"You ask about my opinion on vivisection. I quite agree that it is justifiable for real investigations on physiology; but not for mere damnable and detestable curiosity. It is a subject which makes me sick with horror, so I will not say another word about it, else I shall not sleep tonight."
Charles Darwin, Correspondence vol. 19, letter to E. R. Lankester, March 22, 1871

"Facts are stubborn things, but statistics are pliable."
Mark Twain, 1835-1910

Food for Thought ...

REACH Out-Numbered! The Future of REACH and Animal Numbers

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Abstract

The EU's REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) Regulation requires animal testing only as a last resort. However, our study (Knight et al., 2023) in this issue reveals that approximately 2.9 million animals have been used for REACH testing for reproductive toxicity, developmental toxicity, and repeated-dose toxicity alone as of December 2022. Currently, additional tests requiring about 1.3 million more animals are in the works. As compliance checks continue, more animal tests are anticipated. According to the European Chemicals Agency (ECHA), 75% of read-across methods have been rejected during compliance checks. Here, we estimate that 0.6 to 3.2 million animals have been used for other endpoints, likely at the lower end of this range. The ongoing discussion about the grouping of 4,500 registered petrochemicals can still have a major impact on these numbers. The 2022 amendment of REACH is estimated to add 3.6 to 7.0 million animals. This information comes as the European Parliament is set to consider changes to REACH that could further increase animal testing. Two proposals currently under discussion would likely necessitate new animal testing: extending the requirement for a chemical safety assessment (CSA) to Annex VII substances could add 1.6 to 2.6 million animals, and the registration of polymers adds a challenge comparable to the petrochemical discussion. These findings highlight the importance of understanding the current state of REACH animal testing for the upcoming debate on REACH revisions as an opportunity to focus on reducing animal use.

1 Introduction

We have once again (Knight et al., 2023) attempted to bring animal numbers into the policy discussion around Europe's Chemicals Regulation REACH. The American science fiction writer Daniel Keys Moran said, "*You can have data without information, but you cannot have information without data*". We shared a personal 20-year history of this journey earlier (von Aulock et al., 2022). Policy impact analysis is needed for evidence-based policy-making. Numbers are not always fun. "*Statistics are the triumph of the quantitative method, and the quantitative method is the victory of sterility and death*" is a quote from the Franco-English writer Hilaire Belloc (1870-1953). Bad policies are even less fun.

There is a tremendous difference between statistics and predictions. With more data becoming available, we can fine-tune predictions, but this requires us to look at where the forecasts were off.

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The first forecast of the number of animals that would be needed to fulfil the REACH requirements was compiled by van der Jagt et al. (2004). There were many reasons for this forecast to underestimate the number of animals that would be needed for REACH testing: data on chemical industry from 1991-1994 that included only 12 member states (Hartung and Rovida, 2009), over-optimistic expectations as to the use of (Q)SAR, etc. Our own analysis (Rovida and Hartung, 2009) was too impressed by the number of pre-registrations received by ECHA a year earlier and underestimated how many lower-tonnage substances were never registered and the (at least initial) use of read-across. We could also not foresee Brexit where an important part of European chemical industry exited the EU.

Already in 2011, we analyzed actual registrations relevant for the developmental and reproductive toxicity studies identified in the 2009 analysis (Rovida et al., 2011). Assessment of 400 randomly selected REACH dossiers and reports on the use of animal testing for reproductive and developmental toxicity found that animal testing was the primary method for assessing these toxicities, with few *in vitro* studies being used, that read-across opportunities were not being fully utilized, and that testing on a second species was more frequent in developmental than in reproductive toxicity testing. The report concluded that the contribution of read-across was probably underestimated and that the high number of animals that would be used for testing purposes in REACH was a cause for concern.

More recently, we studied cosmetics-only substances (Knight et al., 2021), which are in principle exempted from REACH. The Cosmetics Regulation ban on *in vivo* testing took effect on March 11, 2009 for skin and eye irritation, acute toxicity, and genetic toxicity, and on March 11, 2013 for all other human health endpoints. We identified 419 REACH dossiers for 413 unique substances in the European Chemicals Agency (ECHA) database. Of the 419 dossiers, 233 (55%) were for substances with volumes of 1-10 tons/year (REACH Annex VII) and 124 (30%) were for substances with volumes of 10-100 tons/year (REACH Annex VIII). 82 dossiers (20% of the 419 analyzed) contained no *in vivo* studies and fully relied on alternative methods for all human health and ecotoxicity endpoints considered in the dossier. However, 63 cosmetics-only substances had *in vivo* tests for human health endpoints done after the Cosmetic Regulation deadlines for *in vivo* tests for the respective endpoints, making a total of 104 tests. This suggests that most of these tests were performed to comply with REACH. While not directly relevant for the overall use of animals for REACH here, the study illustrates the complex relationships of different legislations and the difficulties of strategically reducing animal testing.

2 4.2 million and counting... the toll of REACH on animals used for systemic toxicity studies

REACH (Regulation EC 1907/2006) requires a registration dossier for all substances manufactured or imported into the EU in a quantity ≥ 1 ton per year (t/y). Among the many aspects of REACH, there is the request to receive a full toxicological and ecotoxicological characterization of each substance as well as requests that increase with the tonnage band of the registered substance with thresholds defined at 1 t/y (Annex VII), 10 t/y (Annex VIII), 100 t/y (Annex IX), and 1000 t/y (Annex X).

Before REACH approval in 2006 and its entering into force in 2008, there was a long discussion in the EU about its potential toll on animal numbers (van der Jagt et al., 2004; Rovida and Hartung, 2009), since most of the toxicological and ecotoxicological endpoints required new *in vivo* tests. After about 15 years, no further official assessment of the number of animals used for REACH purposes has been made. The regular ECHA reports on alternative methods are mainly focused on the application of alternative strategies to adapt the standard information requirements compared to experimental studies but with no reference to the number of animals used for the new experimental tests. Rovida et al. (2011) estimated the use of up to 1.6 million animals for reproductive toxicity tests for the substances registered in 2010 while Taylor (2018) concluded that over 2.2 million animals were used at the end of the third REACH deadline of 2018. No other real follow-up was registered, not even from the European Commission although the implementation of alternative methods is requested in Article 1 of REACH: "*The purpose of this Regulation is to ensure a high level of protection of human health and the environment, including the promotion of alternative methods for assessment of hazards of substances*".

To gain a more accurate picture of the situation, we have begun to count the number of animals that have been used for REACH purposes and released results for the endpoints counted to date, which are the three major endpoints for human systemic toxicity: repeated dose toxicity, reproductive toxicity, and developmental toxicity (Knight et al., 2023). The calculation of the number of animals used for these endpoints is based on animal data retrieved from the public ECHA database, which contains the publicly available part of the dossiers of the registered substances[1].

The analysis applied strict rules for the inclusion of studies in the calculation and was focused only to studies performed for the three human systemic toxicity endpoints to date. The evaluation of the other endpoints requiring *in vivo* tests would have taken too long to complete. According to previous estimates (Rovida and Hartung, 2009; Taylor, 2018), the three systemic toxicity endpoints are responsible for the majority of the animals used, so we believe that they provide a good picture of the total number of animals used for REACH purposes.

The summary of the number of animals already used for REACH purposes is presented in Table 1. In addition to these, many tests are scheduled or under way as a result of specific requests by ECHA. These tests primarily result from ECHA decisions related to testing proposal evaluation or other compliance checks performed directly by ECHA or by other committees involving representatives from all EU Member States. These ongoing tests are not yet present in the public database. The full list of

[1] https://echa.europa.eu/information-on-chemicals/registered-substances

Tab. 1: Total animal count for endpoints found in existing registration dossiers in the public ECHA database (December 2022) plus the animal estimate for pending reproductive toxicity, developmental toxicity, and sub-chronic toxicity tests
Pending tests are new tests required by an authorized testing proposal or a compliance check that were not yet present in the dossiers at the time of the analysis. Data from Knight et al. (2023).

Endpoints	Total animals used in tests
Reproductive toxicity	1,479,084
Developmental toxicity	1,292,023
Repeated dose toxicity	131,700
Total animals in submitted completed studies	**2,902,807**
Total animals in pending tests	**1,271,026**
TOTAL	**4,173,833**

approved tests is presented on the ECHA website[2]. Based on this list, the number of animals that are in use in ongoing studies was estimated, and the result is also shown in Table 1.

The main findings of the study on animal testing in REACH are as follows: 4.2 million animals have been used to fulfil the REACH requirement for the three systemic endpoints, repeated dose toxicity, reproductive toxicity, and developmental toxicity. Of these, approximately 2.9 million animals have been used for REACH testing as of December 2022 and additional tests requiring at least 1.3 million more animals are currently in progress. Compliance checks are ongoing, and it is anticipated that more animal tests will be required.

Readers might wonder why this is not leading to a visible increase in the EU animal use statistics. As discussed earlier (Busquet et al., 2020), the main reason is that pups are excluded from the statistics: "*What remains outside of the scope of annual statistical reporting, even if covered by the scope of the Directive, are: a) Foetal forms of mammals*" stated in the EU statistics, on which we commented "*Reproductive and developmental toxicity include far more pups than adult animals, e.g., a two-generation study treats only 20 male and 20 female, but in total on average 3,200 animals are involved in case of rats (factor 80) and 2,100 in case of rabbits (factor 53). Similarly, the one generation study OECD TG 414 treats 40 animals, but 784 rats (factor 20) or 560 rabbits (factor 14) are involved. The developmental toxicity screening test OECD TG 422 treats 20 animals but involves on average 412 (factor 21). Applying this to 140,513 animals for reproductive toxicity testing or 97,671 animals for developmental toxicity in 2017, several million animals would need to be added*". Another reason why there is no impact on EU animal use statistics is that many of these tests are performed outside the EU, typically in India or in the USA. This is due to a limited capacity of EU contract research organizations (CROs), lower costs of non-EU CROs, or trust in CROs that are routinely used by companies. With the American author Tim O'Reilly (1954-) we might conclude, "*Statistics are like bikinis. What they reveal is suggestive, but what they conceal is vital*".

3 Endpoints excluded from the Knight et al. (2023) evaluation

The Knight et al. (2023) study considers only data for the three systemic toxicity human health endpoints. Although these tests are responsible for most of the animals required to accomplish REACH provisions, we must not forget the contribution from other endpoints.

In the Rovida and Hartung (2009) analysis, 92% of all animals were expected to be used for the three endpoints covered in the Knight et al. (2023) report. There are many differences between that prediction and the situation today, but for a rough estimate of the contribution of other endpoints to REACH so far, we can use that assumption, leading to an estimated contribution for the other endpoints of 372,331 animals. This is without the increasing use of *in vivo* tests for "*in vitro* first" endpoints, implementation of the REACH amendments or the proposed REACH revision, as explained below.

When REACH was published in 2006, *in vivo* skin and eye irritation tests were requested starting from Annex VIII, and *in vivo* skin sensitization tests for all registered substances starting from Annex VII, including transported intermediates exceeding 1000 t/y. In 2016, REACH was amended to set *in vitro* as a first choice for those endpoints (Regulation EU 2016/863 and 1688 respectively). Nevertheless, the number of new *in vivo* tests is still increasing, even for cosmetic ingredients for which *in vivo* tests should be avoided according to Regulation EC 1223/2009 (Knight et al., 2021).

Other *in vivo* tests are for acute toxicity endpoints, which require a limited number of animals per test, but with a procedure that is generally quite severe (acute lethality), and for this reason, these tests should be avoided as much as possible, with specific indications in the ECHA guidance on information requirements R.7a (ECHA, 2017a) to reach that goal.

In vivo genotoxicity studies will probably increase in future as a consequence of a recent REACH amendment (Regulation EU 2022/477) that requires *in vivo* tests whenever one of the three *in vitro* tests requested in Annex VII and VIII is positive. This aspect is examined further in Section 5.

Other tests such as carcinogenicity or chronic tests were not analyzed in the main paper because they are rarely performed for REACH purposes. In fact, these tests are required only under exceptional circumstances, and their inclusion in the estimate would have had little effect, even if the number of animals involved per study is high.

Regarding environmental toxicity assessment, the use of fish in *in vivo* tests for REACH purposes is remarkable. When REACH was published, the fish acute test was included in An-

[2] https://www.echa.europa.eu/information-on-chemicals/dossier-evaluation-status; list downloaded 29 January 2023

Tab. 2: Estimate of animals used for REACH until 2022 for endpoints other than repeated dose toxicity, reproductive toxicity, and developmental toxicity

The number of animals per tonnage band is taken from Rovida and Hartung (2009). The number of registrations per tonnage band is taken from the ECHA evaluation report[a]. A simple multiplication leads to 3.2 million animals if all testing needs were satisfied by new tests. In the absence of better estimates, the over-optimistic assumptions of 2009 were applied, resulting in about 590,000 animals.

Breakdown by tonnage band	Number of animals for non-covered endpoints (2009 estimates) # of animals	REACH registration[a] # of substances	Animal use estimate for doing all tests # of animals	Animal use estimate with 2009 assumptions for actual tests # of animals
≥ 1000 t/y (Annex X)	651	2,353	1,531,803	264,006
100 - 1000 t/y (Annex IX)	577	2,334	1,346,718	236,201
10 - 100 t/y (Annex VIII)	69	2,738	188,922	58,046
1 - 10 t/y (Annex VII)	24	4,054	97,296	30,405
TOTAL			**3,164,739**	**588,658**

[a] https://echa.europa.eu/progress-in-dossier-evaluation

nex VIII, and the long-term test on fish plus the bioconcentration (BCF) test on fish started from Annex IX. This situation was changed by the recent amendment, Regulation EU 2022/477, as described in Section 5. The request for BCF may increase also as a consequence of Regulation 2023/707, an amendment to the Regulation on Classification, Labelling and Packaging (CLP, Regulation EC 1272/2008), which introduces new hazard classes for the environment, as described in Section 6. The calculation of the number of fish is not trivial as the final reports are usually based only on the number of eggs at the start of the test, and the number of adult fish at the end of the study must be extrapolated with a complex procedure. This aspect will need to be considered when the implementation of the revisions becomes visible in the ECHA public database, presumably in 8-10 years.

In addition to the standard tests, the evaluation program can ask for additional tests that can be either tests that are now included in Annex IX or X and are requested for Annex VII and VIII substances or other more specific tests such as OECD TG 426 for neurodevelopmental toxicity. The Endocrine Disruptor Assessment program[3] also requests many new *in vivo* tests, both for the human health and the environment sectors. This program is general, not restricted to REACH substances, and many substances fall under the scope of the Biocide Product Regulation (BPR), Regulation EU 528/2012, which controls the sale and use of all types of biocidal products used to protect humans, animals, materials, or articles against harmful organisms.

In the Rovida and Hartung (2009) analysis, 92% of all animals were expected to be used for the endpoints covered in the Knight et al. (2023) report. For a rough estimate of the contribution of other endpoints to REACH so far, a number of assumptions have to be made: No waiving, read-across or existing data were assumed; carcinogenicity studies were assumed for 1% of Annex X studies; and further mutagenicity studies were not included (discussed further below). Table 2 first shows that if all tests were carried out on these registrations, 3.2 million additional animals would be used. Using the 2009 estimates for existing data, QSAR, read-across and waiving, we end up at more than 590,000 animals. The latter is probably an underestimate given the low current acceptance of read-across and low use of QSAR. It therefore seems safe to assume that about 1 million animals must be added to the 4.2 million animals so far (+23%) for other endpoints.

3.1 Registration of petrochemicals and other UVCBs

Most safety evaluations conducted by industry or regulatory bodies concern "mono-constituent" substances, meaning those with a single major constituent making up at least 80% of the weight, even after considering impurities (ECHA, 2017b). Other substances are considered either multi-constituent substances or UVCBs (unknown or variable composition, complex reaction products, and biological materials). UVCBs account for approximately 20% of all recent substance registrations in the EU (ECHA, 2017c) under the REACH regulation. The regulatory decision-making process for UVCBs is complicated due to a lack of well-established evaluation frameworks within current chemical regulatory systems (ECHA, 2017c).

Petrochemicals serve as prime examples of UVCBs (Clark et al., 2013) with enormous production volumes: In 2022, 889 million tons of petroleum substances were registered for REACH (manufactured or imported into the EU)[4]. 96% of this tonnage is currently used as either fuel or as an intermediate in chemical synthesis. In terms of volume, petroleum substances represent ~25% of all chemicals placed on the EU market (Ketelslegers et al., 2020). They pose problems for REACH with their complex and varying nature resulting from their production processes including the distillation of petroleum feedstocks and

[3] https://echa.europa.eu/ed-assessment

[4] https://www.concawe.eu/reach/

possible additional processing steps such as solvent extraction, hydro-desulfurization, or hydrogenation (McKee et al., 2015). Consequently, these complex substances include a multitude of individual hydrocarbon molecules, which can be aliphatic/paraffinic (straight or branched chain), alicyclic/naphthenic (primarily comprising saturated cyclo-paraffinic constituents), or aromatic. Petrochemicals can significantly vary in their chemical complexity and diversity based on the source of raw material and degree of refinement containing any or all of these types of constituents in different concentrations, depending on the specific manufacturing process, often restricted to specific ranges determined by the technical specifications of each product. The task of categorizing petroleum substances is made more intricate by the current substance naming system, which is not directly linked to chemical composition but relies on the manufacturing process, related physical-chemical properties, and product performance specifications. By early 2019, there were 185 petroleum substances registered through ~4,500 unique registrations (Ketelslegers et al., 2020) in 18 categories[5]. Petroleum substances that are heavier and have high boiling points, starting from certain gas oils, contain increased levels of polycyclic aromatic constituents (PACs), which have a higher potential for inducing systemic toxicity as well as carcinogenic and reproductive toxicity (Roth et al., 2013; Feder and Hertzberg, 2013; McKee and White, 2014; McKee et al., 2014) and were included in California's Preposition-65[6]. Murray et al. (2013) in the context of the EPA High Production Volume (HPV) Chemical Challenge Program summarized reproductive toxicity data on high-boiling petroleum substances (HBPS) using the results of 39 repeated dose and 59 developmental rat dermal toxicity studies. They found clear evidence of developmental toxicity but low potential to produce male or female reproductive toxicity in rats. McKee and White (2014) summarized information on about 400 petroleum-derived substances in the same HPV program, concluding, "*Higher boiling substances may contain polycyclic aromatic constituents (PACs) that can be mutagenic and carcinogenic and may also cause developmental effects*".

Because of their high tonnage production levels, they fell under the 2010 REACH deadline, and several data gaps were identified, which were in part addressed by read-across, grouping, and test proposals (Concawe, 2019). However, ECHA expressed concern over the scarcity of available information on chemical composition and subsequently questioned the read-across assumptions in petroleum substance submissions (ECHA, 2020), leading to demands for additional analytical chemistry, further toxicology data to better characterize similarity, additional justification for the read-across, and addressing data gaps. The practice of grouping petroleum substances for regulatory decision-making and read-across traditionally hinges on the physical/chemical properties, manufacturing process, and similar end uses (McKee et al., 2015). However, due to the inherent chemical complexity of petroleum substances and the lack of regulatory guidance on what data could definitively show substance similarity, defining chemical groupings and applying read-across remains a challenge.

In 2016, ECHA formed the Petroleum and Coal Stream Substances Working Group (PetCo WG) together with representatives from the European Commission, EU member states, and industry stakeholders including Concawe (European Oil Company Organisation for Environment, Health and Safety)[7] with a mandate to develop an approach for the assessment of petroleum substances with potential substances of very high concern (SVHC) status (ECHA, 2017c). Concawe, a division of the European Fuel Manufacturers Association, addresses the challenge of reproductive toxicity testing on their website[4]: "*reproductive toxicity data is a standard requirement in REACH, comprising of two endpoints: i) Pre-Natal Developmental Toxicity (PNDT) and ii) fertility (more recently by an Extended One-Generation Reproductive Toxicity Study (EOGRTS)). As permission from ECHA is required before conducting a higher-tier toxicity test in vertebrate animals to satisfy these endpoints, in 2010 Concawe submitted testing proposals to conduct two PNDT and six EOGRT studies covering the identified data gaps in six substance categories firstly assessed including Gas Oil categories. A more recent re-evaluation of the Concawe dossiers indicated that further testing will be needed to address endpoints in additional petroleum substance categories. The current paradigm expects all testing proposals for non-CMR* [carcinogenic, mutagenic and reprotoxic] *substances to* [be] *submitted by 2030. This is part of the overall Concawe REACH strategy for Human Health, which consists of reducing and refining animal testing and the development (and application) of (in-vitro) alternative approaches e.g., to assess biological coherence and to strengthen RA justifications*."

Why does this matter? First, large amounts of these petrochemicals are traded: "*In Europe alone in 2018, around 600 million tonnes of refined petroleum products were manufactured and the refining industry collected some €281 billion of duties for EU economy and generated over €23 billion in added value to local and national EU economies (Concawe data, based on Eurostat and EU Commission data)*" (Ketelslegers et al., 2020). Tonnage is used in REACH to determine testing needs. Thus, the maximum testing needs apply to these products. Secondly, it is difficult to group or distinguish different petrochemical products. However, the number of distinct mixtures determines the resulting testing needs. ECHA has included petroleum substances in the restriction roadmap (pool 2)[8], which means that test data are critically important. The attempt to reduce the number of tests by grouping through biological similarity by House et al. (2022) did not really work out, showing that "*transcriptomics data provide … only modest additional value for grouping*". In other words,

5 https://www.fuelseurope.eu/reach

6 https://www.p65warnings.ca.gov/fact-sheets/petroleum-products-environmental-exposure-refineries

7 Most petroleum and coal stream substances under REACH are managed by the following consortia: Petroleum stream substances (Concawe), Coal stream substances (R4CC), Lower Olefins and Aromatics (LOA), Hydrocarbons Solvents REACH consortium (HCSC), Higher Olefins & Poly Alpha Olefins (HOPA)

8 https://echa.europa.eu/documents/10162/17228/petco_19_notes_en.pdf/c9c64575-c6d2-6a5a-a109-9c10cf80887f?t=1673448243650

Tab. 3: Comparison of the number of expected registrations and the estimated number of animals in different studies
Data from Pederson et al. (2003), the pre-registered substances and the forecast of the possible registered substances (Rovida and Hartung, 2009), and the ECHA data retrieved from the webpage on progress evaluation or provided in the REACH registration statistics (30/11/2022). The actual number of animals is taken from Knight et al. (2023).

	Number of registered substances	Estimated number of animals
Pedersen et al. (2003) estimation	29,342	1.3 - 3.9 million
Pre-registered substances (ECHA Press Release, 2009)[a]	143,835	141 million (when testing all, Rovida et al., 2009)
Estimation considering market increase 1994 to 2008 (Rovida et al., 2009)	68,208	54.4 million
ECHA registered substances (dossier evaluation 2009-2022)[b]	11,479	so far 4.2 million for only three endpoints (Knight et al., 2023) – ongoing process
ECHA registered substances (Feb. 2023)[c]	20,516	

[a] ECHA Press Release (2009). 27 March 2009 ECHA/PR/09/03; https://echa.europa.eu/documents/10162/17096/pr_09_03_list_prereg_substances_20090327_en.pdf (accessed 17.07.2009); [b] https://echa.europa.eu/progress-in-dossier-evaluation; [c] https://echa.europa.eu/registration-statistics

the biological effects are so different that they cannot be grouped. Testing of 185 petrochemicals for REACH has been estimated to consume one million animals and €600 million (Ketelslegers et al., 2020). In the absence of an accepted grouping strategy, this must be seen as the lower estimate. Depending on the number of individual UVCB identified, this number could increase up to the number of registrations, resulting in roughly 24 million animals[9] for ~4,500 registered products. This illustrates the critical importance of finding a workable path for these chemicals.

4 Comparison with previous estimates

The number of 4.2 million animals is different from previous estimates. Before REACH was approved, the EU Commission asked for a possible impact on the number of animals used for testing. Pedersen et al. (2003) estimated the number of necessary new tests and their costs, considering the number of registered substances and the rate of existing studies with the possibility to adapt non-standard information. Using the Pedersen et al. (2003) results, van der Jagt et al. (2004) concluded that the full implementation of REACH on existing substances would require 1.3 to 3.9 animals in new *in vivo* studies. Noteworthy, these estimates were based on data from before 1994, and both expansion of the EU and growth of the chemical industry were not considered. When REACH entered into force, the registration of the substances was preceded by a transition phase when almost 144,000 substances above 1 t/y were pre-registered. Acknowledging that this number was overstated, we based our estimation on the chemical market increase, concluding that the toll could reach 54 million vertebrate animals (Rovida and Hartung, 2009). Fortunately, only a small portion of the pre-registered substances was later registered (Tab. 3). ECHA has also reported other numbers for registered substances (see Section 4.1), but all in this range.

To compare the actual number of registrations with those used for the estimations, the data from the REACH webpage on evaluation are taken as reference, leading to the conclusion that the number of registered substances represents 39% of the estimation of Pedersen et al. (2003) and 16.8% of the estimates based on market increase (Rovida and Hartung, 2009).

Our 2009 analysis assumed "*All preregistered phase-in substances, i.e., substances classified in EINECS* [European Inventory of Existing Commercial Chemical Substances] *and NLP* [No-longer Polymers] *database, are included in the estimate, assuming that companies know their trade and production volumes*." This would have led to 47,858 substances present in the EU in a quantity > 1000 t/y (Annex X) and 53,048 in a quantity > 100 t/y (Annex IX). An alternate methodology in the 2009 study began by utilizing the estimated number of chemicals from the study by Pedersen et al. (2003), based on the EU's situation from 1991 to 1994. These figures were then adjusted by a multiplier derived from the growth of the chemical industry, as portrayed by CEFIC, the European Chemical Industry Council, in 2009, including a factor for the general growth of the chemical industry and accounting for the EU's expansion. Both approaches seemed reasonable at the time but strongly overestimated the actual registration numbers. Or, quoting Niels Bohr (1885-1962), "*Prediction is very difficult, especially about the future*".

The interesting question is, was the characterization of chemical industry in the early 1990s inflated, were the growth numbers of industry exaggerated or are chemicals under-registered? It is surprising that the number of chemicals in low tonnage bands is quite low, which might indicate some underreporting.

[9] 4,500 divided by 185 multiplied by 1 million animals

Even if production in Europe ceased, import as bulk material or in products from outside Europe would count. Noteworthy, ECHA's Enforcement Forum agreed in November 2022 that the next REACH enforcement project will investigate how companies fulfil the registration, authorisation and restriction obligations for products and chemicals they import into the EU[10]. The project will run from 2023 to 2025 and will require close cooperation between REACH enforcement and national customs authorities in the EU Member States. This could considerably increase the number of substances to be registered in lower tonnage bands.

EU production of chemicals is continuously increasing, by market value from 2011 to 2021 according to EuroStat[11] from €537 billion to €769 billion. Production increased from 2005 to 2021 by an average of 0.75% per year[11]. CEFIC reports[12] that sales in 2011 reached €506 million and €594 million in 2021, but the share of the world market, which was 27% in 2001, decreased from 19% to 15% in that time. The authors have not been able to find data on how many chemicals are on the European market. Wang et al. (2020) analyzed 22 inventories of the 19 most developed countries: Their key findings were that over 350,000 chemicals and mixtures of chemicals have been registered for production and use, up to three times as many as previously estimated, and with substantial differences across countries/regions. Many chemicals remain publicly unknown because they are claimed as confidential (over 50,000) or are ambiguously described (up to 70,000). The inventories that were included focus primarily on industrial chemicals, with few exceptions, and do not include unintentionally produced chemicals. This characterization of the global chemicals market is difficult to align with the registration numbers under REACH.

To move from the number of registered substances to the number of animals that were used during the registration process, we need to consider three more factors: i) the number of substances that are registered in the different tonnage bands; ii) the number of animals that each test requires, and iii) the number of new tests that are necessary, i.e., the rate of adaptation to the standard information.

4.1 Number of substances that are registered in the different tonnage bands

On March 11, 2023 there were 26,809 results in the ECHA database for registered substances[13]. By exporting the whole list into Excel, some further analysis was possible. The first step was the identification of substances with registration status "active" by eliminating about 3,000 chemicals with the status "no longer valid" or the status "cease manufacture". The latter substances still exist, but no update is required until a registrant reactivates them.

Tab. 4: List of registered substances under REACH
Data were directly retrieved from the ECHA database using ECHA's Export to .xlxs (Excel file) tool. Among the 26,809 substances of the ECHA database, there are 23,925 dossiers for active substances. These are subcategorized into full substances, NONS, and intermediates.

Registration status	TOTAL
All	26,809
No longer valid	751
Cease manufacture	2,133
Total active	**23,925**
– Full	11,908
– NONS	3,622
– Intermediate	8,395

Some of these seem to be the result of Brexit, others might indicate that manufacturers stopped production in face of the effort and costs of registration. Noteworthy, registration needs do not end with Brexit but essentially move from the UK producers to the EU importers, so we should expect reactivation of many of these registrations. The remaining 23,925 substances are distributed as listed in Table 4. NONS (Notification of new substances) are the substances that were notified according to an amendment of Directive EEC 67/548, which required new marketed substances to be notified with a set of physicochemical data and toxicological information. These substances were considered registered according to REACH even if the dossiers were far from being compliant with REACH. As soon as these substances are updated to the new provisions, they lose "NONS" status.

Further considerations are impossible. In fact, many substances have neither CAS (Chemical Abstracts Service) nor EC registration numbers, and NONS are difficult to characterize because, as a result of the transposition from the old system, their records are almost empty. The database shows the total tonnage band for each substance, but this is generic, including the contributions of all registrants, and there is no direct relationship with the highest tonnage band of the registration dossier. Transported intermediates have no data in the total tonnage band column, and there is no clue about how many of them were registered in the tonnage band ≥ 1000 t/y, requiring the application of Annex VII. In conclusion, the public database of registered substances offers no help in the determination on how many substances have been registered in the different tonnage bands.

10 https://echa.europa.eu/-/next-eu-wide-reach-enforcement-project-to-focus-on-imported-products

11 https://ec.europa.eu/eurostat/statistics-explained/index.php?title=Industrial_production_(volume)_index_overview#Development_of_main_industrial_groupings_and_individual_industries

12 https://cefic.org/app/uploads/2023/03/2023-Facts-and-Figures.pdf

13 https://echa.europa.eu/information-on-chemicals/registered-substances

Tab. 5: Number of registered substances as published on the ECHA webpage on progress in the evaluation[a] and in the 5th Report on the use of alternatives to testing on animals for the REACH Regulation (ECHA, 2023)
According to ECHA, each substance is counted once (i.e., only at the highest tonnage band it was registered for). Substances registered for intermediate use only and substances registered under the previous legislative regime (NONS) are excluded from this count.

Tonnage band (t/y)	Registered substances	
	ECHA Evaluation Report[a]	ECHA report on the use of alternative methods (ECHA, 2023)
≥ 1000 t/y (Annex X)	2,353	2,335
100 - 1000 t/y (Annex IX)	2,334	2,346
10 - 100 t/y (Annex VIII)	2,738	2,857
1 - 10 t/y (Annex VII)	4,054	4,901
TOTAL (not on the webpage)	**11,479**	**12,439**

[a] https://echa.europa.eu/progress-in-dossier-evaluation (accessed 15.06.2023)

The number of registered substances appears on other pages of the ECHA website. For example, the page on the progress in the evaluation[14] indicates the number of registered substances in the different tonnage bands, excluding intermediates and NONS (Tab. 5). In this document, the total of these substances is only 11,479, which is only about half the number of 23,925 active substances in the database. These numbers are similar to those reported in the latest ECHA report on the use of alternatives to testing on animals for the REACH Regulation (ECHA, 2023), also listed in Table 5.

On a regular basis, there is also the publication of the REACH registration statistics, released in the section about "Information on chemicals" (update of 28/02/2023[15]). Table 6 reports the number of registered substances in different breakdowns present in this ECHA document on REACH registration statistics, which explains that the reported total number of substances and registrations in the different columns is variable as one dossier may contain up to three registration types (full, transported isolated intermediates, and on-site isolated intermediates). The two numbers, 25,229 and 32,396, are quite different, and there is also a mismatch with the total number of substances reported in the header of the document (22,331) as well as the total number of substances in the ECHA database, which was 26,708 on 20/12/2022. The number of registrations (Tab. 6) is the number of the dossiers that have been submitted, and it counts the dossiers from all co-registrants.

For the aim of evaluating the impact of the number of new *in vivo* tests that REACH is requiring now and in the future with the revision, we need to consider the tonnage band for each substance. However, it seems that there is no precise number on which it is possible to reasonably base the estimation. There are many possible reasons. In addition to the fact that a substance can appear up to three times, as a full substance, as isolated and transported intermediate or only as isolated intermediate, there is also the situation when a substance was registered according to a tonnage band and downgraded later and the confounding aspect that dossiers submitted by UK companies were deactivated after Brexit.

This shows that robust data are difficult to obtain. William Edwards Deming (1990-1993), a leading management thinker in the field of quality, wisely said, "*Without data, you're just another person with an opinion*."

4.2 Number of new substances per year

The number of registered substances is evolving. In the ECHA database, it is possible to retrieve the number of substances according to the date of their first submission (Fig. 1). Even though these numbers are not representative of the total number of substances in the different REACH Annexes, they can provide an approximate idea of the growth of the chemical industry in the EU.

The first three points of the graph in Fig. 1 relate to the three REACH deadlines, corresponding to:

- 2010: Registrations of substances manufactured or imported in quantity ≥ 1000 t/y plus substances classified as CMR in category 1 and substances classified persistent, bioaccumulative and toxic (PBT) or very persistent and very bioaccumulative (vPvB) in the tonnage band ≥ 100 t/y
- 2013: Registrations of substances manufactured or imported in quantity ≥ 100 t/y
- 2018: Registrations of substances manufactured or imported in quantity ≥ 1 t/y

In the table below the graph, the search criteria period was extended one month after the first two deadlines, and the period covering the third deadline was extended until the end of the year, to take into account possible delays in the submissions or in uploading them into the ECHA database. There were serious problems with the third deadline because many tests were not available on time, and the corresponding substances were registered late.

From 2020 onward, there is a steady condition, with an average of 646 new substances registered per year. This is in line with the prediction of the market expansion of the chemical sec-

14 https://echa.europa.eu/progress-in-dossier-evaluation (accessed 15-06-2023)
15 https://echa.europa.eu/documents/10162/2741157/registration_statistics_en.pdf/

Tab. 6: Number of registered substances in different breakdowns present in the ECHA document on REACH registration statistics[a] (28/02/2023)
The total in the column of REACH registration statistics is not present in the original documents, but in the header of the document the number (#) of registrations is 102,066 but the number (#) of substances is 22,331, which is different from both the totals (25,229 and 32,396) reported below.

	# of registrations	# of substances
Breakdown by registration type		
Full registration	80,230	13,029
Intermediates	20,199	9,947
NONS	2,796	2,253
TOTAL (not in the ECHA report)	**103,225**	**25,229**
Breakdown by tonnage band		
≥ 1,000 (Annex X)	21,447	2,360
100 - 1000 t (Annex IX)	15,410	3,838
10 - 100 t (Annex VIII)	18,493	5,546
0 - 10 t (Annex VII)	24,880	8,772
SUBTOTAL (not in the ECHA report)	**80,230**	**20,516**
Intermediates	19,040	9,627
NONS (confidential)	2,796	2,253
TOTAL (not in the ECHA report)	**102,066**	**32,396**

[a] https://echa.europa.eu/documents/10162/2741157/registration_statistics_en.pdf/

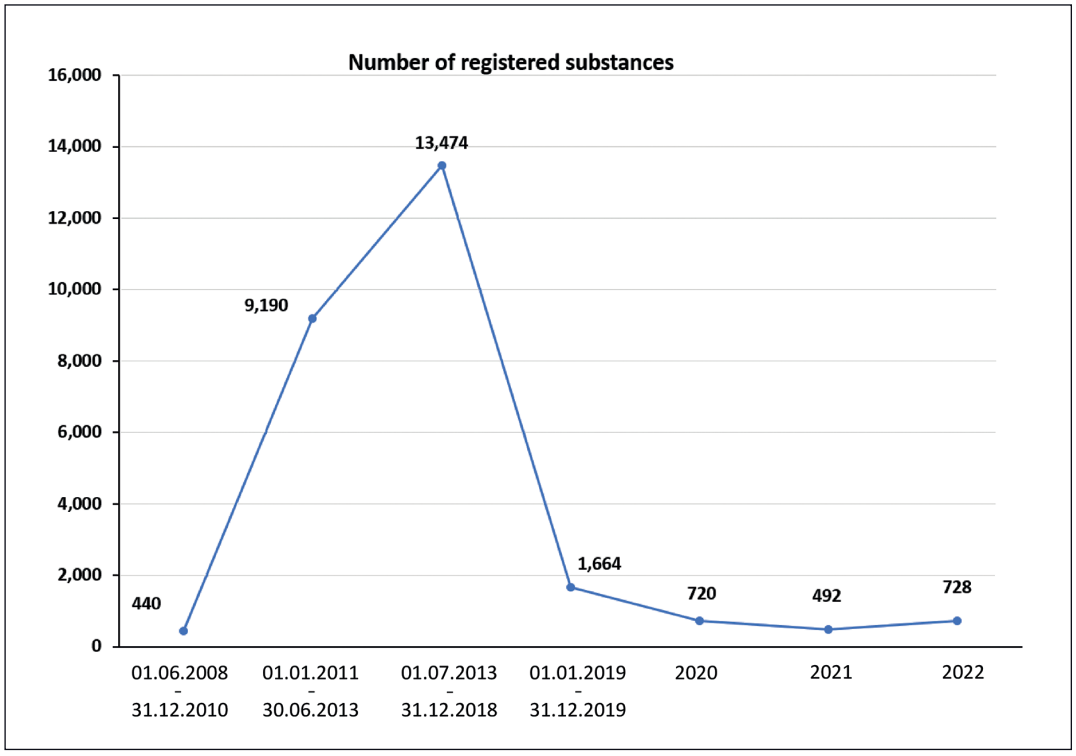

Fig. 1: Number of substances retrieved from the ECHA database according to the date of first submission

Tab. 7: Average number of laboratory animals per test for the three main endpoints for human systemic toxicity, repeated dose toxicity, reproductive toxicity, and developmental toxicity

Endpoint	Average number of animals per test in van der Jagt et al., 2004	Average number of animals per test in Rovida and Hartung, 2009	Average number of animals in Taylor, 2018	Average number of animals in Knight et al., 2023
OECD TG 407	50	40	60	81[a]
OECD TG 410	50	40	60	98[a]
OECD TG 412	50	40	60	90[a]
OECD TG 408	80	80	100	122[a]
OECD TG 411	NR[b]	80	120	117[a]
OECD TG 413	NR[b]	80	120	139[a]
OECD TG 421	80	560	400	802/598[c]
OECD TG 422	-	412	500	808/604[c]
OECD TG 414 (rat)	100	784	900	1,459
OECD TG 414 (rabbit)	100	560	900	1,094
OECD TG 416	448	3,200	2,200	3,099
OECD TG 443	-	-	960	2,733/1,830[c]

[a] Excludes recovery groups. About 30% of short-term repeated dose tests and 35% of sub-chronic tests included recovery groups, which typically added a total of 20 or 40 animals to the test. [b] Not reported, due to the variability of interim animals, i.e., animals that are added and sacrificed before the end of the exposure period. [c] In these paired numbers, the first number is when the dose range-finding test used mated animals (with offspring) and the second number is when it used unmated animals (no offspring).

tor, and this number can be used to estimate the impact of future amendments to the REACH legislation.

The number of registered substances in 2010 is too low, and it does not represent the number of substances registered for the the first REACH registration deadline on November 30, 2010. On November 1, 2010, ECHA announced the successful submission of 3,400 substances, a number that is different from the 440 in Figure 1. This number may be the result of eliminating all the double submissions that were possible in 2010 plus the substances that now have a different EC number and required a new registration recorded some years after the first. This is the case when a substance is registered with an EC name that is found to be incorrect after the analytical characterization of the substance or simply because the REACH regulation requires specific conditions for naming UVCB substances. It is also clear that the number of registered substances in 2019 was probably affected by delays from the previous deadlines. ECHA reports that in 2020, only 346 new substances were registered (ECHA, 2021). The reason for the mismatch is that ECHA considers only substances that were not pre-registered, while our numbers retrieved from the ECHA database consider all substances registered for the first time.

4.3 Average number of laboratory animals per test

Having a precise estimate of the average number of animals per test is necessary to evaluate the impact when a new *in vivo* test is requested. This can also be used to measure the consequences that any actions towards reducing animal test burden may have. REACH claims the promotion of alternative methods in its Article 1, but this has never been measured in terms of animal numbers used. In 2018, Taylor performed an accurate analysis of the evaluation procedure implemented by ECHA. The conclusion was that it was too early for a definitive conclusion on the number of animals that REACH will require, and probably this number is going to constantly increase as long as *in vivo* tests are the standard procedure for submitting toxicological information on chemicals.

Table 7 summarizes previous estimates of the average number of animals per test, including the averages calculated in Knight et al. (2023). Compared to the other averages, the Knight et al. (2023) averages are not an indirect estimation (e.g., inferred from test guideline requirements), but are a direct calculation from reported animal data for tests performed for REACH purposes since 2009. For this reason, these should represent the new reference for future estimates, showing that in general earlier analyses were conservatively underestimating the number of animals per test. Table 7 limits this comparison to the three main endpoints for human systemic toxicity, repeated dose toxicity, reproductive toxicity, and developmental toxicity as they are the only three that were analyzed in Knight et al. (2023).

4.4 Number of new *in vivo* tests required during the evaluation process

Van der Jagt et al. (2004) calculated the number of animals that the REACH implementation would require, considering the availability of existing studies and the possibility to adapt

non-standard data, as explained in column 2 of REACH Annexes VII through X and the provisions described in REACH Annex XI.

The decision on the number of new tests that are necessary is not definitive. The initial dossiers often contained read-across studies, but many of these were rejected during the evaluation process, which is still ongoing. ECHA (2021) reported that 75% of evaluated dossiers containing read-across had been rejected because they were not compliant. To make the situation worse, there are also cases where existing *in vivo* studies were rejected because they were too old and either not compliant with the latest standard or not performed under GLP conditions. For example, a substance received the request for a new OECD TG 443, the Extended One Generation Reproductive Toxicity Study (EOGRTS) in spite of the presence of a three-generation reproductive toxicity study included in the dossier[16].

The ECHA reports on the use of alternative methods state the percentage of adaptations present in the registration dossiers, but there is little information on the total rate of rejection during the evaluation process and its impact on the total number of animals requested by the REACH implementation.

5 Regulation (EU) 2022/477 amending REACH Annexes VI to X

Regulation (EU) 2022/477 amending REACH Annexes VI to X, which entered into force on October 14, 2022 defines the type of strategy that needs to be applied in some cases. This regulation limits the possibility to adapt the standard information with non-animal tests and enlarges the request for *in vivo* tests for substances registered in a lower tonnage band. In fact, this amendment implements what was already formulated in the ECHA guidance document (ECHA, 2017a), but the weight of a text written into a regulation makes it mandatory.

The amendment applies to new substances, to any update of existing substances, and to substances that are evaluated by ECHA. The details of the text of this amendment compared to the previous version of REACH are shown in the supplementary file[17], while this section provides a glimpse of the main consequences in terms of new *in vivo* tests that are required. It is not possible to quantify exactly the impact that this new amendment will have on the number of animals used for REACH purposes and how long it will take to fully implement it. This is because the number of substances that are registered in the different tonnage bands is uncertain (see Section 4), and it is also difficult to estimate the prevalence of the specific property that will trigger additional tests. Moreover, many of the tests that are requested in this amendment are not commonly applied, and there is not enough experience to estimate the average number of animals that each of them may require.

Nevertheless, the following subsections present possible scenarios derived from the full implementation of this amendment on registered substances, using the numbers reported in the latest ECHA evaluation report[14]. These numbers exclude substances registered as transported intermediates in quantity ≥ 1000 t/y that should comply with Annex VII provisions. The number of nanoforms from the ECHA database of registered substances, selecting "substance has nanoforms" + "Registration type: full" + "registration type: active"[18] and 145 corresponding to the 150 used in our calculations.

The minimum and maximum percentage of substances requiring new tests are guesses based on our experience and hints in the literature, just to provide an idea of the possible impact that this new regulation may have on the number of animals that REACH requires. The aim is to provide a demonstration that a tool to measure the impact of a new regulation in terms of animal numbers is available, and it should be used to assess the potential impact on animal numbers before adopting a new legislative act. A more detailed analysis will be possible in 5 to 10 years, though there is hope that a new guideline from the European Commission will allow use of non-animal methods to fulfil the information requirements.

Taken together, these "guesstimates" detailed below suggest that the 2022/477 amendment to REACH added between 3.6 to 7.0 million animals. We are wondering whether policymakers were aware of the consequences of these "clarifications"?

5.1 *In vivo* genotoxicity test

This amendment specifies that a new *in vivo* test is necessary if any *in vitro* test is positive, starting from Annex VII. The Ames test (OECD TG 471) is mandatory in Annex VII, while additional tests to further measure non-mutagenic genetic toxicity are requested with one test to be selected among the mouse lymphoma assay, the chromosomal aberration test or the *in vitro* micronucleus test combined with another mutation test on mammalian cells. According to this amendment, nanoforms are tested immediately *in vivo*. So far, mutagenicity was tested *in vivo* 786 times with 11,744 registrations (6.7%) in the higher tonnage bands. So, the *in vivo* test has been applied rarely to date. The *in vitro* genotoxicity battery, however, is notorious for false-positive findings (Kirkland et al., 2005, 2007; Walmsley and Billinton, 2011), with Kirkland et al. (2005) reporting, "*When all three tests were performed, 75-95% of non-carcinogens gave positive (i.e., false positive) results in at least one test in the battery*". Snyder and Green (2001) reported that about 50% of non-carcinogenic marketed pharmaceuticals had positive results in at least one of the regulatory *in vitro* genotoxicity tests, while Brambilla and Martelli (2009) found 30% positive non-carcinogens. Thus, we must expect a relatively large number of additional tests. Notably, these assays also have some reproducibility issues: A retrospective analysis of 237 compounds

16 https://echa.europa.eu/documents/10162/0f26eda1-9a01-5076-f41a-30283d6bef36

17 doi:10.14573/altex.2307121s

18 https://echa.europa.eu/information-on-chemicals/registered-substances?p_p_id=dissregisteredsubstances_WAR_dissregsubsportlet&p_p_lifecycle=1&p_p_state=normal&p_p_mode=view&_dissregisteredsubstances_WAR_dissregsubsportlet_javax.portlet.action=dissRegisteredSubstancesAction (accessed 01.07.2023)

with multiple Ames screen test results found that 49 (21%) had discrepant results (McCarren et al., 2011).

To measure the impact of this amendment, we assume that the vast majority of compounds are non-carcinogens and use the Kirkland et al. (2005) Ames test specificity of 33% false-positives, and apply this to 4,054 Annex VII substances (row 1, Tab. 8) resulting in 66,900 animals; notably, transported intermediates registered in quantity ≥ 1000 t/y with the same requirement were not included as their number is unknown. The combined number of positive outcomes of the *in vitro* test battery performed in substances registered in Annex VII-X of about 43% (50% total positives from the 30, 50 and 75-95% cited above, minus 7% already done), and the number of nanoforms that are registered in any tonnage band to be all tested *in vivo*, resulting in 167,150 animals. The latter were introduced for registration in 2020 and number just 150 according to an undated website[19], while ECHA had expected twice as many; we use 150 as a conservative estimate. Together this makes 4,681 tests with 234,029 animals. The *in vivo* tests use an average number of 50 animals per test as provided in Taylor (2018).

5.2 OECD TG 443, the Extended One-Generation Reproductive Toxicity Study (EOGRTS)

In Annex X, the OECD TG 443 (EOGRTS) including the extension to the second generation is mandatory. Substances in Annex IX need it only in case of mild concern, while it is requested for substances in Annex VIII only in exceptional circumstances, for example when the screening test presents a possible endocrine disruptor activity. Nanoforms in Annex IX and X must have a test by inhalation[20], where the average number of animals per test is taken from Knight et al. (2023). The guessed percentage of substances requiring additional tests is taken as min.1% and max. 5% for Annex VIII.

OECD TG 443 is more extensive than OECD TG 416 and contains additional endpoints, such as the full screening of endocrine activity. During the evaluation procedure a new EOGRTS test is often required if the existing OECD TG 416 was performed before 2000 or it is not GLP compliant. We calculate the number of animals considering a minimum of additional 30% (total minimum 35%, Tab. 8) and a maximum of 50% (total maximum 55%, Tab. 8), i.e., 824 to 1,294 of substances in Annex X missing this information. In this case, extension including the second generation is necessary. A minimum of 1% and a maximum of 5% of tests for substances in Annex VIII and the 150 nanomaterials may require this test. We include only 1% substances in Annex IX because there is little difference to the previous version of REACH. Using 1,830 to 2,733 for animal numbers (see Tab. 7), we arrive at a minimum of 1.9 and a maximum of 4.3 million animals.

5.3 Developmental toxicity testing with OECD TG 414

The *in vivo* test following the OECD TG 414 on developmental toxicity is now requested in Annex VIII if there is some specific concern from the screening test, such as the possibility that the substance is a developmental neurotoxicant; we estimate a minimum of 1% and a maximum of 5% of substances (of 2,738 Annex VIII substances, i.e., min. 27 to max. 137 chemicals to test, Tab. 8) that may require this test for substances in Annex VIII. In Annex IX, the test is mandatory and repeated on rabbit now if there is some concern on rats (estimated at 15-25%), while in Annex X, the second species is always mandatory. The average number of animals is taken from Knight et al. (2023) as 1,459 rats and 1,094 rabbits (Tab. 7), respectively. From Annex IX, the test must be by inhalation for nanoforms. We estimate again 150 nanoforms with the same average number of animals per test as derived from studies performed by gavage, even if the inhalation route generally requires more animals. According to the REACH amendment, the possibility for waiving the first test on rats for substances in Annex IX or the confirmation on rabbits for substances in Annex IX are reduced; this will increase animal use but was not factored into our estimate as the extent of use of these provisions is not clear. This results in tests in a total of a minimum of 641,806 animals (383,009 of these rabbits) and maximum of 1,056,936 animals (638,349 of these rabbits).

5.4 Long-term fish tests

The new amendment modifies Annex VIII requesting a long-term study on fish if the substance is not water-soluble (< 1 mg/L) and not biodegradable. In these cases, the acute short-term toxicity test on fish, which was the standard information requirement in Annex VIII, is waived. This change has a strong impact on the number of animals that are necessary for REACH compliance as the short-term test uses one tenth of the animals that are necessary in a long-term test.

To cover this endpoint, there is the possibility to select one of the following tests:

- OECD TG 210 Fish, Early-life Stage Toxicity Test
- OECD TG 212 Fish, Short-term Toxicity Test on Embryo and Sac-fry Stages
- OECD TG 215 Fish, Juvenile Growth Test

According to Article 1(3) of Directive 2010/63/EU on the protection of animals used for scientific purposes, fish are counted as animals that deserve protection when embryos can feed independently (5 days post fertilization[21]). This endpoint is typically covered by applying OECD TG 210, which requires exposure of at least 80 fertilized eggs per test item concentration in test chambers. The hatching rate of zebrafish is about 91% (Farhana et al., 2019). Even if the initial phase of the test is not done on protected animals according to the Directive, the test is continued after hatching for a specific period that is necessary for the fish in the control group to reach a juvenile life-stage, so in the end the

[19] https://nanodb.dk/en/news/echa-may-have-overestimated-number-of-substances-with-nanoform-on-the-eu-market-low-number-of-reach-nano-registrations-bother-echa-blames-industry/

[20] As an additional note, it should be considered the difficulty in performing such a complex study like the OECD TG 443 by inhalation using nanoforms. There are so many practical problems that rise doubts on the reliability of the final outcome.

[21] https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:02012D0707-20140115

test uses sentient vertebrates. The decision on the starting dose is based on the results of a pre-dose finding test that is usually performed on half the eggs of the main test. Just by applying the standard request of OECD TG 210, the total number of fish per test is about 700, exceeding the average considered in van der Jagt et al. (2004) or Taylor (2018), who reported an average of 400 and 420, respectively.

The main uncertainties in this estimate are:

- Number of registered substances in Annex VIII (currently 2,738; Tab. 2) that, depending on their physicochemical properties, require the long-term test instead of, or in addition to, the short-term test.
- Probably, not all substances that are eligible for the long-term instead of the short-term test will be effectively tested, and in many cases, registrants will wait for the formal decision from ECHA or if the dossier requires a spontaneous update for other reasons.

The guessed range of 15% and 25% of substances requiring a new OECD TG 210 was used for substances in Annexes VIII-X (Tab. 2), corresponding to a minimum of 779,625 and a maximum of 1,299,375 fish.

Starting from Annex IX, in cases of concern, the Fish Sexual Development Test (FSDT) (OECD TG 234) can be requested. The starting point of this test includes an average of 30 fertilized eggs per 4 replicates and 7 doses, counting 840 fertilized eggs at the beginning of the study without counting the preliminary DRF study. This test is performed when other data is available, and often the pre-DRF test is not necessary. Considering a hatching rate of 0.91 (Farhana et al., 2019), the total number of fish per test is 764. The OECD TG 234 is not part of the standard information requirement but may be requested in the final decision independent of the tonnage band of the registered substance if the evaluation process deems it is necessary. There is no clue on the number of new tests that will be required. The contribution of new OECD TG 234 is thus not included in our calculation, but 1% requests would result in more than 35,000 animals.

5.5 Bioconcentration factor (BCF) in fish

Starting from Annex VIII, the 2022/477 amendment expands the request for a new test to measure bioconcentration in fish, including all substances that are not biodegradable and for which the estimation or measure of octanol/water partitioning (K_{ow}) is not reliable. This applies to all UVCB substances, surfactants, and organic salts. Regarding UVCBs, this request is applied also to biodegradable substances when it is suspected that one of the components may accumulate.

The bioconcentration factor is measured *in vivo* following the procedure described in OECD TG 305, which advises to use radiolabeled test substances to facilitate the analytical determination in water, food, and fish samples, and may be used to determine whether identification and quantification of metabolites is necessary. For this reason, testing UVCBs is difficult if not impossible because it requires identification of the component of concern in the UVCB. From a scientific point of view, the test should be repeated focusing on each component individually. This is not feasible in practice due to the intrinsic complexity of the UVCBs, the duration of the experiment, and its costs.

The standard BCF test on fish requires more than 280 fish per test (Taylor, 2018) and could be replaced by a test based on fish primary hepatocytes (OECD TG 319) that measures the substance clearance capacity in fish liver. Another alternative is a test that replaces fish as the main organism with *Hyalella azteca*, which is a benthic freshwater amphipod[22]. For a rough estimate of animal numbers, 5 to 10% application to 7,425 Annex VIII to X chemicals was assumed, corresponding to 103,950 to 207,900 fish.

6 Regulation EU 2023/707 amending CLP

Regulation EC 2008/1272 on classification, labelling and packaging (CLP) of chemical substances and mixtures tries to discourage the performance of new tests, in particular if animals are involved. However, classification according to CLP is required for registered substances, so eventually the provision of CLP must be applied in addition to the REACH standard request of information. Without going into the details of all endpoints, many criteria for classification are based on results on animals.

Currently, the main concern is about an amendment of the CLP contained in Regulation EU 2023/707, which introduces the following new hazard classes:

- EUH 380 (Category 1): May cause endocrine disruption in humans
- EUH 381 (Category 2): Suspected of causing endocrine disruption in humans
- EUH 430: May cause endocrine disruption in the environment
- EUH 431: Suspected of causing endocrine disruption in the environment.
- EUH 440: Accumulates in the environment and living organisms including in humans
- EUH 441: Strongly accumulates in the environment and living organisms including in humans

The given definition of "endocrine disruptor" is "*a substance or a mixture that alters one or more functions of the endocrine system and consequently causes adverse effects in an intact organism, its progeny, populations or subpopulations*". In the area of endocrine disruptors, many validated *in vitro* tests are already available, but they are accepted only if the result is positive. This is one of the reasons why companies are reluctant to use *in vitro* methods: fear of having to justify a positive outcome. In conclusion, the main way to fulfil the decision for the classification of a substance or mixture as a human endocrine disruptor is through one of the *in vivo* tests described in the EFSA-ECHA guideline (EFSA, 2018). The impact can be detrimental if the new OECD TG 443 test is used, with its 1,830 to 2,733 average number of animals per experiment (Knight et al., 2023). Exactly the same

[22] Presentation of Kristin Schirmer at the ECHA New approach methodologies workshop: Towards an animal free regulatory system for industrial chemicals (https://echa.europa.eu/-/new-approach-methodologies-workshop-towards-an-animal-free-regulatory-system-for-industrial-chemicals)

considerations are valid for the assessment of environmental endocrine disruptors with the new hazard classes.

Regarding the other hazard classes for PBT (EUH440) and vPvB (EUH441), these endpoints are already part of the requirements in REACH registration dossiers, so at least these new endpoints should not trigger new *in vivo* tests. The concern about the need to assess biopersistence with *in vivo* tests is described in the previous chapter. As CLP is not part of REACH, estimates on impacts on animal numbers were not included.

7 REACH revision

Article 138 of REACH asks for an extensive revision of the regulation by June 1, 2019. Furthermore, the Chemicals Strategy for Sustainability (CSS)[23] proposed by the European Commission in 2020 cross-fertilizes with this process. In 2021, the Commission published an Inception Impact Assessment[24] on the planned revision of REACH and finalized the comprehensive stakeholder consultation activities for the REACH Revision Impact Assessment. The European Commission has prioritized the task of formulating the legal revision proposal(s) and aims to complete it in 2023[25]. The proposal is anticipated to be approved by the fourth quarter of 2023. Following this, it will be forwarded to the European Parliament and the Council to undergo the co-legislative process. The proposal could potentially be implemented between 2025 and 2027. The Commission aims to future-proof REACH by keeping pace with technological advancements in the field of safety assessment, allowing more flexible approaches, and thereby setting in place a transition away from animal testing towards more species-relevant and modern science. However, proposals presented so far have significant negative animal welfare consequences. Among the changes, there are three issues that may have a strong impact by increasing the number of animals used for testing:

1. Extension of a CSA to include substances that are registered in the tonnage band 1-10 t/y (Annex VII)
2. Registration of polymers and combination effects
3. New endpoints, i.e., enhanced information requirements/scrutiny, for example endocrine disruptors, respiratory sensitizers, and effects on the nervous and immune systems

The Commission used the Fit for Future Platform instrument[26] to collect evidence on what could be main suggestions for changes, acknowledging that the evaluation of registration dossiers and substances is too complex and insufficient and that there are still gaps for relevant hazardous endpoints especially for carcinogenicity, neurotoxicity, immunotoxicity, and endocrine disruption. Furthermore, the Commission recommends facilitating the registration and evaluation process for small and medium-sized enterprises (SMEs) since they do not have internal expertise and resources to perform information requirements in comparison to the big players. For this reason, the Commission intends to diminish burden of proof for lower tonnage bands by promoting read-across and making data-sharing mandatory.

7.1 Chemical safety assessment (CSA) for Annex VII and other changes in test requirements

Repeat-dose testing of chemicals is the cornerstone of traditional risk assessments as it is used to establish no-effect levels. It was therefore surprising to many experts that this formerly was only a requirement from Annex VIII (>10 t/y) upwards. In the scope of REACH, the CSA is currently done when the substance is classified according to CLP (only few hazard classes are excluded). The CSA must include an exposure assessment and a risk characterization related to the use of the substance along its whole life cycle. Detailed instructions are described in the relevant ECHA guidance[27]. The exposure assessment requires the values for the derived no effect level (DNEL) for human health and the predicted no effect concentration (PNEC) for the environment. The starting value for the PNEC can be derived from the standard Annex VII tests, but this is not the case for the DNEL. Standard Annex VII tests for human toxicity are the skin/eye irritation, skin sensitization, and acute toxicity tests and the Ames test for genotoxicity. None of these can provide the no adverse effect level (NOAEL) that is necessary to derive a DNEL for the CSA, and therefore data from at least one additional new *in vivo* test would be mandatory. There are no details, but it is probable that the request for the OECD TG 422 *Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test* will be extended to all Annex VII substances plus all intermediates registered in quantity ≥ 1000 t/y[28]. Table 9 reports possible scenarios based only on the 4,054 substances in Annex VII as reported in ECHA (2023), see Table 2, without considering the inputs from intermediates and applying an arbitrary percentage of 50% or 80% of substances requiring the new test. The resulting range is between 1.2 and 2.6 million animals.

Notably, the European Commission in their presentation of policy options[29] talks of 5,800 Annex VII substances, which means another 43% increase over the numbers suggested here.

Additional requirements for endocrine disruptor identification for human health and the environment are under discussion with unclear triggers and waivers at this stage:

- Uterotrophic Bioassay in Rodents (OECD TG 440)
- Hershberger Bioassay in Rats (OECD TG 441)
- Amphibian Metamorphosis Assay (OECD TG 231)
- Fish Sexual Development Test (OECD TG 234)

23 https://echa.europa.eu/hot-topics/chemicals-strategy-for-sustainability

24 https://ec.europa.eu/info/law/better-regulation/have-your-say/initiatives/12959-Chemicals-legislation-revision-of-REACH-Regulation-to-help-achieve-a-toxic-free-environment_en

25 https://www.reachlaw.fi/reach-revision/

26 https://commission.europa.eu/system/files/2022-12/Final%20opinion%202022_SBGR2_06%20REACH_rev.pdf

27 https://echa.europa.eu/guidance-documents/guidance-on-information-requirements-and-chemical-safety-assessment

28 https://chemicalwatch.com/754646

29 https://echa.europa.eu/documents/10162/23930482/20230531_nam_workshop_katrin_schutte_com_en.pdf/8b8d968a-ef07-fc21-662d-ad41d86739ae?t=1685511393161

Tab. 8: Estimated range of animal use as a result of the REACH amendment (Regulation (EU) 2022/477)
Registration numbers from the ECHA evaluation report[a] were used.

Endpoint	Chemicals in tonnage band(s) (Annex)	% min. tested	% max. tested	Number of animals per test (absolute or range)	Min. number of animals	Max. number of animals
In vivo genotoxicity	4,054 (VII)	33%[b]		50	66,891	
	7,425 (VIII-X)	43%[c]			167,138	
	150 (nano)	100%				
OECD TG 443 Extended One-Generation Reproductive Toxicity Study (EOGRTS)	2,738 (VIII)	1%	5%	1,830-2,733[d]	50,105	374,148
	2,334 (IX)	1%	1%		42,712	63,788
	2,353 (X)	35%	55%		1,507,096	3,536,912
	150 (nano)	100%			274,500	
OECD TG 414 Developmental toxicity testing	2,738 (VIII)	1% (rat)	5% (rat)	1,459 (rat) 1,094 (rabbit)[c]	39,947	199,737
	2,334 (IX)	15% rabbit	25% rabbit		383,009	638,349
	150 (nano)	100% (rat)			218,850	
OECD TG 210 Fish, Early-life Stage Toxicity Test	7,425 (VIII-X)	15%	25%	700	779,625	1,299,375
OECD TG 305 Bioconcentration factor (BCF) in fish	7,425 (VIII-X)	5%	10%	280	103.950	207.900
TOTAL					**3,633,823**	**7,047,588**

[a] https://echa.europa.eu/progress-in-dossier-evaluation. [b] False-positive rate Ames test for non-carcinogenic substances. [c] Average ~50% false-positive rate of at least one of the genotoxicity battery tests (see text) minus 7% already done. [d] Animal numbers per test with dose-range finding studies from Knight et al. (2023)

Tab. 9: Two scenarios of the number of animals that the need for the OECD TG 422 for substances in Annex VII may require, starting from 4,054 substances in Annex VII (see Tab. 2)
This excludes the intermediates registered in quantity ≥ 1000 t/y

Scenario	50% will require new test		80% will require new test	
	Without pups in DRF study	With pups in DRF study	Without pups in DRF study	With pups in DRF study
Number of substances	2,027	2,027	3,243	3,243
Average number of animals per test	604	808	604	808
Number of animals required	1,224,308	1,637,816	1,958,772	2,620,344

– Medaka Extended One-Generation Reproduction Test (OECD TG 240)
– Larval Amphibian Growth and Development Assay (OECD TG 241)

Depending on the exact conditions of implementation, and multiplying this with the number of dossiers affected, will add several million animals.

At the same time, some animal-saving changes are under discussion including[30]:

– Replacing short-term fish toxicity test with *in vitro* cytotoxicity (OECD TG 249) or fish embryo toxicity (OECD TG 236)
– Replacing bioaccumulation in fish (Annex IX) by either the *in vitro* test OECD TG 319A/B (i.e., intrinsic clearance in rainbow trout hepatocytes) and *in vitro-in vivo* extrapolation (IVIVE) for estimation of kinetic BCF or bioaccumulation in invertebrates (e.g., *Hyalella azteca* bioconcentration test)

The following tests might be deleted:

– acute oral toxicity in rats (Annex VII)
– acute dermal & inhalation toxicity in rats (Annex VIII)
– skin corrosion/irritation (Annex VIII)
– serious eye damage/eye irritation (Annex VIII)
– assessment of toxicokinetic behavior derived from the relevant available information (Annex VIII)
– further studies beyond the 90-day study (Annex IX column 2)
– long-term repeated dose toxicity study (≥ 12 months) (Annex X)
– pre-natal developmental toxicity study 2nd species (Annex X / trigger in Annex IX)
– carcinogenicity study (Annex X)

Most of these use small numbers of animals and/or are not often used, but this shall not belittle these attempts. However, clearly the new test requirements for endocrine disruption and possibly for developmental neuro- and immunotoxicity, respiratory sensitization, etc. vastly outweigh these savings.

To avoid this situation, the new revision of REACH should replace the standard system with a next generation risk assessment (NGRA) that is defined as an exposure-led, hypothesis-driven risk assessment approach that integrates *in silico*, *in chemico*, and *in vitro* approaches, translating data obtained with NAMs to derive the threshold level for the safe use of chemicals (Pallocca et al., 2022a). There is already an interesting initiative that brings together governmental entities to discuss progress in and barriers to applying new tools for risk assessment to find opportunities for collaboration, i.e., APCRA (Accelerating the Pace of Chemical Risk Assessment)[30].

7.2 Registration of polymers

The consumption of plastics[31], the most recognized array of petrochemical products, has increased more rapidly than any other bulk material such as steel, aluminium, or cement, nearly doubling since 2000. Developed regions like the United States and Europe utilize up to 20 times the amount of plastic and up to 10 times the amount of fertilizer on a per capita basis compared to emerging economies like India and Indonesia. This indicates a substantial potential for global expansion. The surge in demand for petrochemical products means they are anticipated to contribute more than a third to the increase in oil demand by 2030, and nearly half by 2050, surpassing trucks, aviation, and shipping. Petrochemicals are also expected to use an extra 56 billion cubic meters of natural gas by 2030, which is roughly equivalent to half of Canada's current total gas consumption.

The knowledge of human and environmental hazards and risks from chemicals associated with the diversity of plastic products is very limited (Lithner et al., 2011), especially with respect to impact on the environment (Groh et al., 2022). The need to register polymers generates several practical problems. It is reasonable to set rules to exclude some of them from the registration process and make the registration mandatory only for polymers that may present concerns. As there is no official draft REACH revision, the following information is taken from preliminary documents such as the minutes of working groups dedicated to this issue.

Polymers and plastics are terms often used interchangeably in casual conversation because most plastics are a type of polymer. In essence, while all plastics are polymers, not all polymers are plastics. There are many types of polymers, including elastomers, fibers, and biopolymers, that are not categorized as plastics. The main difference lies in their chemical composition and properties. The definition of polymer in Article 3(5) of REACH is: "*polymer means a substance consisting of molecules characterised by the sequence of one or more types of monomer units. Such molecules must be distributed over a range of molecular weights wherein differences in the molecular weight are primarily attributable to differences in the number of monomer units*".

The European plastics industry provided data about the sector in 2019[32]: The plastics industry, with over 55,000 companies, most of them SMEs, gives direct employment to more than 1.56 million people in Europe and has a turnover of more than €350 billion (a positive trade balance of €13.1 billion in 2019). It represents 16% of the world market (China 31%). This illustrates how many new stakeholders will be added to the REACH process.

In the REACH revision, polymers will be split into polymers not requiring registration (non-PRR) and polymers requiring registration (PRR). Polymers requiring registration will be (any of the conditions is valid):

– Polyesters in a specific list that will be an Annex of the new Regulation. There is no official list yet, but the Australian law[33] has something similar.
– All fluorinated polymers, regardless of whether the fluorine is attached to the carbon backbone or part of a fluorinated side-chain
– Cationic polymers or polymers that are reasonably expected to

30 https://apcra.net/#/ (APCRA)
31 https://www.iea.org/reports/the-future-of-petrochemicals
32 https://plasticseurope.org/wp-content/uploads/2021/09/Plastics_the_facts-WEB-2020_versionJun21_final.pdf
33 https://www.industrialchemicals.gov.au/help-and-guides/polymer-low-concern-plc-criteria#polyesters

become cationic in a natural environment (e.g., primary, secondary, tertiary amines, quaternary ammonium salts, phosphonium or sulphonium groups, etc.)
- Polymers with MW < 1000 Da or with MW > 1000 Da but with > 2% oligomers with molecular weight < 500 Da and > 5% oligomers with molecular weight < 1000 Da
- Classified polymers according to CLP
- Polymers with reactive functional groups
- Polymers with surface tension < 45 mN/m
- A polymer that is designed, or can be expected, to substantially degrade, decompose or depolymerize into substances that are concerning

Already the decision on whether a polymer needs registration is challenging and requires in-depth knowledge of the material. This will be most challenging for imported products, where the non-EU manufacturer must disclose sensitive information. After accepting this step, there is the question on how to group the polymers to reduce the number of new registrations. The REACH principle of "*one substance, one registration*" is hardly applicable. Polymers can be complex, and the final result depends on the tuning of the manufacturing process. Each manufacturer has their own, often patented, procedure, and commonly final products are different even if starting raw materials are the same. Other variables are:
- Number of repetitions of the monomer, which can be a few to hundreds to thousands of the same monomer. The polymer, by definition, is composed of a range of different molecular weights, and the Gaussian distribution around this value adds another variable.
- Role of the initiator, which is a different molecule. The same types of polymers can have different initiators plus other additional reactants that can contribute to the formation of the polymer.
- Presence of more than one monomer that can build up the polymers in a regular succession or randomly.
- The polymerization can occur by putting all the reactants together or through a precise sequence of steps.
- Additional reactive functional groups can be linked directly on the polymeric chain, without being part of the monomers.
- Finally, there is cross-linking. One polymer may cross-link with itself or with a combination of different polymers, leading to new shapes, resembling a star, a comb, a brush and so on. The side chains can be identical or different with variable length.

In conclusion, the number of different circulating polymers is uncountable, and each manufacturer typically has tens or even hundreds of different polymers in their portfolio. ECHA will request to notify all manufactured polymers to decide how to group them. At this moment, there are no other details on how to perform this step, and probably the final decision will be taken in a second phase with publication of a specific guideline. From the toxicological point of view, the risk is to group polymers that are very different with new tests that do not represent the toxicological profile of the whole group.

7.3 New endpoints

Pereira et al. (2022) discuss the need to revise REACH to improve safety assessment and protect human health and the environment, highlighting the negative animal welfare consequences of the current REACH regulation and the need to transition away from animal testing towards more species-relevant and modern science.

However, the proposals presented so far have significant negative animal welfare consequences. While they put no numbers behind these concerns, the article concludes that a paradigm shift is needed to promote new approach methods (NAMs) in chemical safety assessment and allow REACH to function efficiently and effectively while fulfilling the promise of the Chemical Strategy for Sustainability[34] (CSS). Thinking about introducing NAMs for the characterization of polymers, waiving new *in vivo* tests as well as deleting old ones with limited scientific weight is probably the only solution to collect toxicological information in a reasonable timeline and without putting too much burden on companies.

Among the endpoints under discussion are (developmental) neurotoxicity, immunotoxicity, respiratory sensitization, and most certainly endocrine disruption. In the US, the endocrine disruptor screening program advanced earlier to a first round (Juberg et al., 2014): Notably, this first screening (tier 1, not a definitive assessment) cost about $5 million per substance; this is about the cost calculated for a full Annex X assessment, illustrating the possible dimensions. The increasing availability of *in vitro* and *in silico* approaches for developmental neurotoxicity (Smirnova et al., 2014; Fritsche et al., 2015), including most recently developed OECD *in vitro* guidance[35], neurotoxicity (Schmidt et al., 2017), immunotoxicity (Wang et al., 2022), endocrine disruption (Manibusan and Touart, 2017), etc. offers opportunities to use NAMs. Notably, there is not even an accepted animal test method for respiratory sensitization but some *in silico* opportunities (Golden et al., 2021).

7.4 Conclusions for REACH revision policy options

Of the different policy options, only the extension of the CSA to Annex VII substances can be easily quantified with a range of 1.2 up to 2.6 million animals. Albert Einstein (1879-1955) is quoted, "*Everything that can be counted does not necessarily count; everything that counts cannot necessarily be counted*". The dimension of the polymer challenge counts but cannot be counted. It is probably best to compare it with the general petrochemical problem. It is adding a similar number of chemicals and stakeholders as well as challenges of diversity. It is fair to assume that several million animals will be needed. For other endpoints, too much depends on what is actually decided with respect to the choice of methods, applicable tonnage levels, triggers, and waivers.

Most importantly, the tools are available to derive forecasts

[34] https://environment.ec.europa.eu/strategy/chemicals-strategy_en

[35] https://search.oecd.org/chemicalsafety/testing/guidance-evaluation-of-data-developmental-neurotoxicity-in-vitro-testing.pdf

of the resulting use of animals for whatever political decision is considered.

8 EU Green Deal and impact on REACH and CLP

The EU Green Deal under the CSS opened wide-ranging revisions of linked policy texts such as REACH, CLP, and cosmetics among others in 2019. The need for a "*zero-pollution and toxic-free environment*" under CSS translated into an increase in regulatory testing requirements (CSA for Annex VII substances) as well as new endpoints (developmental immunotoxicity, developmental neurotoxicity, respiratory sensitizer) and categories (polymers, mixtures).

At the same time, CSS claims to promote alternatives to animal testing as much as possible, and recent activities from politics and regulatory agencies in the European Commission are working towards an increased uptake of NAMs such as:

1) EFSA NAM roadmap (EFSA, 2022) contains a more than €20 million call for tenders to develop a NAM toolbox
2) ECHA NAM workshop 2023[36]
3) EC roadmap to phase out animal testing for chemicals foreseen in Q4 2023
4) Adaptation of REACH Annex VII & XI to incentivize the use of NAMs and ensure legal clarity and certainty
5) Adoption by EC of some 100 new and updated test methods[37] – with the majority being NAMs – for REACH
6) Funding of H2020 integrated projects such as the ASPIS cluster[38], which received €60 million.

These ongoings efforts by EU institutions may be linked to some extent to external pressure from the European Parliament and their motion of resolution[39] and the European Citizen's Initiative: Save Cruelty-free Cosmetics[40].

Similar initiatives are underway in the USA, where the EPA released their NAMs Work Plan in 2020[41] and is since then working towards developing and implementing a roadmap for phasing out animal experimentation for regulatory purposes. These different initiatives alongside academic, industry, and SME activities are driving research for the development and validation of NAMs for (eco)toxicological risk and hazard assessment of chemicals and cosmetics[42].

9 Framing and outlook

The results of Knight et al. (2023) are taken as facts. In the text above, we have attempted some informed guesses ("guesstimates"), which include other non-systemic endpoints already done, most likely around 1 million animals, the open discussion on petrochemicals, adding at least 1 million animals, and the 2022 REACH amendment with at least 3.6 million animals. While the current policy options discussed for a revision of REACH can be roughly characterized for the extension of full CSAs to Annex VII substances with 1.6 to 2.6 million animals, the likely inclusion of polymers and other endpoints such as endocrine disruption will add many millions more animals (Fig. 2).

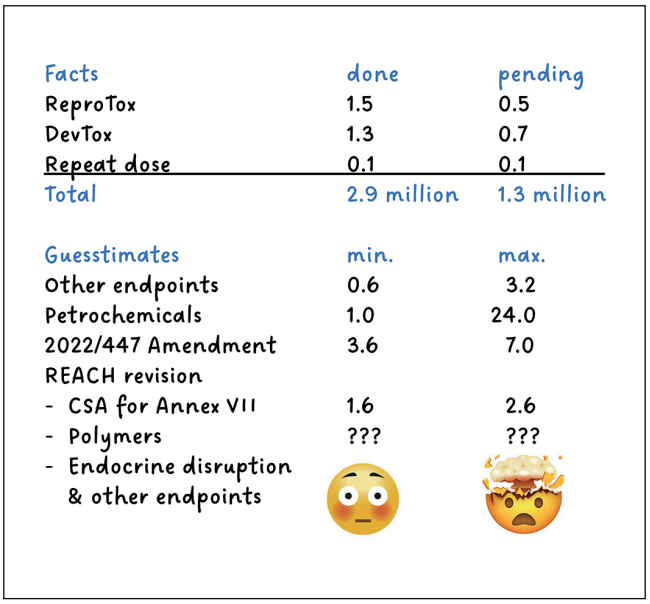

Fig. 2: Summary of main findings of this study

Toward the end of this explainer, it is important to look back at its point of departure – the quote by Charles Darwin, with a balanced, differentiated view on the use of animals. This historical statement is well-aligned with the present legal situation in Europe and many other countries: Animal experiments are legally acceptable if there is a strong rationale for their usefulness and if no alternatives are available. Now, that millions of extra animals have been used (for REACH) and large additional numbers may be used in the future, it is not only reasonable but rather ethically and legally mandatory to ask whether this was useful (Pallocca and Leist, 2022; Pallocca et al., 2022b). The question on usefulness has several dimensions, of which two are of particular relevance here:

(i) Is there a net benefit from having performed these tests (or will there be a gain from new tests)?
(ii) Could the same benefit have been achieved with other methods? More precisely, one may ask whether animal use has

36 https://echa.europa.eu/-/new-approach-methodologies-workshop-towards-an-animal-free-regulatory-system-for-industrial-chemicals

37 https://environment.ec.europa.eu/publications/commission-regulation-amending-purpose-its-adaptation-technical-progress-annex-regulation-ec-no_en

38 https://aspis-cluster.eu/

39 https://www.europarl.europa.eu/doceo/document/B-9-2021-0427_EN.html

40 https://europa.eu/citizens-initiative/initiatives/details/2021/000006_en

41 https://www.epa.gov/chemical-research/epa-new-approach-methods-work-plan-reducing-use-vertebrate-animals-chemical#:~:text=The%20original%20EPA%20NAMs%20Work,Regulatory%20Flexibility%20for%20Accommodating%20NAMs

42 https://www.iccs-cosmetics.org/

a competitive advantage relative to other ways of obtaining the required information.

9.1 Net benefit of millions of animals used

A clear benefit of the experiments performed is that they fulfil legal requirements. This is a strong point, at first sight. In the context of this Food for thought … format, one should also be allowed to ask whether this is also a net benefit. It is not so uncommon that legal requirements conflict and fulfilling one may automatically violate another. For instance, testing of chemicals under REACH also includes compounds being mainly or exclusively used as cosmetic ingredients (Knight et al., 2021) although the Cosmetics Regulation prohibits animal testing. Also, Directive 2010/63/EU forbids animal experimentation unless certain conditions are fulfilled. Until a strategy of NGRA that relies on NAMs is firmly established and implemented (Moné et al., 2020; Pallocca et al., 2022b), it may be a valid argument that animal studies serve a higher value, i.e., they secure the safety of society and its individuals from chemical hazard. This seems to be a conclusive and convincing argument in the "net benefit discussion".

However, it is only conclusive if data show that these animal tests have provided the assumed benefit to the European population. The underlying assumption is that fulfilling the legal requirement of delivering data on certain tests leads to increased safety. If not, the line of argument fails and the justification for the massive use of animals is not given. Is there a comprehensive and conclusive analysis on how much better our safety is after having used several million animals? We are not sure, but legislators and politicians should ask this question – and journalists as well as lobbyists that care for animals, public safety, economic progress or also financial responsibility may also ask such questions. How would chemicals be classified without the use of animals (using available data and prediction tools), and how are they classified after animal data were obtained? How many changes occurred, and do these changes increase safety? It would be embarrassing if we found out that we cannot measure an improvement and animals may have been used for a dubitable phantom benefit. We do not know this, but the data would be interesting.

If this big question cannot be answered, perhaps a more modest benefit might be tested: Did the animal experiments provide general scientific insight to improve toxicological predictions and possibly to reduce a future use of animals? Test data on so many chemicals should provide the core of a spectacular database of toxicological knowledge (Luechtefeld et al., 2016, 2018). It could be used to calibrate prediction mechanisms for new chemical structures or to support read-across procedures as an animal-free approach to classical risk assessment (Rovida et al., 2020; Escher et al., 2019). Has such a database been generated and made transparently available to the public for research purposes? We know of ToxRefDB[43], the US counterpart of such a database (Leist et al., 2008), and also Canadian counterparts, which have been used extensively for calibrating NAM and also for the uncertainty analysis of animal data (e.g., Paul Friedmann et al., 2020; Beal et al., 2022; Ly Pham et al., 2020). Are the European animal testing efforts leveraged by such an approach? Are there any attempts to redeem some of the animal investments, to curtail the potentially further increasing use of animals in the future? To us, these seem to be fair questions. We would be glad to see that the deaths of so many animals had a purpose beyond fulfilling a legal requirement.

9.2 Competitive advantage of animal-based toxicological data

Many discussions on the usefulness of animal experiments stop at the point of having established a net benefit. Yes, animal studies have contributed to a relatively safe chemical environment in Europe. They have for instance shown that organophosphates are extremely toxic for humans, which has led to their nearly comprehensive ban in Europe. They also showed that aminofluorene compounds, which were previously used in the shoe industry, may trigger bladder cancer and that smoking is unhealthy. However, in each of these cases, we would have come to the same conclusions with other available methods: The unhealthy effects of cigarettes only led to regulatory activities when human epidemiological data were solid enough; acetylaminofluorene and related mutagens are easily detected and quantified by NAM, and the same applies to organophosphates. Admittedly, these are only few examples; and yes, there are chronic toxicities not easily identified by NAM, or at least not (yet) with the same specificity and sensitivity. The point is not a quantitative evaluation of the competitive advantage, but to highlight the need to consider alternative, competitive methods at all. There are clearly documented situations where NAM fully match animal data or are even better. Not for all areas, but with time, there will be more areas. Could some of the recent animal studies already have been substituted by other approaches? And how many of the future studies may be replaceable?

These are mandatory questions for responsible politicians and regulators. In simple words, one may ask how it is ensured that at any given time point, only the absolutely necessary and justified number of animal studies is performed. Who makes sure that animal testing is only done where there is a clear competitive advantage. Do we have an instrument, an institution to ask this question with some authority? If we are not sure that the answer is yes, this should be some food for thought on whether the current system works well. Perhaps a test moratorium would be justified to clarify some of the questions?

Lastly, these tests come at a price, not only the price of animal life, but also the costs of these tests and of possibly wrong decisions. In the current work, we have not evaluated the monetary side of REACH, but for example a single EOGRTS costs about €566,000 (Meigs et al., 2018); this means that this part alone will result in €579 to 880 million in testing costs. English mathematician Karl Pearson (1857-1933) said "*Statistics is the grammar*

[43] https://catalog.data.gov/dataset/toxcast-toxrefdb

of Science" – it is also the grammar of economics and should be that of policy-making.

10 Overall conclusion

In our parallel report (Knight et al., 2023), we counted 4.2 million animals that have been used or are in use in new *in vivo* studies performed for compliance with REACH. This number was derived by assessing one-by-one all REACH dossiers in the ECHA database and after analyzing the tests that are ongoing following a decision from the authority but limiting the estimation to the three systemic endpoints (repeated dose toxicity, reproductive toxicity, and developmental toxicity). This number is partial for many reasons. All other endpoints that also require tests on vertebrate animals were excluded from this assessment. Other toxicological tests (acute toxicity, skin/eye irritation, skin sensitization, and genotoxicity) require a limited number of animals while carcinogenicity and other chronic tests are rarely performed, and therefore their contribution in terms of animal numbers is limited. On the other hand, the ecotoxicological tests may require a high number of fish. A rough estimation of these contributions here increases that number by 0.6 to 3.2 million additional animals (Fig. 2). The evaluation of the registration dossiers is still ongoing, and the trend towards new requests is increasing. A major question is still how petrochemicals will be handled. If no grouping can be agreed upon, up to 24 million animals will be needed for 4,500 registered products. The originally suggested grouping would bring this down to 1 million, but the industries' own studies challenge this approach.

But this is not the only factor that has an impact on the toll of REACH in terms of animal numbers. A recent REACH "clarification", i.e., the 2022/477 amendment, is worsening the situation with 3.6 to 7.0 million animals as well as new CLP hazard classes in 2023/707. In fact, both amendments are making the possibility to adapt the standard information requirement with non-animal strategies more difficult, with additional cases when new animal tests are deemed necessary.

Furthermore, a new REACH revision is under discussion, and if the current approach is maintained, the new requirements could be unbearable in terms of animal numbers and burden for SMEs. This is not the only problem: the need for new tests will be massive and eventually unmanageable, leaving many chemicals without characterization. The aim of zero pollution and a toxic-free environment will become impossible to implement if this stays as it is.

George Box (1919-2013) has warned "*Statisticians, like artists, have the bad habit of falling in love with their models*". Are our numbers correct? No, they are estimates in a what-if-scenario. But they may allow to design the future of REACH and its implementation a bit better or at least to raise a consciousness of the consequences, which many citizens do not like. Why are statistics as attempted here necessary? The author Andrew Lang (1844-1912) stated, "*Most people use statistics like a drunk man uses a lamppost; more for support than illumination*". We feel they are both illuminating and supporting the case for NAMs. The time to incorporate NAMs has come. NAMs could help to solve these problems and constitute the only possibility to screen large numbers of chemicals and provide a good toxicological profile that is more human relevant. It is a matter of quitting the traditional approach to embrace a modern science (Fentem et al., 2021).

"*Numbers have an important story to tell. They rely on you to give them a voice*" said data visualization expert Stephen Few. We hope to fuel policy discussions with an evidence base. The EU should pursue leading the change and modernizing safety assessment with courageous EU legislation paving the way to better human and environmental safety. To do so, the institutions and the stakeholders should be as ambitious as possible in setting their threshold of satisfaction.

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Conflict of interest

The authors declare that they have no conflicts of interest.

Data availability

No original data was generated for this manuscript. All data used in this manuscript is publicly available and sources are cited. The raw data retrieved from the ECHA database is available from the corresponding author upon reasonable request.

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