

Article

Key Properties for the Toxicity Classification of Chemicals: A Comparison of the REACH Regulation and Scientific Studies Trends

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Abstract: In the last half century, the improvements in quality of life owing to the development of the chemical industry are indisputable. However, despite global improvements, there has also been a large increase in pollution at the environmental level and this has caused relevant harmful risks not only to wildlife and the environment but also to human health. In response, governments have begun to regulate and control chemicals to prevent environmental pollution. At the European level, REACH (Registration, Evaluation, Authorization, and Restriction of Chemicals) was created with the aim to protect human/animal health and the environment from chemicals. Additionally, this regulation shows the main experimental tests that are needed to classify a chemical from a physicochemical and toxicological point of view. The main objective of this study is to compare the tests or experiments stipulated by the European REACH regulation with the studies carried out by the scientific community. To obtain this comparison, an exhaustive bibliographic review was carried out, analyzing the physicochemical properties and the (eco)toxicological information established by the European REACH regulation and scientific articles published in the Web of Science (WOS) database. The results obtained indicate that, although there are many authors who conducted tests indicated by the regulation, there are others whose essays or studies are not in line with the regulation; this may be because, on many occasions, the purpose of the information to be obtained is quite different.

Keywords: REACH; environment; physicochemical properties; toxicity; risk; minimization



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1. Introduction

There is no doubt about the improvements in quality of life that the development of the chemical industry has provided: solvents, paints, pharmaceuticals, cosmetics, additives, and new materials. Chemistry surrounds us and has allowed the development of society as we know it today. However, chemistry has another face closely related to pollution and the health risks associated with the use of chemical substances.

The environmental movement has evolved exponentially in recent years; however, it was not until the end of the 1950s that there was a true awareness of the harmful effect that industrialization and the massive and uncontrolled use of chemical substances could cause, not only to the environment but also to humans. The turning point was perhaps in 1962, with the publication of *Silent Spring* by Rachel Carson, when the North American population became aware of the serious problem that the lack of control over chemical substances could cause. In her famous book, Carson detailed the effects of certain pesticides on the eggs of various birds, illustrating how dichlorodiphenyltrichloroethane (DDT) and other pesticides could spread through the food chain, causing irreparable damage to the eggs and wildlife. At about the same time, in Europe, panic spread because pregnant women were given a drug called thalidomide, used to reduce the effect of nausea. This

molecule was administered in its two enantiomeric forms; one of them produced significant defects in newborn babies. In most cases, babies were born without limbs or with significant limb deformities (about 10,000 cases worldwide and 5000 in Germany alone [1]). In both cases, public opinion became aware that the substances in question, designed by the scientists in whom people had placed their trust, were not as safe as they believed, and the unintended and catastrophic consequences began to be realized.

For this reason, governments began to regulate the use of chemical substances; legislation was increased to control the manufacture, use, and distribution of chemicals and to establish water, cleaning, and wastewater treatment controls.

However, during the 1990s, a new trend arose that considered if it was enough just to regulate and control chemical substances. Was control the only and most effective way to protect humans and the environment from disastrous outcomes? It was concluded that there was another way to pose the problem. Indeed, in 1990, the Pollution Prevention Act was approved. This act regulated the development of the prevention of waste formation at the source. Using various methodologies and techniques, contamination can be prevented, thereby eliminating the need for post-treatment to control chemicals, aligned with Green Chemistry principles [2].

In Europe, a change in the regulatory current also began and was finally established in the form of REACH (Registration, Evaluation, Authorisation, and Restriction of Chemicals). This regulation aims to protect both human health and the environment from chemical substances and mixtures in Europe (European Chemicals Agency, European Commission). REACH is intended not only to mitigate the environmental damage that new compounds can cause but also to protect human health. European companies and industries have been forced to incorporate this type of regulation in their manufacturing and industrialization processes since its implementation (1 June 2007).

Nowadays, there are more than 80,000 chemical substances registered for use in Europe [2].

Even if a huge amount of toxicological data has been collected over the years, there are still chemical compounds for which the toxicological potential is unknown. Furthermore, there is generally a high lack of knowledge about the environmental and health implications of many of the compounds already on the market [3].

REACH applies to all substances produced or manufactured in more than one ton per year in the EU. Marketing specifications become higher the higher the production in tons per year is. However, as mentioned above, toxicological and ecotoxicological tests remain insufficient and poorly defined.

The REACH regulation itself contemplates the possibility of resorting to tabulated data from handbooks and other sources. In this sense, the need to minimize animal experimentation is emphasized. This is why experimentation carried out *ex officio* at the scientific level should be aligned with the legal requirements. Although the objective of the measurement and study of many properties of chemical substances, whether well-known or newly synthesized, may not be the safety or the minimization of risk in the use of the substance, science constantly generates information that can be used for the purposes of REACH. Coordination between both interests, regulatory and scientific, should always be present. However, do we know if this alignment is taking place? Is there any divergence between science and regulation? If so, what are the critical points? Where are the information gaps?

In this study, we compare in depth the trends of science in the generation of new information on chemical substances and the legal requirements regarding the European REACH regulation. For this, we will carry out a quantitative study, analyzing the available literature and reviewing in the first place the type of trials or experimental tests required by European regulations and the trend of the scientific community. Secondly, when it comes to the same experiments, we will be analyzing if the conditions carried out to carry out the experiments are coincident. The objective of this study is to draw conclusions that allow us to be aware of the coincidences and discrepancies between European regulations and the

scientific trend in terms of obtaining and analyzing information for the determination of the toxicity or green character of a chemical substance.

2. Material and Methods

The systematic review method was used to obtain information related to the objective of the study. This was carried out by conducting a literature search to compile the information based on two distinct parts.

Initially, data related to the generation of new information on chemical substances and the legal requirements regarding the European REACH regulation were consulted on the website of the European Chemicals Agency (ECHA), specifically in sections related to legislation and regulation [4]. The information in this part of the study serves as a reference for the tests that the European REACH regulation establishes for the registration of new chemical substances and their mixtures.

Subsequently, in order to identify the tests used by the scientific community to determine physicochemical, ecotoxicological, and toxicological properties, scientific databases such as Web of Science and PubMed were consulted. The searches for scientific articles were carried out based on the three types of properties mentioned above (physicochemical, ecotoxicological, and toxicological), considering the following inclusion criteria for each of them:

- Controlled vocabulary thesaurus was used;
- Published safety data were updated by searching publications in peer-reviewed journals;
- Articles had to be published in the period from 2007 (start of the application of the REACH regulation) to August 2022;

The following general exclusion criteria were also considered:

- Research areas excluded in the analysis: Film Radio Television OR Audiology Speech Language Pathology OR Religion OR Criminology Penology OR Ethnic Studies OR Rehabilitation OR History Philosophy Of Science OR Astronomy Astrophysics OR Women's Studies OR Area Studies OR Emergency Medicine OR Medical Ethics OR Mathematical Methods In Social Sciences OR Substance Abuse OR Critical Care Medicine OR Family Studies OR Operations Research Management Science OR Art OR Architecture OR Robotics OR International Relations OR Arts Humanities Other Topics OR Philosophy OR Demography OR History OR Telecommunications OR Psychiatry OR Public Administration OR Mineralogy OR Communication OR Sociology OR Orthopedics OR Anthropology OR Automation Control Systems OR Government Law OR Mining Mineral Processing.

For each of the properties, the searching query in the above-mentioned databases was based on "measurement OR assessment OR evaluation" followed by the Boolean operator "AND" and the following specific major terms:

- Physicochemical studies:
 - For the total number of studies related to the evaluation of physicochemical properties, the searching query was "physicochemical properties".
 - For the studies related to the environment AND sustainability, the searching query was "physicochemical properties" AND (environmental OR "environmental risk" OR green OR sustain*).
- Ecotoxicological studies: ecotox* AND (short-term OR acute//long-term or chronic) and subheadings: (aquatic//terrestrial//aerial).
- Toxicological studies: consideration was given to avoid the appearance of ecotoxicological studies on these properties, discarding environmentally related terms: tox* NOT environment* AND (in vitro//in vivo) and other subheadings such as acute toxic*, subchronic, chronic, and mutagen*, related to toxicological studies.

In most cases, the large number of publications led to the consideration of those articles with the best match of the search query.

3. Chemical Properties and Safety Assessment Tests Required under the REACH

With the aim of determining the toxicity of a substance and its potential danger, as well as establishing the conditions of safe use and other issues such as safety pictograms, for example, it is important to carry out different studies. First of all, it is important to determine the quantities per year of the substance to be analyzed, this is essential to determine what type of tests must be done. Once this has been determined, a human health, physicochemical, and environmental hazard assessment should be realized. Additionally, persistent, bioaccumulative and toxic, and very persistent and very accumulative assessments have to be conducted (<https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32006R1907&from=ES> accessed on 8 November 2022).

Bearing this in mind, the physicochemical properties and the safety study (ecotoxicological and toxicological) required by REACH are presented. The necessary studies to be carried out, depending on the tons of substances that are marketed, are shown in Table 1. These studies are presented in detail below.

Table 1. Physicochemical properties and ecotoxicological and toxicological information proposed by REACH to classify a chemical.

Tons/Year	Physicochemical Properties	Ecotoxicological Information	Toxicological Information
>1000		<ul style="list-style-type: none"> - Degradation (biotic, further testing) - Fate and behavior in the environment (further information) - Effects on terrestrial organisms: invertebrates, plants (long term) - Effects on sediment organisms (long term) - Effects on birds (long term or reproductive) 	<ul style="list-style-type: none"> - Reproductive toxicity (developmental, one species) - Reproductive toxicity (two generations, one species) - Carcinogenicity study
100–1000	<ul style="list-style-type: none"> - Stability in organic solvents and identity of relevant degradation products (if substance stability is considered to be critical) - Dissociation constant - Viscosity 	<ul style="list-style-type: none"> - Aquatic toxicity in invertebrates and fish (long term) - Degradation (biotic, soil, sediment, and identification of degradation products) - Fate and behavior in the environment (adsorption/desorption screening, bioaccumulation in aquatic species) - Effects on terrestrial organisms (short term: invertebrates, plants, and soil microorganisms) 	<ul style="list-style-type: none"> - Repeated dose toxicity (28 days, one species) - Repeated dose toxicity (90 days, one species, rodent) - Reproductive toxicity (pre-natal development, one species) - Reproductive toxicity (two generations, one species)
10–100		<ul style="list-style-type: none"> - Aquatic toxicity in fish (short term) - Degradation (abiotic, hydrolysis function of pH) - Fate and behavior in the environment (adsorption/desorption screening) 	<ul style="list-style-type: none"> - Skin and eye irritation (in vivo) - Mutagenicity (in vitro, cytogenicity mammalian cells or micronucleus) - Mutagenicity (in vitro, gene mutation mammalian cells) - Acute toxicity (inhalation and dermal route) - Repeated dose toxicity (28 days, one species) - Reproductive toxicity (screening, one species) - Toxicokinetics (assessment from available information)

Table 1. Cont.

Tons/Year	Physicochemical Properties	Ecotoxicological Information	Toxicological Information
1–10	- State of the chemical at 20 °C and 1013 kPa		
	- Melting/freezing point		
	- Boiling point		
	- Relative density		- Skin irritation or corrosion (in vitro)
	- Vapor pressure		- Eye irritation (in vitro)
	- Surface tension	- Aquatic toxicity in invertebrates and aquatic plants (short term)	- Skin sensitization (in vitro)
	- Solubility in water	- Degradation (biotic, ready biodegradability)	- Mutagenicity (in vitro, gene mutation bacteria)
	- Partition coefficient n-octanol/water		- Acute toxicity (in vivo, oral route)
	- Flash point		
	- Flammability		
	- Explosive properties		
	- Self-ignition temperature		
	- Oxidizing properties		
	- Granulometry		

3.1. Physicochemical Properties

For REACH, the physicochemical characterization is one of the fundamental parts in the acceptance of marketing and production of chemical substances. The properties melting and boiling, partition coefficient (log P), surface tension, vapor pressure, water solubility, relative density, dissociation constant, oxidizing information and explosive properties, flash point self-ignition temperature, flammability, stability, viscosity, and granulometry must be obtained (Table 1). Standard test methodologies, normally OECD, are required for this purpose. The properties needed to be measured according to REACH, with the recommended methodologies and conditions of performing the test are given in <https://echa.europa.eu/support/registration/what-information-you-need/information-requirements-100-tn> (accessed on 8 November 2022).

The importance of the physicochemical properties required by REACH is related to the need to identify the risks associated with their use, as well as to provide correct information to the user. Much of this is reflected in the labeling of the substance once placed on the market. Analyzing the properties required, the information to be given can be grouped considering the nature of the properties. For instance, melting point, boiling point, vapor pressure, and density are quite important physicochemical properties that give information about the physical state of the substance and are related to how humans are most likely to be exposed to the substance and its behavior in the environment. Concretely, relative density explains the tendency to disperse or settle (for gaseous materials) and sink or float (for liquid or solid substances) once in nature. Alternatively, properties such as surface tension, water solubility, and partition coefficient give information about the behavior of the substance in solution; while surface tension is important for other physicochemical testing, water solubility and partition coefficient give information on how the substance is found in the environment and report on the risks of exposure to humans and the environment, as well as bioaccumulation in living organisms. Granulometry, even though it is not a physicochemical property, it is important to measure solid substances since it also provides toxicological information. Properties such as stability in organic solvents, dissociation constant, and degradation give information on the tendency of the molecules to degrade and transform into other products. This information, together with the viscosity (a transport property), is requested for chemical substances marketed in Europe in large quantities (100–1000 tonnes per year) and are crucial to determine their behavior in the environment. Finally, there are another set of properties that should be measured to evaluate the potential hazard associated with the use of chemical substances and safe handling: explosive and oxidation properties, flash point, flammability, and self-ignition.

However, from the point of view of the advancement and development of physicochemical disciplines, the determination of these properties *ex officio* from the scientific level is usually associated with, for example, the knowledge of molecular behavior in the

different states of matter [5–7]; the obtaining of derived properties [8,9], which through different thermodynamic relationships, provide very valuable information to advance the knowledge of the behavior of the molecules that make up matter; or the collection of data for the development of predictive methods and equations of state, among many other objectives [10–13].

3.2. Ecotoxicological Information

Most chemical waste, both industrial and domestic, ends up in the environment. Thus, it is essential to develop regulations that minimize the impact in the environment derived from the use of chemical substances and demand minimum requirements to ensure environmental safety. It is important to determine all environmental characteristics and subsequently analyze abiotic and biotic factors [14]. In this sense, abiotic factors are related to non-living physicochemical properties such as air, soil, water, sunlight, or minerals. On the contrary, biotic factors are living microorganisms living in ecosystems (animals, plants, fungi, etc.)

With this in mind, REACH uses different tests with the aim to study the toxicity of different chemicals, their behavior in the environment and, additionally, how biotic and abiotic factors can be modified. The use of these tests depends on the tones per year of each substance marketed in the European Union. In Table 1, the ecotoxicological tests recommended by REACH are presented. These tests can be divided into a) biomodels; b) degradation (biotic, abiotic, and ready biodegradability), fate, and behavior in the environment (adsorption or desorption and bioaccumulation in aquatic species).

Biomodels are widely used in ecotoxicology because the use of some indicators, generally from different trophic levels, can provide valuable information on how chemicals behave in the environment [15,16]. REACH is mainly focused on aquatic and terrestrial toxicity.

For evaluating the environmental impact, several biomodels are proposed. For instance, algae are used to represent the primary producers (first step in the food chain). The OECD TG 201 (freshwater alga and cyanobacteria, growth inhibition test) is recommended. The aim of this bioassay is to determine the effects of a chemical on microalgae or cyanobacteria exposed to the chemical for 72 h. Then, the growth inhibition is analyzed. The limit concentration corresponds to 100 mg/L [17]. Another required biomodel is invertebrates such as crustaceans, *Daphnia magna* status, classified as primary consumers. REACH recommends carrying out the OECD TG 202 (acute immobilization test). In this test, chemicals are in contact with young daphnids aged less than 24 h. Immobilization is observed at 24 h and 48 h and compared with the control [18]. Finally, vertebrates (normally fish) are widely known as secondary consumers. For the evaluation of chemical toxicity towards fishes, it is recommended to use the OECD TG 203 (fish acute toxicity test). In this test, one or more species can be used, and fishes are exposed to the chemical for 96 h and mortalities are analyzed at 24 h, 48 h, 72 h, and 96 h. After this period, a lethal concentration 50% (LC50), which is the concentration of a chemical expected to produce an effect in 50% of test organisms, value is obtained [19].

For terrestrial studies, invertebrates, plants, and sediment organisms are used. In the case of plants, the OECD 208 terrestrial plant test: seedling emergence and seedling growth test, is usually used. This assay can evaluate the effects of a chemical on seedling emergence and early growth of higher plants. At the end of the assay, measurements are visual based on seedling emergence, biomass concentrations, shoot height, and different visual effects on different parts of the plant. Values of NOEC, which are defined as no observed effect concentration or the LOEC, determine the lowest observed effect concentration [20]. In the case of earthworms, the recommended tests are OECD 207: earthworm, acute toxicity tests and OECD 222: earthworm reproduction test (*Eisenia fetida* / *Eisenia andrei*). In the first one, the recommended species is *Eisenia fetida* and the test consists of putting the worms in contact with the chemical in an artificial soil and analyzing the results after 7 and 14 days of exposition [21]; in the second one, the main aim is to analyze the reproductive output

in *Eisenia fetida* or *Eisenia andrei* in a 4-week period. Finally, values of NOEC and EC₅₀ can be obtained [22]. Additionally, some studies on terrestrial microorganisms such as soil microorganisms or plants are required depending on the tones marketed per year. In the case of soil microorganisms, the OECD 216 soil microorganisms: nitrogen transformation test and OECD 217: soil microorganisms: carbon transformation test, is used. These tests are used to analyze the long-term effects of chemicals on nitrogen and carbon transformation activity of soil microorganisms, respectively [23,24].

Furthermore, ecotoxicological biomodels can be divided into short term and long term. The first one provides ecotoxicological information, usually in 24–96 h of exposition, and EC₅₀ values are given. The typical biomodels are: aquatic invertebrates (daphnids, crustacean, algae, and bivalve mollusks), aquatic plants, fishes, plants, and soil microorganisms. On the other hand, long-term studies are required if tons of chemicals per year are marketed in higher volumes. These tests are based on the use of invertebrates, fishes, terrestrial organisms, sediment organisms, and birds.

In addition to biomodels, REACH requires the use of other types of studies. These are related to degradation (biotic and abiotic as a function of pH, sediments, identification of degradation products, and fate and behavior in the environment). For this, the OECD TG 301 A-F (ready biodegradation test) is recommended. In this test, six different methods can be used: DOC Die-Away, CO₂ evolution (Modified Strum test), MITI (I) (Ministry of international trade and industry, Japan), closed bottle, modified OECD screening, and manometric respirometry [25]. On the other hand, hydrolysis as a function of pH is also recommended (OECD TG111, EU TM C.7, hydrolysis as a function of pH). This method assesses abiotic hydrolytic changes in substances at environmental pH (4–9). Chemicals are incubated without light and at constant temperature; after that, the buffer solutions are analyzed for the test chemical and for hydrolysis products [26]. In the case of adsorption/desorption screening, the preferable tests are OECD TM 106, EU TM C.18 and OECD TM 121, EU TM C.19. In the first one, the objective is to obtain a sorption value with the aim to predict partitioning under different environmental conditions. Several equilibrium adsorption coefficients of a chemical are obtained in different soil characteristics (clay content, organic carbon, soil texture, and pH) [27]. The second one calculates the adsorption coefficient in soil and in sewage sludge [28]. Activated sludge, which is usually present in biological sewage treatment plants (STPs), degrades the substances in municipal and industrial wastewater (biodegradation). This is another test recommended by REACH, which analyses the effect of a chemical on STP microorganisms. It determines the oxygen use by microorganisms in activated sludge during a period of 3–4 h [25]. To carry out this test, OECD TG 209, EU TM C.11, activated sludge, and the respiration inhibition test (carbon and ammonium oxidation) are used [29].

3.3. Toxicological Information

Humans may contact a wide variety of chemicals, either by direct contact or by release into the environment. The use of chemical substances requires examining the potential toxicity and, ultimately, their possible impact on the contribution to human diseases. Knowing the effects of chemicals allows making decisions about the use of chemical products. In most cases, it is required to carry out a battery of tests to evaluate the potential hazard of a chemical substance and thus, be able to make evidence-based decisions on whether to use the substance. REACH guidance establishes the requirements for the study of toxicological properties related to human health depending on the amount of annual production of the substance.

As can be observed in Table 1, REACH regulates the need to obtain toxicological information on substances through different tests with increasing complexity depending on annual substance production.

In the first range of measurement, REACH regulations require the obtainment of test information based on acute oral toxicity, skin or eye irritation, and mutagenicity. It is important to note that in the registration of a substance with higher tonnage ranges, the

performance of *in vivo* tests is determined by the lack of clear results in *in vitro* ones in terms of risk assessment or classification of the substance [25]. It could be the case that some studies are only conducted *in vitro*. Thus, at higher substance production ranges, other endpoints are required. Specifically, *in vivo* studies on acute and subchronic toxicity at repeated doses, *in vivo* mutagenicity and genotoxicity, and assessment of reproductive effects are included. All these necessary tests are presented in Table 1 and most of them are based on standard tests recommended by OECD guidelines and are determined by REACH as stated preference methods. Therefore, the information required for the chemical risk evaluation is clearly defined.

To explore the damage caused by chemicals on the skin, a wide range of standard tests can be performed in accordance with OECD guidelines. Until a few years ago, the assessment of the possible corrosive effect of chemicals on the skin involved the use of laboratory animals (OECD TG 404); however, nowadays the use of other standard tests prevails. Thus, OECD TG 430, 431, 435 and 439 are recommended to evaluate *in vitro* skin corrosion and irritation using different methods. Therefore, test OECD TG 430 is based on the rat skin transcutaneous electrical resistance (TER) method, OECD TG 431 is based on the reconstructed human epidermis (RHE) one, and OECD TG 435 contributes an *in vitro* membrane barrier test method that allows to identify corrosive substances. The OECD TG 439 test includes several validated methods that allow skin irritation assays to be assessed. Moreover, several standard methods are recommended by REACH to assess skin sensitization and some of them used laboratory animals such as guinea pigs and murine (OECD TG 429, 442A, 442B, and 406). Thus, with the aim to improve animal welfare, other mechanistically based *in vitro* and *in chemico* test methods (OECD TG 442C, 442D, and 442E) have taken the lead in the assessment of skin sensitization [30]. These standard methods allow measurement of different events that lead to skin sensitization and quantify the possible allergic response, such as the inflammatory responses generated in keratinocytes and gene expression related to specific cellular signaling routes. All these methods should be used for risk assessment associated with first-tier substances (1–10 tons) [30].

In the case of eye irritation assessment, several *in vitro* and *in vivo* tests based on OECD guidelines are recommended by REACH. The *in vitro* tests OECD TG 491 and 492 are based on cytotoxicity measurements by the MTT assay using a confluent monolayer of *Statens Seruminstitut* Rabbit Cornea (SIRC) cells and reconstructed human cornea-like epithelium (RhCE), respectively. Moreover, the *in vitro* OECD TG 460 method is performed by using a confluent monolayer of Madin–Darby Canine Kidney (MDCK) and a fluorescent dye, allowing comparison of the difference in fluorescence between untreated cells and those treated with the evaluated substances. On the other hand, *in vitro* OECD TG 437 and 438 tests are based on qualitative analysis of the opacity and permeability of bovine and chicken eyes, respectively, and allow to relate it to eye irritation. For the measurement of *in vivo* ocular irritation, the use of the test OECD TG 405 is recommended and intended preferably with albino rabbit. In each animal, one untreated eye and the other treated with the substance are used to measure and compare the damage after 1 h, 24 h, 48 h, and 72 h, evaluating fully the magnitude and reversibility of the effects observed [25,30].

Considering mutagenicity effects of substances, several methods can be used to assess the effects under REACH requirements. At the first level, *in vitro* gene mutation in bacteria based on OECD TG 471 is used frequently. This test is commonly known as the Ames test and allows to determine if a substance can cause genetic mutation in bacteria which could induce it to occur in humans as well [31]. Moreover, *in vitro* mutagenicity in mammalian cells can be measured through the assessment of cytogenicity (OECD TG 473, *in vitro* mammalian chromosome aberration test) or micronucleus-forming ability (OECD TG 487, *in vitro* micronucleus test). These tests can determine the capacity of a chemical to interfere with the genetic material of mammalian cells. Moreover, a substance can provoke a change in the genetic material and OECD TG 476 and 490 can be followed to measure this property. Both tests determine gene mutation using *Hprt* and *xprt* genes or the Thymidine Kinase

gene, respectively [32,33]. Information obtained from all these *in vitro* tests in mammalian cells has an impact on identifying other human toxic properties.

When there is a positive result in any of the *in vitro* gene mutation studies in bacteria or mammalian cells, the *in vivo* mutagenicity must be determined. To do this, REACH recommends carrying out several OECD standard tests (OECD TG 475, 474, 486, 488, 489, 483, and 478). Some of them are based on a chromosomal aberration (chromosome and chromatid) test using different types of cells such as mammalian spermatogonial and bone marrow cells of rodents. Other tests indicate that the animals must be exposed to the substances to be tested and the different mutations generated are checked. It is remarkable the fact that one of the OECD guidelines to follow, specifically OECD TG 486, has not been updated in at least 20 years, which leads one to think that it will not be carried out as much as other tests [30]. Moreover, the *in vivo* chromosome aberration test and the *in vivo* micronucleus test are considered the most adequate tests to follow up on the concern of chromosomal aberrations.

In addition, the toxicokinetic profile of a substance allows to describe the complete behavior in a living whole organism. This assessment allows to determine the effects from different exposure routes and the distribution of the substance through the body. In this sense, REACH recommends following a toxicokinetic test based on OECD TG 417, providing information that allows understanding of the mechanism of toxicity of the tested substance. Moreover, this test provides information on mass balance, absorption, bioavailability, tissue distribution, metabolism, excretion, etc. These basic toxicokinetic parameters, together with complementary screens, provide useful information on the toxicokinetics of molecules [34].

The acute oral toxicity of a chemical is the only *in vivo* test recommended at the first level of tonnage production. The health effects of this event may even occur following ingestion of the substance. Acute oral toxicity can be determined following several methods of OECD guidelines (OECD TG 420, 423, and 425). In all these tests, rodents are the animals used to evaluate the toxic effects and initial doses must be selected accordingly to avoid severe toxic effects or mortality.

Other *in vivo* standard tests can be carried out to assess acute toxicity due to a short term and single exposure of a substance. Some of them are related to an inhalation process which may induce health effects. Again, several OECD guidelines are recommended by REACH. In this sense, OECD TG 403 allows to obtain an estimation value for a median lethal concentration (LC₅₀) or non-lethal threshold concentration (LC₀₁) establishing a possible quantitative risk assessment [35]. Moreover, OECD TG 433 is used when evident toxicity exists and moderately toxic concentrations must be probed in rodents, avoiding lethal concentrations. Finally, OECD TG 436 allows to classify a substance depending on its acute inhalation toxicity. Other acute toxicity tests are related to dermal contact with the tested substance, which follow the OECD guidelines recommended by REACH (OECD TG 402) and provide health information, allowing for classification of the substance [36].

On the other hand, it should be considered that a substance can generate health effects after multiple contacts. For this, tests related to assessing repeated dose toxicity must be performed based on OECD guidelines. All of them are *in vivo* tests and can be carried out in the short term (usually 28 days, OECD TG 407, 410, 412, and 422) or for a long time (usually 90 days, OECD TG 408 and 409). The latter tests tend to be performed when initial toxicity information has been obtained from acute toxicity tests or 28-day repeated dose tests and with the aim to detect subchronic effects on human health [30].

To complete the assessment of chemical substances, it is also essential to detect developmental or reproductive toxicity. All *in vivo* tests recommended by REACH for this assessment focus on reproductive and developmental effects and, depending on the tonnage of production, the number of generations involved is different. Reproductive toxicity screening tests provide primary information on potential problems related to fertility and reproductive ability. Most of the recommended *in vivo* tests follow OECD guidelines such as 421, 422, and 443 [30].

Attending to the nature of these proposed tests, a division is observed between those carried out following *in vitro* and *in vivo* methods. In fact, *in vitro* assays are more extended at the first level of tonnage, with *in vivo* tests (acute and subchronic) being those that must be carried out in the upper ranges. However, there is an increasing interest in avoiding animal experiments. Indeed, REACH establishes that animal experimentation should be avoided whenever possible and information about substances can be gathered from available data [37].

4. Chemical's Toxicity Assessment Studies Aligned with Regulatory Requirements

After having analyzed the REACH recommendations, this section presents the results obtained on the used studies, in general, by the scientific community. First, the most used physicochemical properties will be presented, and then, the ecotoxicity and toxicity tests will be described. An important point to consider in this study is the OECD guidelines, which indicate concentrations/doses tested, models to use, etc. It is important to highlight that after the search is carried out, on many occasions, these types of guides are not literally followed, although they are based on them. In most studies, the indicator/labeled property is used, but the doses or concentrations depend on the substance to be analyzed and the author, making it very difficult to compare the results obtained.

4.1. Physicochemical Properties

Most of the found manuscripts are classified in environmental sciences, ecology, chemistry, toxicology, zoology, biochemistry and biology, pharmacology, pharmacy, public environmental occupational health, marine freshwater biology, agriculture, or water resources, among others, are the most typical research areas.

Of course, there are numerous scientific studies whose objectives are related to obtaining safer substances and processes or the rational design of chemicals, with a green, sustainable, and health promotion approach. However, these represent a small percentage (approximately 25%) of the totality of the studies about physicochemical properties from 2007 to date.

On the other hand, it should be noted that there is a large group of physicochemical properties available that are provided by the scientific community, with the aim of being used to characterize the risk associated with the use of these substances or their sustainability and health nature, which are not required for the REACH regulation.

For example, the dissociation constant (pKa), the overall persistence or half-life, is a measurement related to degradation and formation of byproducts [38,39]. Properties such as organic carbon sorption coefficient (log K_{oc}) or distribution coefficient (log D_{ow}) are measured for their connection with the capacity to remove contaminants in natural matrices [38]. Typical thermophysical properties are also used: refractive index, density, specific heat capacity, and viscosity [40] as well as properties not so common such as contact angle [41], total soluble solids, titrable acidity and firmness [42], or long-range transport potential [39].

All properties aimed at characterizing chemical substances from the point of view of chemical and molecular analysis are also important for environmental and human health risk minimization. Thus, we find numerous studies that carry out spectroscopic measurements [43,44], microscopy [45], X-ray diffractometry [46], or elemental chemical analysis [45] in order to analyze the environmental impact of certain substances or justify their safe use.

It is worth mentioning that, for the environmental risk evaluation of nanomaterials, other types of properties are measured and analyzed. For instance, properties such as particle diameter, specific surface area, crystallinity, weight and weight loss, transformation rate, zeta potential, or Hamaker constant are normally measured and/or predicted [47–50]. For this type of material, surface chemistry, morphology, and sedimentation behavior are factors of importance and provide important information when analyzing the environmental impact of nanomaterials. Other properties that are also usually measured when evaluating

the green character of nanomaterials are those related to the ability of the particles to form aggregates (coating processes or state of aggregation) [48]. In fact, critical aggregation concentration is another property that is used for evaluating the environmental impact of chemicals in general [51,52]. This property has been related to toxicity, bioavailability, and the ability of molecules to cross biological membranes [52,53].

It is also remarkable that, to evaluate the potential risk of chemical substances in soil, very different properties are used, which are not required by REACH. For example, sorption degradation and mobility potential [54], rates of degradation [55], soil sorption coefficient [56], pH [55,57,58], electrical conductivity [57], soil aggregate stability, and soil organic matter [58] are very commonly used.

Finally, there is another important group of physicochemical properties that, although they are not used directly for the environmental or risk assessment of chemical substances, are measured indirectly since they are key properties for the development and application of predictive methods of impacts on the environment or toxicity, as well as Quantitative Structure Activity Relationship (QSAR) or Quantitative Structure Property Relationship (QSPR) methods. In this case, a long and varied set of different properties can be used, from parameters that characterize molecules (topological parameters) [59,60] to bulk properties [61], whether they are volumetric [62], thermodynamic [63], or transport properties [64]. To illustrate this, we will mention some examples: in the work of Schenker et al., degradation half-life, partition coefficient, and energy of phase transition are the properties used to describe the toxicological behavior observed [65]. In the work of Hernandez-Altamirano et al., a total of ten molecular descriptors, related in one way or another to biological activities, were explored: dipolar moment, GAP, C5 atomic charge of NBO, molecular volume, E(HOMO), E(LUMO), C5 Mulliken atomic charge, partition coefficient log P, electrostatic potential, and $\Delta(13)C(C5)$. In this case, only three parameters or properties (log P, electrostatic potential, and $\Delta(13)C(C5)$), seemed to be the tracking variables in the system [66]. It is also common to mix several types of descriptors or properties to construct the predictive model. For instance, in the work of Zuriaga et al., the multivariable regression analysis carried out showed that log P, E(LUMO), and heat capacity were the minimum set of variables that led to the best correlation between experimental and predicted values of toxicity [64].

4.2. Ecotoxicological Information

REACH provides the necessary guidelines for the classification of chemicals. However, the scientific community, while relying on the REACH proposals, does not only use this type of testing. With the intention of verifying the correlation between the studies marked by the regulation and the trends followed in terms of environmental impact assessment of chemical substances by the scientific community, a bibliographic review was carried out.

The results of this review of the literature are shown in the next paragraphs. In Table 2, the distribution between short-term OR acute and long-term or chronic studies is shown. The number of manuscripts of each category published in WOS is quite similar, being a little bite higher for short-term studies with 53% against long-term studies with 47%. Additionally, for short- and long-term studies, aquatic assays predominate over terrestrial and aerial ones.

For the short-term and long-term biomodels, several tests are used. If the percentages obtained for short-term and long-term biomodels are analyzed, it is observed that there are no significant differences and that, in general, the type of biomodel in both short and long term tests are practically the same as well as the percentage of each one. The fundamental difference between some tests and others lies in the time of the test and in the end point of each of them. For both cases, the most frequently used biomodels in aquatic medium are: fish, plants, microorganisms, crustaceans, bacteria, algae, mollusks, planktons, amphibians, and protozoos. For terrestrial analysis, the most used biomodels are plants, microorganisms, worms, fungi, and mites. Finally, in the case of aerial quality evaluation, the main biomodels used are birds, bees, salamanders, ferns, and lichens.

Table 2. Ecotoxicological studies found in the literature (WOS on 9 September 2022).

Short-Term or Acute Biomodels (53%)			Long-Term or Chronic Biomodels (47%)		
Aquatic Quality (80%)	Terrestrial Quality (14%)	Air Quality (6%)	Aquatic Quality (87%)	Terrestrial Quality (10%)	Air Quality (3%)
Amphibians (3%)			Amphibians (2%)		
Algae (10%)	Sediments (10%)		Planktons (2%)		
Rotifers (1%)	Microorganism (30%)	Bees (13%)	Protozoos (2%)	Microorganisms (20%)	Birds or eggs (81%)
Bacteria (7%)	Mites (3%)	Ferns (3%)	Algae (9%)	Mites (2%)	Bees (13%)
Microorganisms (13%)	Earthworms or worms (20%)	Lichens (1%)	Bacteria (8%)	Earthworms/worms (14%)	Ferns (2%)
Plants (19%)	Fungi (2%)	Salamanders (4%)	Microorganisms (14%)	Plants (50%)	Lichens (2%)
Mollusks (3%)	Plants (45%)	Birds or eggs (79%)	Plants (25%)	Sediments (9%)	Salamanders (2%)
Crustaceans (18%)			Mollusks (4%)		
Fish (24%)			Crustaceans (11%)		
			Fish (24%)		

In the case of aquatic biomodels, fish is the most popular biomodel. This may be justified since fishes are an important link in the aquatic food chain. The most used tests are short term or acute toxicity, by exposition of fish to high concentrations of chemicals during a short period of time (days). Several enzymatic tests, gene expression, toxicity profile, quantification of EC50, bioaccumulative analysis, toxicokinetics study, pathological effects, swimming behavior, or embryo development, among others, are used. Some of species used are: *Danio rerio* [51,64,67–71], *Larimichthys polyactis* [72], *Oreochromis niloticus* [73], *Oryzias melastigma* [74,75], *Cyprinus carpio* [76,77], *Poecilia sphenops* [78], *Bryconops caudomaculatus* [79], *Oreochromis niloticus* [80], *Gasterosteus aculeatus* [81,82], *Pungitius pungitius* [82], or *Rhamdia quelen* [83] among others.

In the case of plants, the most common tests evaluate the effect on growing, biosorption, bioaccumulation, or toxicity. Some typically used species are: *Lemna minor* [84,85], *Vallisneria natans* [86], *Lepidium sativum* [87,88], and *Myriophyllum spicatum* [89].

Crustaceans are frequent species worldwide and are used to carry out acute and chronic toxicological tests. In general, the used tests are related to reproduction, immobilization, biomarkers, etc. The most common crustaceans are: *Daphnia magna* [87,90–94] and *Artemia salina* [95].

Algae, as plants, are an important part of the aquatic food chain. If this biomodel is altered by a chemical, a negative effect can be observed in other organisms. Algae are preferred to use because their maintenance is easy and they can predict the hazard in sediments or in other biomodels. The most widespread tests are growth inhibition test, measurement of chlorophyll values, or evaluation of several biomarkers that can be used to determine if chemicals can affect the algae community. Some species used are *Chlorella pyrenoidosa* [96] and *Raphidocelis subcapitata* [93,94,97,98].

In the case of bacteria, the most common species used in ecotoxicological studies is *Allivibrio fischeri* [60,87,93,94,98]. In the case of protozoa, *Tetrahymena pyriformis* [99], *Stentor coeruleus*, *Stylonychia lemnae* [100], or *Paramecium caudatum* [101], among others, are used. These tests are quite popular among the scientific community because these biomodels are more sensitive than vertebrate biomodels.

In general, amphibians are not specifically considered in risk assessment because it is assumed that they are covered by the toxicity studies for aquatic invertebrates, fish, mammals, or birds. However, many endpoints have been analyzed using amphibians such as mortality, growth rates, behavior, or time spend feeding. These tests are usually conducted towards embryos and larvae [102]. Experimental tests with amphibians are related to absorption, toxicity, hormonal studies, and analysis of insecticides and their metabolites [103]. Some species that have been used include: *Xenopus laevis* [104–106], *Bufo americanus* [103,107,108], *Rana pipiens* [109–111], *Plethodon glutinosus* [112], *Bufo woodhousii* [113], *Pristimantis taeniatus* [114], *Rana temporaria* [115–117], or *Ambystoma gracile* [118,119] and *Ambystoma tigrinum* [120,121] among others.

On the other hand, mollusks are also good biomodels to analyze aquatic contamination. Some studies related to oxidative stress, immunotoxicity, neurotoxicity, or genotoxicity have been carried out [122]. Some of the used species are: *Corbicula fluminea* [123,124], *Ruditapes decussatus* [125], *Ruditapes philippinarum* [126,127], *Scapharca subcrenata* [128], or *Crassostrea virginica* [129,130].

Rotifers are abundant in aquatic medium, and they are very useful to evaluate the full life cycle and population level effects when they are in contact with chemicals [131,132]. In general, acute, and chronic toxicity is analyzed and additionally, effects on the feeding behavior and reproduction parameters can be also studied. Some species used for toxicity tests are: *Proales similis* [133], *Brachionus ibericus* [133], *Brachionus calyciflorus* [134], *Brachionus plicatilis* [135], or *Brachionus koreanus* [136].

Regarding terrestrial biomodels, plants, microorganisms, earthworms, sediments, and mites are normally used. The terrestrial plant tests have been carried out in different species such as *Zea mays*, *Allium tuberosum*, *Solanum lycopersicum*, *Lactuca sativa*, *Glycine max* [137], *Mucuna aterrima*, *Blossica oleracea* [138], *Tripleurospermum inodorum*, *Stellaria media* [139], *Brassica rapa* [140], or *Allium cepa* [141]. These species have been used for toxicity studies and determining vegetative and reproductive endpoints [142]. In the case of earthworms, toxic profiles, reproductive toxicity analysis, or even histopathological effects can be evaluated; the main species used are: *Eisenia andrei* [143,144], *Eisenia fetida* [145–147], *Dendrobaena veneta* [148], *Eudrilus eugeniae* [149], *Pontoscolex corethrurus* [150], *Aporrectodea caliginosa* [151], and *Eudrilus eugeniae* [151] among others.

The studies of sediment toxicology began to increase in popularity from the 1970s onwards, driven by growing environmental concerns and the ability of sediments to influence ecosystems [152]. Most sediment tests consist of acute toxicity experiments [153]. It is important to consider, when studying sediments, how they interact with the system as well as the deposition and resuspension [152]. Therefore, adsorption and desorption screening are necessary to determine the distribution of a substance in the ecosystem and to estimate the toxicity in sediments. For these tests, the methodology OECD TM 106: Adsorption–desorption using a batch equilibrium method is followed [27]. OECD guides have also developed a method to determine the toxicity of chemicals in the freshwater dipteran of sediments [154]. According to Simpson et al. benthic organisms such as algae, bacteria, mollusks, annelids, and nematodes, are the most adequate ones to predict the complexity of toxicity in sediment–water systems [155]. On the other hand, the most widely used bacteria for evaluating the acute toxicity in sediments is the already mentioned *A. fischeri* [156]. It is also common to test on algae or aquatic plants using as endpoints the effect on the photosynthesis process (chlorophyll measurement) or enzymatic inhibition [157]. The species *Entomoneis ch punctulata* [158] has also been used to evaluate acute toxicity. For sublethal toxicity essays, mussels such as *Mytilus galloprovincialis* [159] or snails such as *Hydrobia ulvae* [160,161] can also be used.

Finally, the most used biomodels for testing air quality are birds followed by bees or lichens. The experimentation in the case of birds is quite complicated since they are constantly moving animals. The most frequent studies analyze the presence of different chemicals in birds and how these substances affect their organs [162,163]. In the case of bees, the endpoints analyzed are survival, longevity, toxicity, and changes in gut microbiome composition. The most common species are *Apis mellifera* L. [164–167] although others such as *Megachile rotundata* [168], *Apis mellifera anatoliaca* [169], or *Bombus impatiens* [170] can be used.

Lichens are also important for evaluating air quality because of their sensitivity to various environmental factors and thus several physiological parameters can be used to evaluate environmental damage [171]. However, these studies are limited. Some works evaluate the air concentrations of toxicants and how chemicals can affect these organisms. Some species such as *Parmotrema tinctorum* [172–175], *Lobaria pulmonaria* [176] or *Corticolous lichens* [177], and *Leptogium* sp. *Lichens* [178] are used.

To finish the ecotoxicity section, as previously mentioned, it is also important to analyze other types of studies. Biodegradation of a compound is directly related to the persistence of the substance in the environment. These biodegradation studies are usually performed for organic substances [179–181]. Additionally, other kinds of studies including adsorption/desorption [182,183] or bioaccumulation [184–188] studies are carried out. The results of these types of experimentation give an idea of how compounds behave in the environment and, therefore, how they can affect different ecosystems. Adsorption/desorption screening is used to describe the tendency of a substance to bond to a solid (adsorption) and the tendency of a substance to be released into another system (desorption) [189]; the combination of these two is referred to as the sorption potential [190]. The most commonly used parameter to measure the sorption potential is the log K_{oc} (organic–water partition coefficient) [191], which was mentioned previously as an important property to be measured for obtaining information on the environmental behavior of soils. This parameter is normally used together with aquatic toxicity data to predict the hazard potential of a substance in soil/sediment. The most commonly used technique for this screening is the adsorption–desorption method using the batch equilibrium method [27]. This technique consists of the calculation of the adsorption percentage as a function of time and the estimation of the plateau equilibrium as well as the estimation of the soil adsorption coefficient *k_d* and its relationship to K_{oc}.

4.3. Toxicological Studies

The scientific community also carries out toxicological assessments associated with exposure to different substances, irrespective of whether they meet the minimum annual production tonnage. This community is also aligned with this premise of REACH regulations and is working to develop alternative methods for the study of human toxicological properties [192].

Indeed, Table 3 represents the proportions of each measure which are used by the scientific community. As mentioned above, the toxicological information is obtained through *in vivo* and *in vitro* tests. As can be observed in the table, *in vitro* tests are more frequently completed than *in vivo* ones (62% vs. 38%). This may be due in large part to the greater ease of carrying out this type of test and to following the premise of avoiding animal experimentation.

Table 3. Toxicological information found in the literature (WOS on 11 September 2022).

In Vitro (38%)	In Vivo (62%)
Carcinogenicity (11%)	Skin sensitization (1%)
Toxicokinetics (4%)	Irritation (4%)
Irritation (5%)	Cytotoxicity (69%)
Reproductive tests (18%)	Mutagenicity or genotoxicity (21%)
Oral toxicity (32%)	Acute toxicity <i>in vitro</i> (5%)
Inhalatory assays (7%)	
Mutagenicity or genotoxicity (23%)	

By analyzing the ratios for each type of test (*in vivo* and *in vitro* ones), a search for the main endpoints determined for toxicological properties related to human health was carried out. As can be observed in Table 3, cytotoxicity evaluations (69%) are the most frequent ones for *in vitro* assays and acute oral toxicity (32%) for the *in vivo* ones. Both are two of the most frequent endpoints to determine when a substance needs to be registered at the first levels of tonnage [25].

Therefore, the use of *in vitro* assays is widespread because this kind of test promotes reduction in animal testing. The main objective of the *in vitro* approach is to estimate the effects that chemical substances might provoke on human health.

In general, *in vitro* assays and cell-based assays function as great biological systems for the detection of the toxicity of chemicals for the assessment of their potential biological

activity and effects on toxicity pathways [193]. Therefore, cell viability, proliferation, and cytotoxicity assays are broadly used for this type of approach on the effect of human health, even though it is not one of the tests required by REACH [194]. In fact, these tests are frequently evaluated on a wide range of different products, such as solvents, drugs, and bioactive compounds, when an initial toxicological profile is to be established [195]. In this sense, one of the most versatile and popular tests is MTT assay, a colorimetric test that allows the determination of small changes in metabolic cell activity, specifically by the mitochondrial reductase enzyme [196,197]. Other similar in vitro assays for measuring cell viability that are widely used are the resazurin-based method, such as PrestoBlue [198,199]. Respective of the assay used for this endpoint, the toxicity of the substances may be different depending on the origin and cell type used [200]. Considering the work of scientific cytotoxicity measures are the most frequent endpoint used to explore basic human toxicology, as mentioned previously.

Even though cytotoxic assays provide a good approximation of the effects of substances, other in vitro assays can be performed to evaluate toxicological properties. Assessment of damage caused by chemicals to the skin allows to predict their hazard. Conducting in vitro tests of skin corrosion and irritation using reconstructed human epidermis models allows the identification of corrosive substances [201,202] and most of them follow OECD guidelines recommended by REACH. In vitro tests of skin corrosion are not usually performed in isolation for the evaluation of toxicity of substances but tend to be combined with other tests such as skin sensitization, absorption, genotoxicity, and eye corrosion [203,204].

On the other hand, these tests are not as widely used in the scientific community and represent a low proportion of the measures related to toxicological properties of substances.

With respect to in vitro mutagenicity or genotoxic evaluation, the use of the bacterial reverse mutation test (Ames test mentioned above) is recommended by REACH and used worldwide. Again, this test is used in a wide range of substances such as drugs, chemicals, solvents, food additives, and pesticides [205–207]. This test is usually combined with other in vitro tests that allow detection of in vitro mammalian chromosomal aberrations or other chromosome damage [208]. As commented above, genotoxic and mutagenic effects are frequently determined by the scientific community (the second in vitro type of assessment after cytotoxicity, as shown in Table 3) and are usually completed with in vivo assays when there are previous positive results [207].

On the other hand, the correlation between in vitro and in vivo studies is not always good, so other assessment methods are required with the aim to estimate toxicological properties [202]. There are some requirements for human health properties which could be studied both in vitro and in vivo.

Skin corrosion/damage has only been tested in vitro and following OECD guidelines (OECD 439). On the other hand, eye damage or irritation can be studied in vivo. REACH studies for eyes damage/irritation uses a rabbit in vivo test (OECD TG 405). There are in vivo studies conducted by some authors that involve eyes and cutaneous damage [209,210].

Taking into consideration the in vivo models used by the scientific community, *Caenorhabditis elegans* (*C. elegans*) is a good model to evaluate toxicity assays. This testing model could be an intermediate between in vitro and mammalian studies. The scientific community has studied the toxic effects on reproduction with *C. elegans* toxicity assays [211]. *C. elegans* models have been widely used for determining acute LC₅₀, showing that developmental toxicity in *C. elegans* could be as predictive as rat or mouse models [212–216]. *C. elegans* has a combination of advantageous traits (e.g., short life and reproduction cycle, robust, and easy and cheap to maintain large populations) which allow it to be widely used in toxicology [211,217–219].

OECD Guidelines for the Testing of Chemicals have evaluated acute toxicity on Wistar rats that are also widely used by scientific community [220–222]. However, other animals can be used as in vivo models with the aim to assess acute toxicity such as beagle dogs [223].

In vivo models in *Artemia salina* (*A. salina*) and zebrafish have been used to evaluate acute toxicity based in the lethality test of Meyer et al. (1982) modified for *A. salina* and the Fish Embryo Toxicity test (FET) for zebrafish (*Danio rerio*) [224–226].

The zebrafish genome presents a 70% homology with the human genome. The characteristics of the zebrafish model are: small size, external fertilization, short life cycle, reproductive capacity, and rapid development which make it ideal to study embryonic development [227]. Heart from zebrafish shows similar electrocardiographic patterns as humans, which makes it possible to study neurological and cardiac physiology [227]. However, it is important to keep in mind the specific differences between these species.

Chemical exposure and neurotoxicity have been studied in in vivo models of *A. salina* and zebrafish [228]. The great osmoregulation capacity for *A. salina* make this marine crustacean a good in vivo model [229]. The presence of monoaminergic neurons in the outer medulla and different areas of the brain in *Artemia salina* characterize its nervous system [230]. It is important to note that the *A. salina* model could be used to limit the concentrations to be evaluated in the zebrafish animal model.

Several models have been used to study pulmonary toxicity (i.e., inhalation and lung instillation bioassay studies). These models facilitate the comparison of the lung hazard impacts following in vivo exposures [231]. Warheit et al. evaluated acute lung toxicities of intratracheally instilled compounds and particles using a pulmonary bridging methodology [232].

Inhalation is the major exposure route for humans. The respiratory epithelium is the first tissue that inhaled substances directly interact with. OECD test guidelines described acute inhalation toxicity testing performed only in rats and/or mice. Such tests are based on the differences in the respiratory tract architecture and function across species, making it difficult to draw conclusions on the potential hazard of inhaled compounds in humans. Research efforts have been therefore focused on developing alternative, human-relevant models, with emphasis on the creation of advanced in vitro models. Currently, regulatory agencies have not accepted an in vitro model as a stand-alone replacement for inhalation toxicity testing in animals [233–239].

Finally, an in vivo study for toxicokinetics has been developed in plasma or bone marrow exposure. Relevant toxicology species (usually rat or mouse) have been used to conduct a mammalian in vivo micronucleus (MN) test to detect damage to the chromosomes or the mitotic apparatus of erythroblasts. This test identified test substances that may cause cytogenetic damage through formation of MN arising from chromosomal fragments or chromosomes that are not incorporated into daughter nuclei at the time of cell division [240]. Nallani et al. focused on target tissue exposure testing in support of in vivo MN [241], using an in vivo rodent erythrocyte micronucleus test.

5. Conclusions

The great development of industry and above all the incessant design of new chemicals makes it necessary to consider the impact that these substances have on the environment and on human health. For this reason, REACH has been regulating the use of chemicals in Europe in recent decades based on a comprehensive study of the physicochemical and toxicological properties of these chemical substances. On the other hand, the scientific community is also working on the development and study of chemical molecules of interest to society, either synthetic or from natural origin. In this manuscript, we have reviewed the coherence between regulation and the trends in the experimental study of chemicals made *ex officio* by the scientific community.

Analyzing the tests recommended by the REACH regulations for physicochemical and (eco)toxicological properties, it was observed that the scientific community tends to be aligned with REACH requirements. However, the purpose of the information obtained is quite different.

With respect to physicochemical properties, the objective of the scientific community is based on the knowledge of the behavior of the molecule or the provision of data for

predicting effects. For this reason, there are many other properties, of different nature than those required by REACH. This is especially remarkable for the evaluation of the potential risk of chemical substances in soil.

Regarding the study of ecotoxicological properties, it has been observed that the scientific community performs tests beyond those recommended by REACH. In addition, it prioritizes testing related to the aquatic environment over terrestrial or aerial testing. The wide variety of organisms used as biomodels for the different tests is also remarkable, as it is much broader than the REACH guidance tests.

Finally, this same trend has been observed for the study of toxicological properties, as there are studies that the scientific community carries out with the aim of finding out the effects of chemical substances on human health that are not included in those recommended by REACH. Indeed, it is worth mentioning that the scientific community makes extensive use of in vitro versus in vivo tests in order to reduce the use of laboratory animals, but when it does use them, the variety of models used is very wide.

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