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The added value of the 90-day repeated dose oral toxicity test for industrial chemicals with a low (sub)acute toxicity profile in a high quality dataset

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ABSTRACT

A survey conducted on the EU Notification of New Substances (NONS) database suggested that for industrial chemicals with a profile of low toxicity in (sub)acute toxicity tests there is little added value to the conduct of the 90-day repeated dose study. Avoiding unnecessary animal testing is a central aim of the EU REACH chemicals legislation; therefore we sought to verify the profile using additional data. The OECD's eChemPortal was searched for substances that had both a 28-day and a 90-day study and their robust study summaries were then examined from the ECHA CHEM database. Out of 182 substances with high quality 28-day and 90-day study results, only 18 reported no toxicity of any kind in the (sub)acute tests. However, for 16 of these there were also no reported signs of toxicity at or close to the limit dose (1000 mg/kg bw/d) in the 90-day study. Restricting the 'low (sub)acute toxicity in a high quality dataset' profile to general industrial chemicals of no known biological activity, whilst allowing irritant substances, increases the data set and improves the prediction to 95% (20 substances out of 21 substances). The low toxicity profile appears to be of low prevalence within industrial chemicals (10–15%), nevertheless, avoidance of the conduct of a redundant 90-day study for this proportion of the remaining REACH phase-in substances would avoid the use of nearly 50,000 animals and save industry 50 million Euros, with no impact on the assessment of human health.

1. Introduction

All new and existing chemical substances that are manufactured or imported in the European Union must now be registered under EU chemicals legislation REACH (Regulation (EC) No. 1907/2006). The difference between REACH and previous EU chemicals legislation is that the information requirements for existing chemicals (so called phase-in substances) are the same as for new (non-phase-in) substances. Companies had to register all the substances they manufacture or import in quantities above 1000 tonnes per year by 1 December 2010. All the substances they manufacture or import in quantities above 100 tonnes per year were also registered by 1 June 2013. A complete data package for a REACH chemical registration at these tonnages can involve the conduct of at least 10 different animal studies and can cost between 800 and 1600 k Euros (for Annex IX and X, respectively) (based on the most recent figures available; Fleischer, 2007).

An example of the heavy burden of safety information required under REACH is that for substances manufactured or imported at levels of 100 tonnes per year or above (Annex IX requirements), the results of both a 28-day and a 90-day repeated dose toxicity study in rodents is required. This requirement was based on the general assumption that the No Observed (Adverse) Effect Level (NO(A)EL) of a substance decreases as the length of the study increases. For substances that are produced in high quantities it was therefore decided that a 90-day study should be required in addition to the 28-day study. However, in order to reduce unnecessary animal testing, for those substances that have neither a 28-day nor a 90-day study, it is permitted to provide the results of the 90-day study only (REACH Annex IX 8.6.2, column 1). Table 1 outlines the repeated dose and reproductive toxicity requirements of substances being registered under REACH.

Not all existing substances will have the necessary information requirements and it is quite possible that many substances will actually have the results of the 28-day but not the 90-day study. In fact, a review by the European Commission estimated that 93% of relevant substances would not have the 90-day test prior to

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Table 1
REACH requirements for repeated dose and reproductive toxicity studies.

Annex	VII	VIII	IX	X
Tonnage	1 tonne or greater	10 tonnes or greater	100 tonnes or greater	1000 tonnes or greater
Repeated dose toxicity	None	28 day (most appropriate route)	28 day (unless already conducted or the 90 day is proposed) 90-day study should be proposed (most appropriate route)	28 day (unless already conducted or the 90 day is proposed) 90-day study should be proposed (most appropriate route) Longer term studies may be proposed if serious or severe toxicity effects of particular concern were observed in the 28-day or 90-day study for which the available evidence is inadequate for toxicological evaluation or risk characterisation A carcinogenicity study may be proposed if there is evidence from the repeated dose study(s) that the substance is able to induce hyperplasia and/or preneoplastic lesions (and there is wide dispersive use of the substance or frequent human exposure)
Reproductive toxicity	None	Screening study (OECD TG 421)	Prenatal developmental toxicity should be proposed (most appropriate route) Two generation reproductive toxicity study should be proposed, if the 28-day or 90-day study indicates adverse effects on reproductive organs or tissues	Prenatal developmental toxicity should be proposed (most appropriate route) A second species prenatal developmental toxicity should be considered and proposed (most appropriate route) Two generation reproductive toxicity study, unless already provided or proposed

REACH (Pedersen et al., 2003). This is because previous regulatory regimes such as the predecessor to REACH, the Dangerous Substances Directive (Directive 67/548/EEC Annex VIIA) and the voluntary US High Production Volume Challenge Programme only required the 28-day study within the basic data package. Many REACH registrants are therefore now in the position of having to submit 'proposals' to conduct 90-day studies on their substance if the test cannot be waived for other reasons (read across, exposure based arguments, etc.; see Annex XI of REACH for adaptations to the standard testing regime).

Testing proposals must be submitted, not the test result, because REACH says that a proposal for tests required under Annex IX or X, such as the 90-day study, must be first evaluated by the agency responsible – the European Chemicals Agency (ECHA) – before it is conducted. This is a test reduction measure put in place to allow third parties to notify ECHA that they have existing information on the substance, thereby avoiding duplicate animal studies. Testing proposals are published on the ECHA website for a 45 day comment period and then a decision is made via a bureaucratic process that can take at least a year.

In this period of intense data collection to satisfy REACH requirements it is crucial to seek to maximise efficiency, not only of animals but of cost to the industry at large. Despite the animal reduction measures written into the legislation, within a complete REACH submission for which the results of several toxicological studies of different durations and covering a range of endpoints are required, it is entirely possible that there will be tests that are less useful than others. Due to the similar nature of the repeated dose tests – the only difference being duration of the dosing – it is feasible that there may be some element of duplication to the conduct of both the 28-day and the 90-day study for example.

Identifying studies that add little in terms of information on the hazardous properties of the substance, and are in effect 'redundant', is a relatively easy way to reduce animal testing and costs to industry, without adversely impacting on the level of protection of human or environmental health. Examples of where this approach has proved fruitful include a review of the need for dermal acute toxicity studies for industrial chemicals and pesticides (Creton et al., 2010) and a review of the need for single dose acute toxicity studies for medicinal compounds (Robinson et al., 2008) and for industrial chemicals (Chapman et al., 2010) all by the UK National Centre for the 3Rs, a review of the need for the second generation in reproductive toxicity studies by the Dutch National Institute for Public Health and the Environment (Janer et al., 2007), and a review of the need for carcinogenicity studies for medicinal products by the German Federal Institute for Drugs and Medical Devices (Friedrich and Olejniczak, 2011) and also by the US drug industry (Sistare et al., 2011).

Based on their experience with the Notification of New Substances (NONS) system – the chemical registration system that predated REACH – some members of the UK Competent Authority for REACH [the Health and Safety Executive (HSE)] felt that there may be redundancy in the 90-day repeated dose toxicity study. This is because when they reviewed NONS dossiers with low toxicity in the 28-day study they consistently found that the 90-day study also demonstrated low toxicity. To demonstrate their hypothesis, the HSE performed a systematic analysis of the NONS database, which was not publicly available at the time. They found that out of 110 substances with results for both 28-day and 90-day studies via the same exposure route, 17 substances (15%) were identified that had a NO(A)EL close to or greater than 1000 mg/kg bw/d in the 28-day study. In all these substances the 90-day NO(A)EL was also equivalent to or higher than the limit dose of 1000 mg/kg bw/d. The limit dose of 1000 mg/kg bw/d is a pragmatic value introduced to prevent the use of excessive dose levels in toxicity studies, which would be likely to result in effects of no relevance to the human risk assessment. The results of their analysis therefore strongly suggested that there was no added value to the conduct to the 90-day study in these situations, with no margin of error. As a precautionary approach they recommended that all acute endpoints including acute toxicity (by any route), mutagenicity, skin sensitisation and skin and eye irritation should also be negative for the substance to satisfy the 'low toxicity profile'. However, this would have reduced their dataset to 14 substances with obviously no improvement to be gained in predictivity.

The HSE presented their results to the Member State Committee (MSC) at the ECHA in January 2011 (HSE, 2011). The MSC is ECHA's committee that agrees on the need for new toxicity tests when conducting compliance checks of registration dossiers and testing proposals made by registrants who have already identified that they have a data gap. The MSC members expressed interest in

the results but were concerned about the small dataset used to test the hypothesis and the use of potentially low quality 28-day studies to waive what is generally considered to be the more robust 90-day study.

Unfortunately, the ECHA or other Member States have not, to date, investigated this issue further using other databases available to them. Since the HSE proposal, however, the database of registered substances under REACH has been populated with information; primarily on Annex X substances produced in excess of 1000 tonnes per year. This public database called ECHA CHEM (available from the ECHA website www.echa.europa.eu) is European Chemicals Agency's dissemination portal with information on chemical substances registered under REACH and contains information on the composition and use of the substance as well as the 'robust study summaries' of the toxicity tests included in the registration dossier.

In the interests of avoiding the future conduct of unnecessary animal tests we therefore sought to substantiate the HSE hypothesis using additional substances now within the ECHA CHEM database. Our hypothesis was the same as the HSE; where a substance has both a 28-day and a 90-day study result and exhibits a profile of low toxicity in the 28-day study and other acute tests, the 90-day study result will also be 'low toxicity' (NOAEL defined as close to or greater than 1000 mg/kg bw/d). In order to respond to the reservations of some of the MSC members we applied strict inclusion and exclusion criteria to the dataset to ensure it was of high quality.

2. Method

2.1. Initial selection of data set

We used the OECD's eChemPortal (www.echemportal.org) to search for substances for which there was both a 28-day and a 90-day study. The eChemPortal is an effort of the Organisation for Economic Co-operation and Development (OECD) in collaboration with the European Commission, ECHA, the United States, Canada, Japan, the International Council of Chemical Associations, the Business and Industry Advisory Committee, the World Health Organization's International Program on Chemical Safety, the United Nations Environment Programme and environmental non-governmental organisations. eChemPortal provides free public access to information on properties of chemicals. It allows simultaneous searching of reports and datasets by chemical name and number and by chemical property. At the time of the survey, the participating databases with information on chemical properties were CCR (Canadian Categorization Results), ECHA CHEM, J-CHECK (Japan CHEmicals Collaborative Knowledge database) and the OECD SIDS IUCLID (OECD Existing Chemicals Screening Information Data Sets (SIDS) Database). The eChemPortal was used for the initial search as it is not yet possible to search for substance by property type in the ECHA CHEM database. However, since ECHA CHEM is the main database with information on substance properties, a property search in eChemPortal is currently effectively a search of the ECHA CHEM database. At the time of the survey it was not easy to do a combined query in eChemPortal so we chose to search firstly for substances with a 90-day study, since this was regarded to be the least common of the two repeated dose tests.

At the time of our analysis, according to ECHA at their annual stakeholder day in May 2012 the database was populated with the data from approximately 4335 substances, including most of the Annex X substances registered by December 2010. There had been some delay to publication of information on these substances by ECHA and further delay to the publication of this paper was done so as not to preclude any decisions they would make on the information needs of some of these (see Section 4.10). The search was conducted as follows:

1. Selected "Chemical Property Data Search".
2. Under the subheading "Toxicological Information", selected "repeated dose toxicity: oral".
3. For the search criteria in the Query Block, under "study result type" dropdown menu, checked the box next to "experimental result" (left all other boxes unchecked).
4. Under "Test guideline, Guideline" dropdown menu, checked the boxes next to the following guidelines (i.e. all 90-day oral studies in case any were misreported) and added them to the search criteria:
   a. EPA OPP 82-1 (90-day oral toxicity)
   b. EPA OPPTS 870.3100 (90-day oral toxicity in rodents)
   c. EPA OPPTS 870.3150 (90-day oral toxicity in non-rodents)
   d. EPA OTS 798.2650 (90-day oral toxicity in rodents)
   e. EU Method B.26 (sub-chronic oral toxicity test: repeated dose 90-day oral toxicity study in rodents)
   f. EU Method B.27 (sub-chronic oral toxicity test: repeated dose 90-day oral toxicity study in non-rodents)
   g. OECD guideline 408 (repeated dose 90-day oral toxicity in rodents)
   h. OECD guideline 409 (repeated dose 90-day oral toxicity in non-rodents)
5. Selected "Save" to save search criteria then selected Execute Query.

eChemPortal returned a total of 964 substances that reportedly had experimental data for a 90-day study in any species by the oral route. This number is not representative of the actual number of substances with 90-day study results as we subsequently found that eChemPortal cannot recognise test result data from other information such as 'test proposed' or 'read across'.

2.2. Inclusion criteria

The dataset generated under this search was then manually analysed in order to identify those substances which actually had experimental data on the substance itself for both the 28-day and 90-day study by the oral route, in rats. This involved manually clicking through from the eChemPortal search result to the record for the substance, which in all cases was located in the ECHA CHEM database. Substances for which there were no 28-day or 90-day studies on the substance itself, i.e. read across was used or no actual studies were included, were rejected at this stage. This reduced the dataset to 182 substances with both 28-day and 90-day studies by the oral route in rats.

2.3. Exclusion criteria

2.3.1. Key 28 day study

The dataset was then reviewed to strictly exclude all those that had a NOAEL reported to be less than 1000 mg/kg bw/d in their key 28-day study. Where the NOEL (only) was reported this had to also be 1000 mg/kg bw/d (the NOAEL would be at least this value in these cases). Where the NOAEL was 1000 mg/kg bw/d and the NOEL was reported to be a lower value, then such substances would also be excluded. Substances with more than one key 28-day study were rejected if any rodent study did not conform to this (in practice this was rare). This reduced the dataset to 34 substances. It is important to note that in this survey the reports of the registrant regarding the NO(A)EL value were accepted, i.e. there was no reanalysis on our part of the significance of the biological results and respective adjustment of the NO(A)EL.

2.3.2. Quality of data

The dataset was then further reduced in order to remove substances with poor quality datasets. These were defined as where the 28-day and 90-day studies were:

- Not equivalent to the corresponding OECD guideline (TG 407 (28-day repeated dose) or TG 408 (90-day repeated dose)) in terms of duration, species (rats), numbers of animals or main parameters measured. Studies were included if they measured body weight, clinical signs, mortality, histopathology of at least all major organs, clinical chemistry and haematology. Neurobehavior, immunology, urinalysis, ophthalmology were optional since these were not included in the original 1981 versions of OECD TG 407 and 408. Range-finding studies of shorter duration or reduced numbers of animals, the combined repeated dose/reproductive toxicity screening study (OECD TG 422) or published studies that looked at limited parameters were excluded.
- Not conducted up to the limit dose (1000 mg/kg bw/d).
- Conducted prior to 1981 (the date both OECD guidelines were created).
- Apparently equivalent to OECD TG 407 or TG 408 but given a Klimisch score 3 or 4 for whatever reason (A score of 3 or 4 indicates that there are problems with the data quality and reporting as well as not being GLP compliant, Klimisch et al., 1997).

For the purposes of this review, the 90-day result also had to conform to this. One substance (EC 931-203-0) was removed because there had been a bacterial infection in the test animals in the 90-day study such that the registrant was not sure of the biological significance of the effects seen. This further reduced the dataset to 25 substances with high quality 28-day and 90-day study summaries.

2.3.3. Acute toxicity profile

Substances were further excluded if they did not have test data (no instances) or had test data that met the criteria for classification for:

- Acute toxicity (by any route) (0 substances).
- Irritation (skin or eye) (5 substances).
- Skin sensitisation (2 substances, one of which was also a mild irritant).
- Genotoxicity (a positive alert *in vitro* that was not followed up with a negative *in vivo* result) and/or a positive *in vivo* result (0 substances).

The acute toxicity profile for each substance was reviewed using the information provided in the ECHA CHEM database. The registrant's summary reports were accepted as to whether they met the criteria for classification. The Classification and Labelling (CLP) database on the ECHA website was not used for this purpose as at the time it provided inconsistent information on the classification of substances. The final dataset of substances with low (sub)acute toxicity profiles with high quality datasets was 18 substances (10% of 182).

The 90-day study result was then reviewed to determine if the NOAEL was reported to be close to or greater than 1000 mg/kg bw/d.

3. Results

25 substances were reported to have a NOAEL in the 28-day study equal to or greater than 1000 mg/kg bw/d, see Table 2. These substances did not appear to have any common features, some were simple molecules, and others were complex UVCBs (of unknown or variable composition or biological origin). [Physical chemical properties for each substance can be found by typing in the EC number into the ECHA CHEM database at www.echa.europa.eu]. They included:

- Natural dietary components or substances that are metabolised to dietary components (e.g. EC 205-538-1);
- Endogenous molecules (e.g. EC 211-519-9);
- Simple mineral salts (e.g. EC 232-094-6).

Five of the substances were reported to be skin or eye irritants (only) and two were reported to be skin sensitisers (one of which was also a mild skin irritant). Out of the remaining 18 substances with a 'low (sub)acute toxicity profile' (i.e. no positive acute toxicity results), 16 (89%) also had a reported NOAEL close to or greater than 1000 mg/kg bw/d in the 90-day study.

For two of the 16 substances the NOAEL was not reported to be 1000 mg/kg bw/d but was very close. Substance EC 203-490-6 was reported with NOAEL of $>812 \leqslant 4113$ mg/kg bw/d, with no adverse effects at the highest dose of 5% in diet. For substance EC 211-519-9, the reported NOAEL was 968 mg/kg bw/d in females, 914 mg/kg bw/d in males (1.5% in diet). Minor effects were seen at the next dose which was 3000 mg/kg bw/d. We considered that these two substances could reasonably be considered to be of low toxicity.

For the two remaining substances, for various reasons, the reported NOEAL was not close to 1000 mg/kg bw/day.

A NOAEL of 500 mg/kg bw/d was reported for a 90-day rat study for substance EC 231-710-0, based on effects on clotting parameters at the highest dose level of 2000 mg/kg bw/d. These parameters were also investigated in the 28-day study but were not reported to have been affected by treatment and the NOAEL in the 28 day study was reported to be 2000 mg/kg bw/d (the highest dose level tested). The substance is vitamin E acetate and is known to be biologically active. It exists as a number of stereoisomers and different forms of the substance are known to have varying biological potency. The material tested in the 28-day and 90-day studies appears to have been of different composition in terms of stereoisomer content. The material used in the 90-day study is known to be of higher potency than that used in the 28-day study, potentially explaining the apparent discrepancy in the results of these studies.

A 90-day toxicity study performed in the rat with substance EC 619-383-6 reported a NOAEL of 1000 mg/kg bw/d for females but a NOAEL of 250 mg/kg bw/d for males. The substance was shown to cause renal toxicity in both sexes at the highest dose level of 1905 mg/kg bw/d and also in one of ten male rats at 1000 mg/kg bw/d. Similar effects were apparent in rats of both sexes at the highest dose level of 2500 mg/kg bw/d in the 28-day studies, but were not seen in either sex at 1000 mg/kg bw/d. A NOAEL of 1000 mg/kg bw/d was therefore proposed for the 28-day study. While a simple comparison of NOAEL values indicates an increase in toxicity following 90-day exposure compared to 28-day exposure, more detailed analysis of the data shows that the increase in toxicity is in fact marginal, and may reflect biological variation. This substance is however used as a pesticide so is considered to be biologically active.

The NOAEL in the 90-day study was also equal than or greater than 1000 mg/kg bw/d for four of the five skin/eye irritants. For one (EC 203-874-3, 2,2′-sulfanediyldiethanol or thiodiglycol), the registrant reported the NOAEL to be 500 mg/kg bw/d because minor effects on body weight and kidney weight were seen at the next dose which was five times the limit dose; 5000 mg/kg bw/d. The study does not definitely demonstrate an absence of toxicity at 1000 mg/kg bw/d as this dose level was not tested; however it would seem unlikely that any toxicity would have been seen at this dose level.

Table 2
Details of the 25 substances identified to have a NO(A)EL of 1000 mg/kg bw/d in an adequate quality 28 day repeated dose toxicity test. More details on the substances can be found via ECHACHEM database www.echa.europa.eu.

EC No.	28 day result	90 day result	Skin sensitisation	Acute tox – oral	Acute tox – dermal	Acute tox – inhalation	Skin irritation	Eye irritation	Mutagenicity – *in vitro*	Mutagenicity – *in vivo*	Comments
201-148-0	(1985) 90 day with interim sacrifice at 28 days NOAEL = 1000 mg/kg bw/d; (1983) Exp NS: LD0 $\geqslant$ 610 mg/kg bw/d (highest dose tested), no mortalities observed, no details on signs of systemic toxicity to determine NOAEL	(1991) NOAEL = 1000 mg/kg bw/d; (1985) NOAEL > 1450 mg/kg bw/d (highest dose tested)	QSAR WoE: not sensitising	LD50 = 3350 mg/kg bw in females, >2830 mg/kg bw in males – toxicity category 5	LD50 = 2460 mg/kg bw in females, >2000 mg/kg bw in males – toxicity category 4	LC50 > 18.18 mg/L air, 6000 ppm	Exp WoE: mixed: corrosive/not irritating	Irritating – category 1 (irreversible effects on the eyes)	Negative – Ames test/mammalian cell mutation assay/*in vitro* mammalian micronucleus test	Negative – micronucleus assay	Skin and eye irritant
203-490-6	(2001) NOAEL > 5771 mg/kg bw/d (highest dose tested)	(2001) No NOAEL identified – between > 812 $\leqslant$ 4113 mg/kg bw/d, at highest dose 5% in diet no adverse effects were seen. Slight effect: some hepatic and haematological changes	Not sensitising – GPMT	LD50 = 11179 mg/kg bw – not classified	N/A	N/A	Not irritating	Not irritating	Negative – bacterial gene mutation assay/*in vitro* MCAT	Negative – micronucleus assay	90-day NOAEL close to 1000 mg, large dose ranges
203-874-3	(1993) NOAEL = 1000 mg/kg bw/d (highest dose)	(1997) NOAEL = 500 mg/kg bw/d, LOAEL = 5000 mg/kg bw/d (highest dose) (effects on body weight and kidney weight at highest dose)	Not sensitising – GPMT	LD50 = 10000 µl/kg bw, 11800 mg/kg bw	LD50 = 23600 mg/kg bw	No mortality in male and female rats exposed to saturated vapour at 20 °C for 8 h	Not irritating	Slightly irritating; very mild, reversible effects	Negative – Ames test/mammalian cell gene mutation assay/*in vitro* MCAT	Negative – micronucleus assay	Slight eye irritant, some effects in 90-day but large dose ranges
205-538-1	(1997) NOAEL = 4800 mg/kg bw/d in females, 5100 mg/kg bw/d in males (1997) NOAEL = 5300 mg.kg bw/d in males, 4900 mg/kg bw/d in females	(2007) NOAEL = 3170 mg/kg bw/d in males, 3620 mg/kg bw/d in females (2007) NOAEL = 1500 mg/kg bw/d in dogs	Not sensitising – GPMT	LD50 = 17.3 g/kg bw in males, 15.8 g/kg bw in females – not classified	LD50 > 2000 mg/kg bw – not classified	N/A	Not irritating	Not irritating	Negative – Ames test/*in vitro* chromosome aberration test	Negative – micronucleus assay	
207-312-8	(1983) NOAEL = 2000 ppm in diet, at the next higher level of 20,000 ppm only slight changes of doubtful toxicological significance (1991) NOEL = 900 mg/kg/d (dogs)	(1985) NOAEL > 24000 ppm (1991) NOAEL 2.5% in diet	Not sensitising – GPMT	LD50 > 10,000 mg/kg bw – Practically nontoxic	LD50 > 2000 mg/kg bw – relatively harmless	LC50 > 259 mg/m^3 (dust) (highest dose tested)	Not irritating	Not irritating	Negative – Ames test	N/A	
208-764-9	(1990) NOAEL $\geqslant$ 1500 mg/kg bw/d	(1991) NOAEL $\geqslant$ 1000 mg/kg bw/d (highest dose tested)	Not sensitising – LLNA/GPMT	LD50 > 5000 mg/kg bw – not classified	LD50 > 2000 mg/kg bw – not classified	LC50 = 8.67 mg/L (aerosol) – toxicity category 3; mortality seen at high concentrations	Not irritating	Not irritating	Negative – Ames test/mammalian cell gene mutation assay/*in vitro* MCAT	Negative – micronucleus assay/unscheduled DNA synthesis	Mildly acutely toxic by aerosol
211-519-9	(1998) NOEL = 1940 mg/kg bw/d in females, 1965 mg/kg bw/d in males (2% in diet) (highest dose tested)	(2003) NOAEL = 968 mg/kg bw/d in females, 914 mg/kg bw/d in males (1.5% in diet) (next highest dose 3000 mg/kg bw/d, minor effects seen)	Not sensitising – GPMT	LD50 = 10600 mg/kg bw – practically nontoxic	N/A	LC50 > 5.51 g/m^3 – practically nontoxic	Not irritating	Not irritating	Negative – Ames test/mammalian cell gene mutation assay/*in vitro* chromosome aberration test	N/A	

213-879-2	(2005) NOAEL = 1000 mg/kg bw/d (highest dose tested)	(2006) NOAEL = 1000 mg/kg bw/d (highest dose tested)	Not sensitising – GPMT/LLNA	LD50 > 10000 mg/kg bw – not classified	LD50 > 2000 mg/kg bw – not classified	LC50 > 3.1 mg/L air – not classified	Not irritating	Not irritating	Negative – Ames test/ mammalian cell gene mutation assay/*in vitro* chromosome aberration test	Negative – micronucleus assay	
221-424-4	(2005) NOAEL = 1000 mg/kg bw/d (highest dose tested)	(2009) NOAEL = 1000 mg/kg bw/d (highest dose tested)	Not sensitising – LLNA/GPMT	LD50 > 5000 mg/kg bw – not classified	LD50 > 2000 mg/kg bw – not classified	LC50 > 3.1 mg/L (dust) – not classified	Not irritating	Not irritating	Negative – Ames test/ mammalian cell gene mutation assay	Negative – micronucleus assay	
231-493-2	(1987) NOAEL = 4160 mg/kg bw/d (highest dose tested), only reversible effects	(1990) NOAEL = 10% in diet (10,0000 ppm; 10,000 mg/kg bw/d) (highest dose tested)	Not sensitising – GPMT	LD50 > 5000 mg/kg bw – practically nontoxic	LD50 > 2000 mg/kg bw – relatively harmless	LC50 $\geqslant$ 4.9 mg/L air (dust)	Not irritating	Slightly irritating	Negative – Ames test/ mammalian cell gene mutation assay/*in vitro* MCAT	Negative – micronucleus assay/ Drosophila SLRL test	Slight eye irritant
231-710-0	(1999) NOAEL = 2000 mg/kg bw/d, some effects were seen but dismissed as not of toxicological significance	(1986) NOAEL = 500 mg/kg bw/d (severe toxic effects at next level of 2000 mg/kg bw/d) (1982) NOAEL > 360 mg/kg bw/d (highest dose tested in dogs) (2000) NOAEL > 2000 mg/kg bw/d (highest dose tested in minipig)	Not sensitising – photoallergenicity test/Draize test	LD50 > 10000 mg/kg bw – not classified	LD50 > 3000 mg/kg bw – not classified	N/A	Not irritating	Not irritating	Negative – Ames test/ *in vitro* chromosome aberration test	Negative – micronucleus assay	Vitamin E acetate, species differences in 90-day results
232-094-6	(2010) NOAEL > 1000 mg/kg bw/d (2009) NOAEL > 1500 mg/kg bw/d (highest dose tested)	(1993) NOAEL = 5% (12830 mg/kg bw/d) in females, 2.5% (5410 mg/kg bw/d) in males (2000) NOAEL > 0.5% (308 mg/kg bw/d)	Not sensitising – GPMT	LD50 > 5000 mg/kg bw – not classified	LD50 > 2000 mg/kg bw – not classified	N/A	Not irritating	Not irritating	Negative – mammalian cell gene mutation assay/*in vitro* MCAT	N/A	
232-482-5	(1985) NOAEL = 1% in feed (highest dose tested)	(1991) NOAEL = 2500 mg/kg w/d (highest dose tested) (1982) NOAEL = 1% in diet (1989) NOAEL = 10,000 ppm for male and female rats (which corresponds to 714.0 and 831.0 mg/kg bw/d (Ester Gum-CGR) and 713.5 and 815.0 mg/kg bw/d (Ester Gum-PGR) in males and females, respectively)	Not sensitising – GPMT	LD50 > 2000 mg/kg bw - not classified	LD50 > 5000 mg/kg bw – not classified	N/A	Not irritating	Slightly irritating	Read across: negative – Ames test/ mammalian cell gene mutation assay/*in vitro* MCAT	N/A	Slight eye irritant
235-186-4	(2004) NOAEL = 2214.5 mg/kg bw/d, effects on body weight at 4228.5 mg/	(2004) NOAEL = 1695.7 mg/kg bw/d, effects on body weight seen at	Not sensitising – GPMT	LD50 > 1410 mg/kg bw	LD50 > 2000 mg/kg bw – read across	N/A	Not irritating	Irritating	Negative – Ames test/ mammalian cell gene	Negative – micronucleus assay	Eye irritant

(*continued on next page*)

Table 2 (*continued*)

EC No.	28 day result	90 day result	Skin sensitisation	Acute tox – oral	Acute tox – dermal	Acute tox – inhalation	Skin irritation	Eye irritation	Mutagenicity – *in vitro*	Mutagenicity – *in vivo*	Comments
	kg bw/d	3372.6 mg/kg bw/d							mutation assay/*in vitro* chromosome aberration test		
247-148-4	(1997) NOAEL = 1000 mg/kg bw/d (highest dose tested), although some effects on the liver	(2001) NOAEL = 1000 mg/kg bw/d (highest dose received)	Not sensitising – GPMT/LLNA	LD50 > 10000 mg/kg bw – not classified	LD50 > 20000 mg/kg bw – not classified	LC50 > 202.14 mg/L air – not classified	Not irritating	Not irritating	Negative – Ames test/ mammalian cell gene mutation assay/*in vitro* chromosome aberration test	Negative – micronucleus assay	Substance is identified as PBT and is on the Authorization list for bioaccumulation
259-910-3	(1990) NOAEL = 1000 mg/kg bw/d	(1992) NOAEL = 1000 mg/kg bw/d	Not sensitising – Buehler test	LD50 = 3100 mg/kg bw in males, 2600 mg/kg bw in females – not classified	LD50 > 2000 mg/kg bw – not classified	N/A	Not irritating	Not irritating	Negative – Ames test	Negative – micronucleus assay	
284-366-9	(1991) NOEL $\geqslant$ 1250 mg/kg bw/d (highest dose tested)	(1992) NOAEL > 1000 mg/kg/day (highest dose tested)	Not sensitising – GPMT	LD50 > 5000 mg/kg bw – practically nontoxic	LD50 > 2000 mg/kg bw – practically nontoxic	N/A	Not irritating	Not irritating	Negative – Ames test/ *in vitro* chromosome aberration test	N/A	On CoRAP list for PBT/ environmental concerns
425-220-8	(1997) NOAEL = 1000 mg/kg bw/d, NOEL = 1000 mg/kg bw/d (oral) (highest dose tested)	(2010) NOAEL $\geqslant$ 1000 mg/kg bw/d (highest dose tested)	Not sensitising – GPMT	LD50 > 2000 mg/kg bw – not classified	LD50 > 2000 mg/kg bw – not classified	N/A	Not irritating	Not irritating	Negative – Ames test/ mammalian cell gene mutation assay/*in vitro* chromosome aberration test	N/A	
475-290-9	(1992) NOEL > 1000 mg/kg bw/d	(2008) NOAEL = 3601 mg/kg bw/d in females, 3129 mg/kg bw/d in males	Not sensitising – GPMT	LD50 > 5110 mg/kg bw – not classified	LD50 > 2000 mg/kg bw – not classified	N/A	Not irritating	Not irritating	Negative – Ames test	N/A	
500-033-5	(1984) NOEL > 1000 mg/kg bw/d	(2000) NOAEL = 50 mg/kg bw/day, decreased body weights at doses higher than 250 mg/kg bw/d	Sensitising: LLNA	LD50 > 15000 mg/kg bw – toxicity category 5	LD50 > 20 mL/kg bw – toxicity category 4	LC0 = 0.000008 ppm (saturated atmosphere)	Slightly irritating	Not irritating	Positive – mammalian cell gene mutation assay, negative – Ames test	Negative – dominant lethal assay	Skin sensitizer and skin irritant
500-183-1	(1994) NOAEL = 6245 mg/kg bw/d in males, 6771 mg/kg bw/d in females	(1994) NOAEL = 1000 mg/kg bw/d (1995) NOAEL = 4159.4 mg/kg bw/d males, 4619.9 mg/kg bw/d females	Not sensitising – GPMT	LD50 > 5000 mg/kg bw – not classified	Read across: LD50 > 2000 mg/kg bw – not classified	LC50 > 5.2 mg/L air – not classified (aerosol)	Not irritating	Not irritating	Negative – Ames test	Read across: negative – micronucleus assay	
619-383-6	(1988) NOAEL = 1000 mg/kg bw/d (toxicological	(1987) NOAEL = 1000 mg/kg bw/d (females),	Not sensitising – GPMT	LD50 > 5000 mg/kg bw –	LD50 > 2000 mg/kg bw –	Effect level = 1.2 mg/L air – not classified	Not irritating	Not irritating	Negative – Ames test/	Negative – micronucleus	

	effects at 2500 mg/kg bw/d)	250 mg/kg bw/d (males); effects on kidneys seen in one male at 1000 mg/kg/bw/d (1988) NOEL = 1000 mg/kg bw/d, LOEL = 5000 mg/kg bw/d (mice) (1989) NOAEL 500 mg/kg/day (females), no NOAEL identified for males, effects on kidneys and liver at 500 mg/kg bw/d (dogs)		practically nontoxic	acute dermal toxicity classified as low				*in vitro* chromosome aberration test	assay	
700-073-5	(2009) NOAEL = 2000 mg/kg bw/d (highest dose tested)	(2010) NOAEL $\geqslant$ 2000 mg/kg bw/d (highest dose tested)	Not sensitising – LLNA	LD50 > 2300 mg/kg bw – practically nontoxic	N/A	N/A	Not irritating	Not irritating	Negative – Ames test/ mammalian cell gene mutation assay	Negative – micronucleus assay	
931-269-0	(2008) NOAEL = 1000 mg/kg bw/d (highest dose)	(1999) NOAEL = 1000 mg/kg bw/d, some haematological effects at this dose which were dismissed	Not sensitising – GPMT	LD50 > 2000 mg/kg bw – not classified	LD50 > 2000 mg/kg bw – not classified	Read across: LC50 > 2100 mg/m^3 air – not classified	Not irritating	Not irritating	Negative – Ames test/ *in vitro* MCAT	Negative – micronucleus assay	
932-215-9	(1993) NOEAL = 51100 mg/kg bw/d in males, 5221 mg/kg bw/d in females (1992) 3 mL/kg bw/d	(2003) NOAEL = 3000 mg/kg bw/d in males, 3300 mg/kg bw/d in females	2/3 LLNA test were sensitising probably should be classified	LD50 > 5000 mg/kg bw	LD50 > 2000 mg/kg bw	N/A	Not irritating	Not irritating	Negative – Ames test/ *in vitro* MCAT/ mammalian cell gene mutation assay	Negative – micronucleus assay	Possible skin sensitiser

Abbreviations: GPMT, guinea pig maximisation test; LLNA, local lymph node assay; MCAT, mammalian chromosome aberration test; NOAEL, No Observed Adverse Effect Level; NOEL, No Observed Effect Level.

4. Discussion

4.1. Overall result

This is the first study we are aware of that utilises the ECHA CHEM database to evaluate the utility of a toxicological endpoint from the perspective of the 3Rs.

Out of a total of 182 substances with a 28-day and a 90-day study via the oral route in rats, a total of 18 substances had a NOAEL in a high quality 28-day study of equal to or higher than 1000 mg/kg bw/d and a profile of low toxicity in all other acute tests. This constituted just 10% of the 182 substances with both studies. All studies had a Klimisch score of 1 or 2 which indicates that they are either GLP compliant guideline studies or otherwise high quality reports. Most of the substances were excluded because they had a reported NOAEL of less than the limit dose, or the NOAEL (or NOEL) could not be identified. A small proportion of these had reported no toxic effects at the highest dose level tested which happened to be less than 1000 mg/kg bw/d. On a precautionary basis it was decided to exclude these substances, as the reasons for not giving the limit dose were not available. As one might expect, none of the substances with low toxicity in a high quality 28-day study were reported to be acutely toxic either. This was also found in the HSE NONS study.

In 89% of cases where the substance had a profile of low toxicity in sub-acute (28-day) studies the substance also had a profile of low toxicity in the subchronic (90-day) study, that is, a 90-day study NOAEL close to 1000 mg/kg bw/d (16 out of 18 substances). The limit dose of 1000 mg/kg bw/d is a pragmatic value introduced to prevent the use of excessive dose levels in toxicity studies, which would be likely to result in effects of no relevance to the human risk assessment. The conduct of the 90-day study in these cases did not add to the hazard assessment of the substance.

On a precautionary basis, skin and eye irritants (as well as two skin sensitisers) were excluded from the 'low toxicity profile' as suggested by the HSE NONS review. However, four out of five substances with some degree of skin or eye irritation but showing low toxicity in the 28-day study also showed low systemic toxicity in the 90-day study. One substance had an equivocal result but could also be considered as showing low toxicity in the 90-day study. In the HSE NONS review, in fact, permitting substances with any alert for mutagenicity, skin sensitisation and skin irritation did not change the 100% prediction of low toxicity in the 90-day study. Including substances with irritation potential expands the proportion of substances for which the 'low (sub)acute toxicity profile' applies and does not affect the prediction. Irritation effects do not seem to necessarily indicate biological activity and could be a result of pH or mechanical effects (especially for eye irritation). In addition, local (site of contact) irritant effects tend to be disregarded in oral studies performed using oral (gavage or dietary) exposure as not of relevance to the risk assessment. It is also worth noting that the results of eye irritation tests *in vivo* in particular have been shown to be notoriously unreliable and inaccurate (Ohno et al., 1999; Lordo et al., 1999; Weil and Scala, 1971) and there is therefore a risk that genuinely non-irritant (and non-toxic) substances may be unnecessarily excluded from the 'low toxicity' rule by relying on the results of this particular *in vivo* test.

The results from the two substances where the reported 90-day NOAEL was not close to 1000 mg/kg bw/d did not in fact disprove the low toxicity hypothesis as the conclusions made by the registrant may have been too conservative, due to different stereoisomers being tested or the wide dose ranges used. However, these substances could have been considered in advance to be 'biologically active'; substance EC 231-710-0 is actually vitamin E acetate and EC 619-383-6 is a pesticide. On a conservative basis from this analysis it could be argued that such substances should not be considered to fit the 'low toxicity profile' anyway.

4.2. Suggested criteria for determination of the 'low (sub)acute toxicity with high quality data' profile

We therefore conclude that, if a substance fulfils the following criteria for a profile of 'low (sub)acute toxicity with high quality data', then the 90-day study is likely to be redundant:

- Experimental data equivalent to OECD TG 407 28-day oral toxicity in rats, on the substance itself, conducted in 1981 or later, with Klimisch reliability score 1 or 2 and conducted to the limit dose (1000 mg/kg bw/d) or beyond, with a study result reported to be a NOAEL of 1000 mg/kg bw/d or higher.
- The substance is not reported to be mutagenic or a skin sensitiser or acutely toxic by any route and there are adequate data to support this (i.e. any positive results from *in vitro* mutagenicity tests are followed up).
- There is no additional evidence based on physical chemical properties, structure or use that the substance could be biologically active.

As adjusted therefore, this profile was found for 21 substances and held true in the 90-day study for 20 (95%) substances, with one outlier being equivocal due to the large range of test doses applied.

4.3. The 'low toxicity' substances

Statistically, there will always be a subset of substances with a low toxicity profile and while it is apparent in this dataset that there is no single structural or physicochemical property of these substances that would predict the profile, the majority of substances did show properties which would indicate low toxicity. For some of the substances, low toxicity can be explained by low oral bioavailability which could have been predicted as a consequence of solubility or molecular weight (e.g. EC 213-879-2; EC 221-424-4; EC 500-183-1, EC 232-482-5); others could be predicted to be subject to rapid metabolism and/or urinary excretion, with limited systemic exposure (e.g. EC 619-383-6); others are naturally occurring dietary components (e.g. EC 211-519-9) and therefore already known to be of low toxicity. For some substances, however, limited testing appears to have been necessary to determine the toxicity profile. It is therefore premature to suggest structures that could predict the low toxicity profile.

4.4. Animal reduction possibility

Our analysis supports the HSE NONS review in that substances with the low toxicity profile tend to be of low prevalence among general industrial chemicals. Within both datasets of substances with both 28-day and 90-day study results the prevalence of low toxicity is in the range of 10–15%. Prior to REACH, which now requires a 90-day study as a standard information requirement for high production substances, companies would perhaps have made a scientific judgement that a 90-day study on a low toxicity substance was not worthwhile. Therefore the 'low toxicity profile' could be higher in the real world.

Given the thousands of substances to be registered under REACH, non-conduct of approximately 15% of 90-day oral studies, however, could still achieve a significant reduction in cost and animal usage, without compromising human health protection. On 1st June 2013, a further 2923 substances were registered as being manufactured or imported in quantities of 100 tonnes or more

per year (ECHA, 2013). These are Annex IX substances and will also require a 90-day study if one has not already been performed or can be waived. The 90-day study uses a minimum of 100 animals and costs an estimated 116 k Euros (Fleischer, 2007). Non-conduct of the 90-day study for 15% of these substances would therefore result in a saving of 43845 animals and cost reductions of over 50 million Euros. Serious consideration of the added value of the conduct of the 90-day study for substances with a 'low toxicity in a high quality dataset' would align with one of the policy aims of REACH to minimise animal testing and to promote alternative methods of obtaining data. It has the added benefit of reducing the costs for registration.

4.5. How to waive the 90-day test in REACH registrations

Two independently conducted reviews of different substances have now concluded that a 'low (sub)acute toxicity profile' is predictive of low toxicity in the sub-chronic 90-day oral study in rats. Substances that appear to fit the profile should be seriously considered as candidates for waiving the 90-day study under REACH (or indeed any other regulatory regime for that substance). Column 2 of Annex IX 8.6.2 already permits the waiving of the 90-day study if ''the substance is unreactive, insoluble and not inhalable and there is no evidence of absorption and no evidence of toxicity in the 28-day 'limit test', particularly if such a pattern is coupled with limited human exposure''. This is a high hurdle to clear and not all the substances included in this review would meet this definition. There may be particular difficulty, for example, in demonstrating limited human exposure or lack of absorption. We suggest that REACH registrants will have to use Annex XI Section 1.2 'weight of evidence' arguments in their submission, referring not only to the 'low (sub)acute toxicity in a high quality dataset' profile and analysis presented here but to substance-specific arguments that support this. This may include the results of other existing subacute or sub-chronic tests, the results of (Q)SARs, whether the substance is 'natural', and whether its structure and physicochemical properties already give confidence that the substance is not bioavailable. *In vitro* absorption tests could also support the waiving of the test if the suspicion is that the substance is unreactive due to lack of absorption (e.g. *in vitro* skin absorption OECD TG 428, OECD, 2004 and *in vitro* gastrointestinal absorption methods using Caco-2 cells, see van Breemen and Li, 2005). In the meantime we strongly urge ECHA and the European Commission to consider the implications of this survey and that of the HSE NONS review, issue guidance and adjust the REACH Annexes, which appear overly-strict in this respect.

4.6. Limitations of the survey

A limitation to both this study and the HSE NONS review is that the review was not done blind. That is, we had awareness of the result of the 90-day study at the point of making the comparison. This is because we had to be sure that we had 90-day studies of sufficient quality to enter into the dataset. In addition, because of the way the data is presented in the ECHA CHEM database the person reviewing the data could see all the test results. We have been transparent in our treatment of the data but future analyses should seek to test our final criteria 'blinded' and follow as much as possible the principles of (retrospective) 'validation', described by the OECD (OECD Guidance Document 34, OECD, 2005). One way to do this would be to ensure that one person creates the database of substances, removes the 90-day study information so that a second person can make predictions based on the criteria developed here, and then compares these to the results of the 90-day studies that have been verified by a third person who is unaware of all other information.

An element of expert judgment will always be required to determine whether the substance fits the 'low (sub)acute toxicity in a high quality dataset' profile. This is because it is necessary to check the (robust) study summary to ensure that the registrant was correct to come to the conclusion they did on the acute toxicity test results and the results of the 28-day study. There is always an element of opinion on whether some findings are biologically relevant or can be dismissed. It may be that for some substances the NOAEL from the 28-day should be adjusted. In addition, there is other information on the substance that should be considered, such as its use, structure, physical chemical properties and any other test results, if available, that would enable the expert to explain why the substance may be of low toxicity.

Studies that used excessively large doses in the top group significantly affected our ability in some cases to come to a conclusion about low toxicity, including for the one remaining outlier. Because of the wide dose ranges either side of the limit dose (defined as our cut off for 'low toxicity') the NOAEL was sometimes lower than 1000 mg/kg bw/d, since effects were (unsurprisingly) seen at the top dose. In this way the test led to a conservative NOAEL. Had the study been conducted up to the limit dose it is entirely feasible that several of the substances would have a NOAEL of 1000 mg/kg bw/d. The use of doses in excess of the limit is of general concern aside from this survey. Excessive doses have animal welfare implications in that (severe) adverse effects will almost certainly be experienced. They are also unnecessary as excessive doses usually result in effects of no relevance to the human risk assessment since such (relative) exposures in humans will never be seen. Internationally agreed OECD test guidelines for both the 28-day and the 90-day oral study permit the use of the limit dose to avoid the use of excessive doses unless human exposure indicates the need for a higher dose level to be used.

4.7. Scope of the recommendation

The MSC expressed concern about the use of old studies in the HSE NONS analysis, even though this did not influence the results (ECHA, 2011). Their concern was that the original OECD TG407 (the key, oral route, 28-day guideline, OECD, 2008) was created in 1981 and revised in 1995 to include more parameters and therefore that studies conducted prior to these dates would not be so sensitive. However, the fact that the prediction model works with old data actually strengthens it, since if old 28-day studies are not very sensitive then there are in theory *less likely* to predict the 90-day result, whereas they predict low toxicity in the 90-day study very well. What is important in the reviews demonstrating the validity of the 'low toxicity profile' is that the 90-day study (TG 408, OECD, 1998) is conducted to modern standards, not necessarily the 28-day study. Nonetheless we responded to the MSC concerns by omitting studies that at least predated the creation of the OECD TG (i.e. studies older than 1981) and ensuring high quality (Klimisch) scores for both 28-day and 90-day studies.

It does appear that those substances with a low (sub)acute toxicity profile are of generally low toxicity in all other toxicity tests. Further analysis identified no additional hazards for these substances (e.g. carcinogenicity), giving confidence that relevant effects are not being missed by the non-conduct of the 90-day study. Indeed, since our analysis found that the 90-day study for these substances also gave a NOAEL of close to 1000 mg/kg bw/d then clearly the 90-day study did not identify any findings of concern that would warrant further investigation in chronic or carcinogenicity studies.

However, it should be noted that the recommendation to waive testing is currently limited to the 90-day study only and does not extend to reproductive studies such as the reproductive toxicity screening study, the prenatal developmental toxicity study or the

two generation reproductive toxicity study. Under REACH, a reproductive toxicity screening study (TG 421, OECD, 1995) is a requirement of Annex VIII, the same point at which the 28-day study is considered and prior to consideration of the need for a 90-day study (see Table 1). For substances for which a 90-day is mandatory (Annex IX), a prenatal developmental toxicity test is also mandatory, so reproductive concerns would need to be considered – separately – at the same stage anyway. A two-generation reproductive toxicity test is required if effects on reproductive tissues are seen in either the 28-day or 90-day test. Since both 28-day and 90-day tests look at the reproductive tissues, only one of these is needed. A two generation reproductive toxicity test is also required at Annex X, irrespective of the presence or absence of any alerts. So, the non-conduct of the 90-day study should therefore really have no bearing on the consideration of reproductive effects.

Two substances used in this study are, however, of concern within the REACH context. EC 247-148-4 is on the candidate list for persistent, bioaccumulative and/or toxic (PBT) concerns, namely bioaccumulation; EC 284-366-9 is on the Community Action Rolling Plan list for its environmental PBT properties. In the context of REACH, persistence, bioaccumulation, reproductive and ecotoxicity concerns would be identified in other tests and therefore have no impact on the applicability of this approach.

4.8. Extending the profile using other data

When sufficient data becomes available it would be interesting to see if the low toxicity rule applies to tests conducted via the dermal and inhalation routes in addition to the oral route. We repeated the review using inhalation as the route and found that only 3 out of 71 gases and vapours with both studies satisfied the criteria for low toxicity in a high quality dataset (difluoromethane, EC 200-839-4; 1,1,1,2,2-pentafluoroethane, EC 206-557-8; 1,1,1-trifluoroethane, EC 206-996-5). All of these also showed no toxicity at the equivalent limit in the 90-day study by the inhalation route. We did not include them in this survey since the inhalation and dermal routes are complicated by site of contact effects as well as systemic toxicity. However, these initial findings suggest that the rule may also apply to the inhalation route but we appreciate that more data may be required.

Substances with results for the combined repeated dose/reproductive toxicity screening study (OECD TG 422, OECD, 1996) instead of the 28-day study (OECD TG 407) were not included in this analysis. A reproductive toxicity screening study (TG421) is a requirement of Annex VIII, the same point at which the 28-day study is considered (see Table 1). In this scenario, TG 422 is a useful animal reduction method. In the combined study males are dosed for at least 4 weeks and females longer, about 53 days. So the dosing length is equivalent to the 28-day study. It is possible therefore that the same rule may apply to a negative OECD 422 result. However, largely due to our desire to keep the analysis simple and the complicating fact that TG422 studies tend to be recorded as reproductive toxicity studies, necessitating an entirely separate eChemPortal search, we cannot make this judgement at this time.

We also restricted the database to only rat data to avoid the complication of species differences. Both the 28-day and the 90-day study tend to be conducted on rats; however there are occasions when mice are used. Some of the substances also had non-rodent data, shown in Table 2. It may be worthwhile expanding the dataset to any 28-day or 90-day test result but this would be for the purposes of expanding the dataset upon which to test the hypothesis only, as most substances would have rat data if they had any data at all.

4.9. Suggestions for future investigations

Given the apparently low prevalence of low toxicity substances with both 28 and 90-day study results, the profile is currently demonstrated with a low number of substances. Clearly the difficulty lies in obtaining databases of substances that have good quality data from both the 28-day and 90-day studies and show low toxicity.

Databases of cosmetics may be a good source of non-toxic substances, however, the EU based CosIng (see http://ec.europa.eu/consumers/cosmetics/cosing/) is not a complete toxicological database. The opinions of the European Commission's Scientific Committee for Consumer Safety (see http://ec.europa.eu/health/scientific_committees/consumer_safety/opinions/index_en.htm), which include study summaries, are usually on substances with potential toxicity issues. Another option may be the REACH Annex IV substances, of which there are over 30, which are substances that do not need to be registered due to no toxicological activity and include naturally occurring substances such as glucose and neon. REACH consortia that may have unused data or data on substances not yet registered are also encouraged to come forward with more data.

Following the submission of the Annex IX substances in June 2013, however, it is likely that the ECHA CHEM database will soon be populated with more substances that have both 28-day and 90-day study results. One recommendation is therefore to redo the analysis on substances not yet used in this or the HSE NONS study, using the criteria developed here. It could be broadened to allow the results of the combined repeated dose/reproductive toxicity screening study (TG422) to be considered in addition to the 28-day study. In addition, rather than focusing on the low toxicity profile alone, it may be of interest to quantitatively correlate the NOAELs of all substances with both 28 and 90-day study results. Although it is generally assumed that the NOAEL decreases with increasing study duration, to date there has been no statistical analysis of this using a large dataset of industrial chemicals. This could help confirm the 'low (sub)acute toxicity rule' as well as provide information on the overall relationship between the 28 and 90-day study. This would be similar to the study by Bulgheroni et al. (2009) that looked at the relationship between the acute oral toxicity test and the 28-day study in an effort to see if the NO(A)EL in the 28-day study could retrospectively predict toxicity in the acute study.

4.10. Prediction of results from substances with 90-day studies proposed

Another way to test the 'low toxicity' hypothesis is to make a prediction of the 90-day study result immediately before it is conducted. It is better in terms of animal welfare to avoid this kind of prospective validation but this scenario already exists as a consequence of the testing proposal system under REACH. From the first REACH deadline, by 1 September 2012 there were 114 substances that had testing proposals on the ECHA website for the 90-day study rather than submission of the data itself. We reviewed this dataset and identified 14 substances (12%) that appeared to fit the 'low toxicity profile in a high quality dataset' profile (see Table 3). These substances were then reviewed by one of the authors (Andrew) to apply a level of expert judgment to the reported results. They made a final conclusion that for 11 substances they would expect a 90-day study result to also be around 1000 mg/kg bw/d; three they were unsure about. The delay to the submission of this paper was done so as not to appear to be interfering with the decision making by the MSC on these substances, a process which typically takes at least a year. The test results of this sample will not be known until the end of 2014, but, should they

Table 3
Substances with 90-day studies proposed by 1 Sep 2012, fitting the low toxicity profile.

EC No.	28 day result, rat, oral	Skin sensitisation	Acute toxicity, oral	Skin irritation	Eye irritation	Mutagenicity – *in vitro*	Mutagenicity – *in vivo*	Comments
480-370-1	(2007) NOAEL = 1000 mg/kg bw/d	N/A	LD50 > 2000 mg/kg bw	Not irritating	Some evidence of irritation	Positive/ negative	Negative	The low water solubility of this substance may limit oral bioavailability. Data indicate rapid hydrolysis (<1 min) to form ethanol and polymeric reaction products, so any systemic absorption is likely to be due to ethanol
432-070-7	(2000) NOAEL = 1000 mg/kg bw/d	Not sensitising	LD50 > 2000 mg/kg bw	Not irritating	Some evidence of irritation	Positive/ negative	Negative	Some effects seen in the 28-day study at the limit dose of 1000 mg/kg bw/d, but these were not considered to be of toxicological significance and/or not clearly related to treatment
470-680-5	(2008) NOAEL = 1000 mg/kg bw/d kg/day, (2007) NOAEL = 1000 mg/ kg bw/d	Not sensitising	LD50 > 2000 mg/kg bw	Not irritating	Irritating, category 2	Negative	Not done	Two 28-day studies; one with minor/adaptive effects at the highest dose level of 1000 mg/kg bw/d; a second study performed at 2000 mg/kg bw/d shows effects on some parameters but these are disputed by an independent reviewer (but in any case are above the limit dose). UNSURE
204-111-7	(1995) NOAEL = 1000 mg/kg bw/d	Not sensitising	LD50 = 2900 mg/kg bw	Not irritating	Slightly irritating	Negative	Negative	Evidence of adaptive effects at the highest dose level of 1000 mg/kg bw/d in the 28-day study and renal effects in the male rat (only) are dismissed as non-relevant; justification for low toxicity would require a more detailed case for dismissing these effects. UNSURE
230-991-7	(1990) NOAEL = 1000 mg/kg bw/d, NOEL = 200 mg/kg bw/d	Not sensitising	LD50 = 4595 mg/kg bw – practically nontoxic	Not irritating	Highly irritating	Negative	Not done	Minor/reversible effects seen at the highest dose level of 1000 mg/kg bw/d in the 28-day study. NB substance is shown to cause developmental toxicity at dose levels not causing maternal toxicity
203-326-3	(2010) NOAEL = 1000 mg/kg bw/d, NOEL = 300 mg/kg bw/d	Not sensitising	LD50 > 2000 mg/kg bw	Not irritating	Not irritating	Negative	Not done	No effects of treatment at the limit dose of 1000 mg/kg bw/d in the 28-day study. OECD QSAR Toolbox predicts no bioavailability which would be consistent with the molecular weight and insolubility in water
211-074-0	(1995) NOAEL = 1000 mg/kg bw/d	Not sensitising	LD50 = 3000 mg/kg bw – practically nontoxic	Not irritating	Not irritating	Negative	Not done	Substance predicted to be bioavailable but rapidly metabolised and incorporated into normal metabolism
203-838-7	(1998) NOAEL = 1750 mg/kg bw/d	Not sensitising	Exp NS: LD50 = 8370 mg/ kg bw – practically nontoxic	Corrosive – category 1	Exp NS: highly irritating	Negative	Not done	Substance is classified for acute inhalation toxicity (borderline) but this is likely to be related to its corrosivity. Toxicity was seen at the highest dose level of 3500 mg/kg bw/d in a 28-day study; effects are most likely related to local irritation caused by gavage. NOAEL of 1750 mg/kg bw/d exceeds the limit dose. Substance is expected to be absorbed and rapidly metabolised (fatty acid)
426-040-2	(1997) NOAEL = 1000 mg/kg bw/d	Not sensitising	LD50 > 2000 mg/kg bw	Not irritating	Not irritating	Negative	Not done	No effects at the limit dose; substance not predicted to be bioavailable based on the molecular weight and low water solubility
641-136-6	(2010) NOEL = 1000 mg/kg bw/d, no NOAEL identified	Not sensitising	LD50 > 2000 mg/kg bw	Not irritating	Not irritating	Negative	Not done	No effects at the limit dose of 1000 mg/kg bw/d in the 28-day study; the substance is likely to be of limited or no bioavailability based on its low water solubility
271-237-7	(2010) NOAEL = 1000 mg/kg bw/d in females and 300 mg/kg bw/d in males	Not sensitising	LD50 > 2000 mg/kg bw	Not irritating	Not irritating	Negative	Not done	28-day reports a NOAEL of 300 mg/kg bw/d in males, apparently based on reduced weight gain. Other effects at the highest dose level are dismissed as adaptive or non-adverse. Substance is a UVCB and only a proportion of the components are likely to be bioavailable. However, the NOAEL in males cannot be disregarded. UNSURE
271-239-8	(2010) NOAEL = 1000 mg/kg bw/d in females and 300 mg/kg bw/d in males	N/A	LD50 > 5000 mg/kg bw	Not irritating	Slightly irritating category 2B	Negative	Not done	28-day NOAEL of 300 mg/kg bw/d for males based on a marginal effect on red blood cell parameters (increased erythrocyte count – not adverse?) at the highest dose level of 1000 mg/kg bw/d
404-370-8	(1995) NOAEL $\geqslant$ 1000 mg/kg bw/d	Not sensitising	LD50 > 2000 mg/kg bw	Irritating	Not irritating	Negative	Not done	Effects at the limit dose of 1000 mg/kg bw/d in the 28-day study are minimal/adaptive. The intact substance is unlikely to be bioavailable, however systemic exposure to the hydrolysis product is likely (hydrolysis at pH 4 is very rapid)
402-140-1	(1996) NOAEL $\geqslant$ 1000 mg/kg bw/d	Not sensitising	LD50 > 2000 mg/kg bw	Irritating – cat 2	Not irritating	Negative/ positive	Negative	Effects at the limit dose of 1000 mg/kg bw/d in the 28-day study are minimal/adaptive. The intact substance is unlikely to be bioavailable, however systemic exposure to the hydrolysis product is likely (hydrolysis at pH 4 is very rapid)

support our hypothesis then this should give significant weight to the validity of the 'low toxicity profile' approach.

5. Conclusion

A review of the ECHA CHEM database identified 18 substances with a 'low toxicity' result in the 28-day study and no reports of toxicity in other acute tests. Of these substances, 16 (89%) also showed no signs of toxicity at, or close to, the limit dose of 1000 mg/kg bw/d in the 90-day study. The conclusion therefore is that if a substance conforms to a number of criteria indicating a 'low (sub)acute toxicity profile' then the 90-day study may be redundant. Based on our analysis we would recommend that substances with only skin or eye irritation are not excluded from the 'low toxicity profile' since these substances appear to also show low toxicity in the 90-day study, but that substances that, based on their use or existing knowledge, are known to be biologically active should be excluded. Our suggestion is that substances are considered for this approach if they satisfy ALL of the following criteria:

- Experimental data equivalent to OECD test method TG 407, on the substance itself, conducted 1981 or later, with reliability score 1 or 2 and conducted to the limit dose (1000 mg/kg bw/d) or higher, with a study result reported to be a NOAEL of 1000 mg/kg bw/day or higher.
- The substance is not reported to be mutagenic or a skin sensitiser or acutely toxic by any route and there are adequate data to support this (i.e. any positive results from *in vitro* mutagenicity tests are followed up).
- There is no additional evidence based on physical chemical properties, structure or use to suggest that the substance could be biologically active.

Retrospectively, this low toxicity profile was found for 21 substances and held true in the 90-day study for 20 of these (95%), with the remaining substance being equivocal due to the large range of test doses applied. Avoidance of redundant 90-day studies for the remaining phase-in REACH substances could collectively avoid the use of nearly 50,000 animals and save industry 50 million Euros. Regulatory authorities and chemical companies must now consider if the 90-day study can be waived on a case by case basis for individual chemical substances on the basis of this 'low toxicity with high quality data' profile.

Conflict of interest

None declared.

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