost of New Agrochemical Product Discovery, Development and Registration in 1995, 2000, 2005-8 and 2010 to 2014. R&D expenditure in 2014 and expectations for 2019. Phillips McDougall Consultancy Study for CropLife International, CropLife America and the European Crop Protection Association. 2016.
2. Sánchez-Bayo F. The trouble with neonicotinoids Science. 2014;346:806-07.
3. Morrissey CA, Mineau P, Devries JH, et al. Neonicotinoid contamination of global surface waters and associated risk to aquatic invertebrates: A review. Environment International. 2015;74:291-303.
4. Sánchez-Bayo F, Goulson D, Pennacchio F, et al. Are bee diseases linked to pesticides ? - A brief review. Environment International. 2016;89-90:7-11.
5. NRC. Toxicity testing: Strategies to determine needs and priorities. Washington, DC: National Academies Press. 1984.
6. http://ec.europa.eu/environment/chemicals/reach/reach_intro.htm
7. http://www.repository.law.indiana.edu/facpub/719
8. Russell WMS, Burch RL. The principles of humane experimental technique. Universities Federation for Animal Welfare, Hertfordshire, United Kingdom. 1959.
9. Goh JY, Weaver RJ, Libby Dixon L, et al. Development and use of in vitro alternatives to animal testing by the pharmaceutical industry. 1980-2013. Toxicol Res. 2015;4:1297.
10. Settivari RS, Ball N, Murphy L, et al. Predicting the Future: Opportunities and Challenges for the Chemical Industry to Apply 21st-Century Toxicity Testing. J Amer Ass Lab Anim Sci. 2015;54:214-23.
11. Tropsha A. Best Practices for QSAR Model Development, Validation, and Exploitation. Mol Inf. 2010;29:476-48.
12. Eriksson L, Jaworska J, Worth AP, et al. Methods for Reliability and Uncertainty Assessment and for Applicability Evaluations of Classification- and Regression-Based QSARs. Environ Health Perspect. 2003;111:1361-75.
13. Tennekes HA, Sánchez-Bayo F. The molecular basis of simple relationships between exposure concentration and toxic effects with time. Toxicology. 2013;309:39-51.
14. Grandjean P. Paracelsus Revisited: The Dose Concept in a Complex World. Basic Clin Pharmacol Toxicol. 2016;119:126-32.
15. Barnes DG, Douglas M. Reference Dose (RfD): Description and Use in Health Risk Assessments. Regul Toxicol Pharmacol. 1988;8:471-86.
16. Calabrese EJ. Muller's Nobel lecture on dose–response

for ionizing radiation: ideology or science? Arch Toxicol. 2011;85:1495-98.

17. Anonymous. Genetic effects of atomic radiation. Summary Report of the Committee on Biological Effects of Atomic Radiation by the National Academy of Sciences, BEAR I Genetics Panel (W. Weaver, Chair). Science. 1956;124:170.
18. Albert RE. Carcinogen risk assessment. Environ Health Perspect. 1989;81:103-05.
19. US Environmental Protection Agency. Health risk and economic impact assessments of suspected carcinogens. Fed Regist. 1976;41:21402-05.
20. US Environmental Protection Agency. Guidelines for Carcinogen Risk Assessment. EPA/630/P-03/001F. 2005. pp. 1-166.
21. Kroes R, Galli C, Munro I, et al. Threshold of toxicological concern for chemical substances present in the diet: A practical tool for assessing the need for toxicity testing. Food and Chemical Toxicology. 2000;38:255-312.
22. Calabrese EJ, Baldwin LA. Toxicology rethinks its central belief. Hormesis demands a reappraisal of the way risks are assessed. Nature. 2003;421:691-92.
23. Calabrese EJ. The road to linearity: why linearity at low doses became the basis for carcinogen risk assessment. Arch Toxicol. 2009;83:203-25.
24. Calabrese EJ. How the U.S. National Academy of Sciences mislead the world community on cancer risk assessment: New findings challenge historical foundations of the linear dose response. Arch Toxicol. 2013;87:2063.
25. Calabrese EJ. An abuse of risk assessment: how regulatory agencies improperly adopted LNT for cancer risk assessment. Arch Toxicol. 2015;89:647-48.
26. Thomas AD, Fahrer J, Johnson GE, Kaina B. Theoretical considerations for thresholds in chemical carcinogenesis, Mutat Res: Rev Mutat Res. 2015;765:56-67.
27. Tennekes HA. The significance of the Druckrey–Kupfmuller equation for risk assessment - the toxicity of neonicotinoid insecticides to arthropods is reinforced by exposure time. Toxicology. 2010;276:1-4.
28. Tennekes HA, Sánchez-Bayo F. Time-dependent toxicity of neonicotinoids and other toxicants: Implications for a new approach to risk assessment. J Environ Anal Toxicol. 2012;S4-001.
29. Pletz J, Sánchez-Bayo F, Tennekes HA. Dose-response analysis indicating time-dependent neurotoxicity caused by organic and inorganic mercury- Implications for toxic effects in the developing brain. Toxicology. 2016;347:1-5.
30. Druckrey H, Schildbach A, Schmaehl D, et al. Quantitative analysis of the carcinogenic effect of diethylnitrosamine. Arzneimittelforschung. 1963;13:841-51.
31. Druckrey H, Preussmann R, Ivankovic S, et al. Organotropic carcinogenic effects of 65 different N-nitroso-compounds in BD-rats. Z. Krebsforsch. 1967;69:103-201.
32. Wunderlich V. On the Origins of the Druckrey-Küpfmüller Papers (1948-1949): Dose-Effect Relations in Carcinogenic Substances. Medizinhistorisches Journal. 2005;40:369-97.
33. Druckrey H, Küpfmüller K. Dosis und Wirkung. Beiträge zur theoretischen Pharmakologie. Editio Cantor GmbH, Freiburg im Breisgau, Germany. 1949.
34. Magee PN, Barnes JM. The production of malignant primary hepatic tumours in. the rat by feeding dimethylnitrosamine. Brit J Cancer. 1956;10:114-22.
35. Magee PN, Farber E. Toxic liver injury and carcinogenesis. Methylation of rat-liver nucleic acids by dimethylnitrosamine in vivo. Biochem J. 1962;83:114-24.
36. Tennekes HA. A Critical Appraisal of the Threshold of Toxicity Model for Non-Carcinogens. J Environ Anal Toxicol. 2016;6:408.
37. Abbink J. The Biochemistry of Imidacloprid. Pflanzenschutz-Nachrichten Bayer, Germany, F R,Serial ID- ISSN: 0340-1723. 1991.
38. Mehlhorn H, Mencke N, Hansen O. Effects of imidacloprid on adult and larval stages of the flea Cfenoaephalides felis after in vivo and in vitro application: a light- and electron-microscopy study. Parasitol Res. 1999;85:625-37.
39. Maus C, Nauen R. Response to the publication: Tennekes, H.A. (2010): "The significance of the Druckrey-Küpfmüller equation for risk assessment: the toxicity of neonicotinoid insecticides to arthropods is reinforced by exposure time". Toxicology. 2011;280:176-77.
40. Tennekes HA. The significance of the Druckrey–Küpfmüller equation for risk assessment- the toxicity of neonicotinoid insecticides to arthropods is reinforced by exposure time: responding to a Letter to the Editor by Drs. C Maus and R Nauen of Bayer CropScience AG. Toxicology. 2011;280:173-75.
41. Liess M, Foit K, Becker A, et al. Culmination of Low-Dose Pesticide Effects. Environ Sci Technol. 2013;47:8862-86.
42. Uğurlu P, Ünlüb E, İpek Satar E. The toxicological effects of thiamethoxam on Gammarus kischineffensis (Schellenberg 1937) (Crustacea: Amphipoda) Environ Toxicol Pharmacol. 2015;39:720-26.
43. Alkassab AT, Kirchner WH. Impacts of chronic sublethal exposure to clothianidin on winter honeybees. Ecotoxicology. 2016;25:1000.
44. http://www.disasterinthemaking.com/.
45. Van Dijk TC, Van Staalduinen MA, Van der Sluijs JP. Macro-Invertebrate Decline in Surface Water Polluted with Imidacloprid. PLoS ONE. 2013;8(5):e62374. doi:10.1371/journal.pone.0062374
46. Hallmann CA, Foppen RPB, van Turnhout CAM, et al. Declines in insectivorous birds are associated with high neonicotinoid concentrations. Nature. 2014;511:341-43.
47. Sorg M, Schwan H, Stenmans W, et al. Ermittlung der Biomassen flugaktiver Insekten im Naturschutzgebiet

Orbroicher Bruch mit Malaise Fallen in den Jahren 1989 und 2013. Mitteilungen aus dem Entomologischen Verein Krefeld. 2013;1:1-5.

48. http://www.oecd.org/env/ehs/testing/adopted-test-guidelines-toxicity-testing-3r-relevance.htm
49. Dresser R. Standards for Animal Research: Looking at the Middle. J Med Philos. 1988;13:123-43.
50. Parasuraman S. Toxicological screening. J Pharmacol Pharmacotherap. 2011;2:74-79.
51. Weber CI, Peltier WH, Norberg-King TJ, et al. Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms. 2nd edition. EPA/600/4-89/001. Environmental Monitoring Systems Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, Cincinnati, OH. 1989.
52. Adema DMM. Daphnia magna as a test animal in acute and chronic toxicity tests. Hydrobiologia. 1978;59:125.
53. Guilhermino L, Diamantino T, Carolina Silva M, et al. Acute Toxicity Test with Daphnia magna: An Alternative to Mammals in the Prescreening of Chemical Toxicity? Ecotoxicol Environ Safety. 2000;46:357-62.

***Correspondence to:**

Dr. Henk A. Tennekes
Experimental Toxicology Services (ETS) Nederland BV,
Frankensteeg 4,
7201KN Zutphen,
The Netherlands
Tel: +31 (0) 575 547717
E-mail: info@toxicology.nl