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(Q)SAR tools for priority setting: A case study with printed paper and board food contact material substances

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ABSTRACT

Over the last years, more stringent safety requirements for an increasing number of chemicals across many regulatory fields (e.g. industrial chemicals, pharmaceuticals, food, cosmetics, …) have triggered the need for an efficient screening strategy to prioritize the substances of highest concern. In this context, alternative methods such as *in silico* (i.e. computational) techniques gain more and more importance. In the current study, a new prioritization strategy for identifying potentially mutagenic substances was developed based on the combination of multiple (quantitative) structure-activity relationship ((Q)SAR) tools. Non-evaluated substances used in printed paper and board food contact materials (FCM) were selected for a case study. By applying our strategy, 106 out of the 1723 substances were assigned 'high priority' as they were predicted mutagenic by 4 different (Q)SAR models. Information provided within the models allowed to identify 53 substances for which Ames mutagenicity prediction already has *in vitro* Ames test results. For further prioritization, additional support could be obtained by applying local i.e. specific models, as demonstrated here for aromatic azo compounds, typically found in printed paper and board FCM. The strategy developed here can easily be applied to other groups of chemicals facing the same need for priority ranking.

1. Introduction

Together with the high and continuously growing number of chemical substances subject to safety assessment, comes the need to establish adequate screening strategies to prioritize those of highest concern for human and/or environmental health. One notable example of a large group of substances urgently requiring a prioritization ranking for in-depth safety evaluation, are those used in food contact materials (FCM). Food contamination due to leakage of substances from FCM has become an increasing source of concern for human health (e.g. Liu et al., 2016; Muncke et al., 2014). Since 2011, an updated list of substances authorized as starting product or additive for the manufacture of plastic FCM is available (European Union, 2011). For non-plastic FCM, however, no harmonized European regulation has been established yet. Although national legislation exists in several Member States for different types of FCM, a broad range of substances currently used in FCM have not been evaluated for their safety (European Parliament, 2016).

Printing inks and paper(board) constitute large groups of non-plastic FCM substances. They are often used in combination and have been at the origin of multiple contamination issues, examples being the isopropylthioxanthone and the 4-methylbenzophenone crises (EFSA, 2005; 2009). Most of the substances that can be present in printed paper and board FCM have not been officially

Abbreviations: AD(I), applicability domain (index); ECHA, European Chemicals Agency; EFSA, European Food Safety Authority; FACET, Flavours, Additives and food Contact materials Exposure Task; FCM, food contact materials; FIG, FACET Industry Group; k-NN, k-Nearest Neighbors; IRFMN, Instituto di Ricerche Farmacologiche Mario Negri; (Q)SAR, (quantitative) structure-activity relationship; RASFF, Rapid Alert System for Food and Feed; SA, structural alert; SMILES, simplified molecular-input line-entry system.

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evaluated for their potential toxicity. Consequently, these non-evaluated substances could give rise to future food crises (Van Bossuyt et al., 2016).

Regarding plastic FCM, the European Food Safety Authority (EFSA) requires a core set of test data in order to be able to evaluate consumer safety of these materials. Genotoxicity data are always requested, regardless of the (estimated) migration level (EFSA, 2012). Indeed, genotoxicity i.e. the ability to cause DNA damage, can induce adverse human health effects including cancer (Claxton et al., 2010). In line with new EFSA Scientific Committee's recommendations on genotoxicity testing strategies, a battery of 2 *in vitro* genotoxicity tests is required, i.e. a gene mutation test in bacteria and an *in vitro* mammalian cell micronucleus test. If one of these tests yields a positive or equivocal result, further (*in vivo*) testing may be needed in order to investigate the genotoxic potential of the substance (EFSA, 2016).

The bacterial reverse mutation assay (Ames test) is the most commonly used *in vitro* test to detect gene mutations (OECD, 1997). Although it is a suitable test to identify gene mutation-inducing chemicals, its technical characteristics (in particular the test duration and the high quantity of test compound required) do not allow testing of >1000 substances in a short period of time at reasonable cost. The same obstacles are also encountered with the other assay required in the genotoxicity testing battery. A promising approach to detect mutagens without animal nor *in vitro* testing lies in the application of *in silico* tools. These computer-assisted methodologies are based on available experimental data, and are increasingly adopted in regulatory toxicology because of their time-, cost- and animal-saving nature. In particular, (quantitative) structure activity relationship ((Q)SAR) systems represent promising predictive computational techniques to evaluate potential genotoxicity and carcinogenicity of chemical substances (Serafimova et al., 2010).

(Q)SARs comprise both statistical QSAR and rule-based SAR systems. Rule-based models perform predictions via detection of so-called 'structural alerts' (SA), i.e. chemical fragments responsible for the toxic effect as determined earlier based on human expert knowledge. Statistical models, on the other hand, predict toxicity using an algorithm obtained by investigating the mathematical correlation between chemical properties (translated into molecular descriptors) and toxic activity (Bakhtyari et al., 2013). In both systems, chemicals are typically processed by means of their simplified molecular-input line-entry system (SMILES) representation. Most commercial (e.g. Derek Nexus®) and free (e.g. Toxtree) *in silico* software programs include statistical QSAR and/or rule-based SAR models to predict the induction of gene mutations in the Ames test ('Ames mutagenicity'). Furthermore, due to the abundance of consistent Ames test results and due to the binary result type: mutagenic/non-mutagenic, robust models for Ames mutagenicity are available and therefore the prediction performance for this endpoint is substantially better compared to other toxicological endpoints (Kamath et al., 2015). Indeed, *in silico* models for genotoxic endpoints other than Ames mutagenicity (e.g. chromosome-damaging potential in the micronucleus test) exist, but until now their accuracy is limited and needs to be improved before these models can become a more reliable screening tool.

Numerous publications on (Q)SAR evaluation of chemicals/chemical groups are available, however mostly in the context of model validation. Besides one study in which 2 SAR models were used to rank heat-generated food contaminants (Cotterill et al., 2008), to our knowledge, no study reports are available on the application of (Q)SARs for prioritization of potential human genotoxicants. In the current study, a screening strategy based on (Q)SAR tools is applied to identify, within the large number of non-evaluated substances that can be used in printed paper and board FCM, those that represent the highest concern for human health. The non-evaluated substances were first selected from a recently compiled inventory containing all substances which may be used in this type of FCM (Van Bossuyt et al., 2016). Next, their potential to induce gene mutations was predicted using a battery of Ames mutagenicity (Q)SAR models. The models were selected by taking into account existing recommendations such as the use of complementary systems (in terms of prediction method). Moreover, the combination of a SAR and a QSAR is already mandatory in certain regulatory domains, for example in the case of impurity testing of pharmaceuticals as described in the ICH M7 guidelines (ICH, 2014). Using the combined (Q)SAR results, a priority list could be composed of non-evaluated printed paper and board FCM substances requiring an urgent in-depth safety evaluation.

2. Materials and methods

2.1. Study substances

Substances that have not been officially evaluated were selected from a recently compiled inventory including 6073 unique substances which may be used in printed paper and board FCM (Van Bossuyt et al., 2016). Out of the 4690 non-evaluated compounds, 1769 single substances were retained for the current analysis. The remaining 2921 non-evaluated substances are not eligible for straightforward *in silico* processing, due to their chemical structure (e.g. polymers, mixtures, complexes, inorganic substances). Subsequently, the ChemSpider (Royal Society of Chemistry, 2016), ChemIDplus (National Institutes of Health (2016a)), PubChem (National Institutes of Health (2016b)) and European Chemicals Agency (ECHA, 2016) databases were consulted to collect missing CAS numbers and SMILES for the 1769 non-evaluated single substances. ChemSpider was used as the primary information source, whereas the ChemIDplus, PubChem and ECHA databases were consulted in case ChemSpider yielded no or ambiguous results. Afterwards, the compound selection was further refined by excluding substances for which no definite CAS number or SMILES could be identified, reducing the final number to 1723 (Fig. 1).

Fig. 1. Selection of study substances.

2.2. (Q)SAR models

The selected (Q)SAR models, specified in Table 1, are diverse not only regarding their prediction method (SAR/QSAR), but also with respect to their availability (free/commercial). For each system, the prediction model(s) related to Ames mutagenicity was (were) applied.

- *Toxtree*

Toxtree (www.toxtree.sourceforge.net) is an open source software application of the Joint Research Centre of the European Union (European Commission, 2016b). Toxic hazard of test compounds is predicted based on a decision tree-approach, constructed through the definition of rules flagging alerts for the selected endpoint. For Ames mutagenicity, 44 SAs are incorporated. A QSAR module for aromatic amines and αβ-unsaturated aliphatic aldehydes is also available and allows to refine the prediction of these specific chemical classes (not considered in the current study). Toxtree does not feature an applicability domain functionality.

- *VEGA*

The VEGA platform (www.vega-qsar.eu) has been developed by the Istituto di Ricerche Farmacologiche Mario Negri (IRFMN) and can be downloaded for free. It comprises an array of toxicity estimation models, including 3 for the evaluation of Ames mutagenicity i.e. CAESAR, SarPy and ISS (IRFMN, 2016a). CAESAR and SarPy, both QSAR models, were developed using the same training set of 4337 compounds. However, their prediction technique differs in the sense that CAESAR combines a machine-learning algorithm with 2 sets of sequential SAs (Ferrari and Gini, 2010), whereas SarPy follows a purely quantitative approach to determine whether test compounds are mutagenic or non-mutagenic (Ferrari et al., 2013). The third Ames mutagenicity prediction model, ISS, contains a set of SAs extracted from Toxtree, more specifically the SAs related to mutagenicity as implemented in the Benigni-Bossa rulebase for mutagenicity and carcinogenicity. In theory, this should result in the same model as the Toxtree model described above. However, in practice the outcome sometimes differs in VEGA/ISS and Toxtree. A possible explanation for these differences may be found in the rebuilding process that was used to translate the Toxtree rulebase into VEGA/ISS.

In the current study, the separate results of the 3 Ames mutagenicity models were combined into 1 final 'VEGA consensus' result, since this approach increases the prediction performance compared to the use of the individual models (Cassano et al., 2014). The output of the single models is integrated through their corresponding applicability domain index (ADI) by means of the following equation:

$$\text{CONSENSUS} = \frac{(\pm 1)*\text{ADI}_{\text{CAESAR}} + (\pm 1)*\text{ADI}_{\text{SarPy}} + (\pm 1)*\text{ADI}_{\text{ISS}}}{\text{ADI}_{\text{CAESAR}} + \text{ADI}_{\text{SarPy}} + \text{ADI}_{\text{ISS}}}$$

Each ADI in the numerator is multiplied by +1 for a positive prediction and by −1 for a negative prediction. In case the final outcome is negative, only prediction results with an ADI of at least 0.75 in all 3 models were considered negative. A similar approach has been proposed by Cassano et al. (2014).

- *Derek Nexus™*

Derek is commercially available as part of the Lhasa Knowledge Suite® (Lhasa Limited, 2016a) and is a SAR tool that runs predictions for, among others, *in vitro* mutagenicity through expert-based rules. The latter were developed from a variety of open literature and confidential data. For this reason and because it is a rule-based system, no defined training set nor applicability domain are available. However, a recently implemented structure classification feature allows to substantiate negative predictions (Williams et al., 2016). In case no alert for Ames mutagenicity is found, the software labels the test compound as 'inactive' (i.e. negative). Additionally, the compound structure is screened for 'misclassified' and 'unclassified' features. If it contains a chemical fragment that is not retrieved in the set of compounds on which the expert rules are based, the graphic display will highlight this part of the molecule and indicate that the structure contains unclassified features. Misclassified features, on the other hand, refer to chemical substructures that are not SAs, but have been found in experimentally positive reference compounds that lack a SA in Derek. Since negative predictivity generally remains high for both (median = 84%), misclassified and unclassified features are regarded as negative predictions that are flagged for expert review. However, for the current prioritization strategy that does not include elaborate expert reviewing, we followed a precautionary approach. Hence only negative predictions without warnings were considered negative.

- *Sarah Nexus™*

The Lhasa Knowledge Suite® also contains a QSAR-based Ames mutagenicity model named Sarah (Lhasa Limited, 2016b). In this statistical tool, the query compound is fragmented, after which the fragments are reviewed for activity *versus* inactivity. A network of hypotheses is then created by arranging meaningful fragments, followed by the application of relevant hypotheses to inform an overall mutagenicity prediction. A confidence score and applicability domain check complete the final conclusion.

Table 1
Model description.

Abbreviation	Software (version)	Model name	Method	AD	Availability
Global (Q)SARs					
Toxtree	Toxtree (2.6.0)	*In vitro* mutagenicity alerts (Ames test) by ISS	SAR	/	Freeware
VEGA	VEGA (1.1.1)	Mutagenicity (Ames test) model (CAESAR) v.2.1.13	QSAR	VEGA ADI	Freeware
		Mutagenicity (Ames test) model (SarPy/IRFMN) v.1.0.7	QSAR	VEGA ADI	
		Mutagenicity (Ames test) model (ISS) v.1.0.2	SAR	VEGA ADI	
Derek	Derek Nexus™ (4.1.0)	Mutagenicity *in vitro*	SAR	/	Commercial
Sarah	Sarah Nexus™ (1.2.0)	Ames mutagenicity	QSAR	Sarah AD	Commercial
Local QSARs for aromatic azo compounds					
CORAL	CORAL	Ames mutagenicity	QSAR	DefectSMILES	Freeware
istKNN	istKNN (0.9)	Ames mutagenicity	QSAR	/	Commercial

AD(I): applicability domain (index); (Q)SAR: (quantitative) structure-activity relationship.

- *CORAL*

CORAL (www.insilico.eu/coral) is a freely available standalone application software for building regression or classification QSAR models based on the Monte Carlo optimization method. It was developed as part of the EU-funded CHEMPREDICT project (IRFMN, 2016b). A complete description of the Ames mutagenicity model for aromatic azo compounds, built using the CORAL software, is provided by Manganelli et al. (2016). In brief, this model was generated using local and global SMILES-based descriptors on 3 random splits of data in training, calibration and validation sets. Test compounds are checked for falling into the applicability domain by calculating their DefectSMILES that should not exceed a predefined threshold value.

- *istKNN*

istKNN is a recently developed commercial software tool that can be used to build, evaluate and apply k-Nearest Neighbors (k-NN) models. The k-NN approach identifies a number (k) of neighboring compounds for the target compound to make a prediction. Each 'neighbor' is assigned a similarity index, allowing to extract the first k molecules with the closest similarity. In addition, specific similarity thresholds are defined, resulting in predictions solely based on molecules with a similarity index higher than the selected threshold. This method and the istKNN program are described by Manganaro et al. (2015). Details concerning the istKNN model for Ames mutagenicity prediction of aromatic azo compounds were recently published by Manganelli et al. (2016).

2.3. Model output processing

Each of the models described in section 2.2. Introduces its own particular denomination method to label negative and positive compounds (or -in the case of Derek- the probability of toxicity). In the present study, the original model-specific classifications were converted into 3 categories (negative, positive or undefined) for reasons of uniformity (Table 2). The undefined category is composed of:

- substances outside domain (in the case of Sarah),
- substances lacking sufficient similar compounds to make a prediction (in the case of istKNN),
- substances for which negative predictions are less convincing due to a low ADI (in the case of VEGA),
- substances with high DefectSMILES (in the case of CORAL) and
- substances with mis-/unclassified features (in the case of Derek).

This conservative approach was adopted in order to minimize the number of false negatives. Indeed, labelling mutagens incorrectly as non-mutagenic should be avoided as much as possible.

2.4. Determination and characterization of priority substances

All compounds were processed in 4 global (Q)SARs (i.e. Toxtree, Vega, Derek Nexus™ and Sarah Nexus™), which are based on structurally diverse compounds, reflecting a range of different action mechanisms (Chaudry et al., 2010). Aromatic azo compounds were also examined in 2 local QSAR models (i.e. CORAL and istKNN), built from structurally similar compounds i.e. all containing an aromatic azo structure. Substances positive in the 4 global tools are considered of highest priority with respect to further safety testing. Among these, priority ranking was refined based on the amount and reliability of available experimental mutagenicity data. This is ideally investigated through database and literature searches. However, a fair amount of information can already be deduced by a more detailed investigation of the (Q)SAR results. For example, substances predicted positive with a confidence score of 100% in Sarah or an ADI of 1 in VEGA are chemicals for which positive experimental Ames test results are already available.

Furthermore, a recently compiled inventory of substances which may be used in printed paper and board FCM was consulted to roughly estimate the likelihood of the high priority substances to migrate into the food and become bioavailable after oral intake. In addition, the Flavours, Additives and food Contact materials Exposure Task (FACET) tool was used to obtain a first indication of their actual use. The inventory and FACET tool have been described earlier (Van Bossuyt et al., 2016).

Also, the application of 2 local QSARs was investigated for substances containing an aromatic azo bond, with the goal of priority ranking refinement. More specifically, aromatic azo substances positive in one or two of the additional QSAR tools were considered of higher priority than those predicted negative by both.

3. Results and discussion

3.1. Individual models

An overview of the prediction outcome of the 1723 study substances as a function of (Q)SAR system used is presented in Fig. 2. At least 229 up to 366 of the substances are predicted mutagenic *in silico*. It must be noted that, in the case of VEGA, most of the substances (758) are outside domain when applying the ADI requirements set out in 2.2. This is due to the differences between the prediction methods and applicability domains of the 3 individual VEGA tools constituting the consensus model. Apparently, several substances do not reach an ADI $\geq$0.75 in all 3 models in order to consider them as negative in the current approach (Table 2). For these substances, together with the undefined substances in Derek

Table 2
Harmonization of positive, negative and undefined predictions.

Model	Positive	Negative	Undefined
Global QSARs			
Toxtree	Structural alert for *S. typhimurium* mutagenicity	No structural alerts for *S. typhimurium* mutagenicity	N/A
VEGA	1	0 with ADI $\geq$0.75 for all models	0 with ADI <0.75 for at least 1 model
Derek	'Equivocal' to 'Certain'	'Inactive without mis-/unclassified features' to 'Doubted'	Inactive with mis-/unclassified features
Sarah	Positive	Negative	Outside domain
Local QSARs for aromatic azo compounds			
CORAL	1	0 with DefectSMILES <1.83485	0 with DefectSMILES $\geq$1.83485
istKNN	1	0	No molecules were suitable for prediction

N/A: not applicable; ADI: applicability domain index.

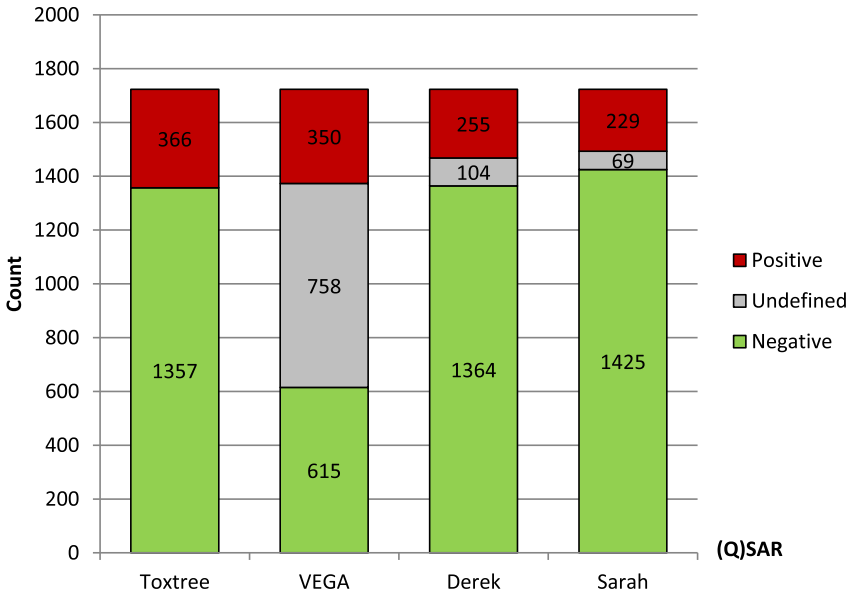

Fig. 2. Number of substances predicted negative (green), positive (red) and undefined (grey) for Ames mutagenicity in a series of (Q)SAR tools. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and Sarah, a positive outcome cannot be ruled out.

The positive rate is most divergent between Toxtree and Sarah. Interestingly, these tools are at the same time most dissimilar with regards to their prediction mechanism: Toxtree is a generic rule-based SAR model without the possibility of AD determination, whereas Sarah is a statistically-based QSAR model in which each compound is checked for being in- or outside a predefined Ames mutagenicity AD. Fig. 2 also shows that the numbers of positives found in the individual models are very similar in Toxtree (366) and VEGA (350) on the one hand, and in Derek (255) and Sarah (229) on the other hand. This suggests that there might be a substantial overlap in the compounds predicted positive by Toxtree and VEGA, and by Derek and Sarah, respectively. As such, we found that for Toxtree and VEGA, 269 of the substances were overlapping. The fact that VEGA has implemented the Toxtree model contributes to this overlap. For Derek and Sarah, the overlap was limited to 119 compounds (Table 3). This observation demonstrates that the different methods lead to a different outcome for several compounds. Detailed examination of the non-overlapping compounds with contradictory prediction results can therefore reveal chemical classes for which Ames mutagenicity prediction needs improvement. Evidently, if a substance is positive in multiple tools - based on various data sets and subsequent prediction rules - this could be expected to imply solid reasoning, in turn associated with increased prediction confidence for experimental mutagenicity. Therefore, substances positive in a variety of (Q)SAR models are of higher concern.

3.2. Combination of models

The combined prediction outcome using the 4 (Q)SAR systems is depicted in Fig. 3. Out of the 1723 non-evaluated substances, 106 are predicted mutagens by all tools, whereas 572 are predicted to be non-mutagenic. A substantial part of 1045 study substances was not clearly identified as mutagenic or non-mutagenic, but either positive in at least 1 but not in all tools, or negative in all tools but with an outside domain notification in at least 1 tool. These substances were considered as 'undefined'. In Fig. 4, a more detailed overview of the prediction results is provided. The majority of substances (1191) do not trigger a positive prediction in any of the

Table 3
Number of substances predicted positive for Ames mutagenicity in a battery of 2 (left panel) and 3 (right panel) (Q)SAR tools.

	Battery of 2 tools			Battery of 3 tools	
(Q)SAR	VEGA	Derek	Sarah	VEGA	Toxtree
Toxtree	269	205	154	183	
VEGA		195	172		147
Derek			119		110
Sarah				112	

Derek	Sarah	*(Q)SAR*

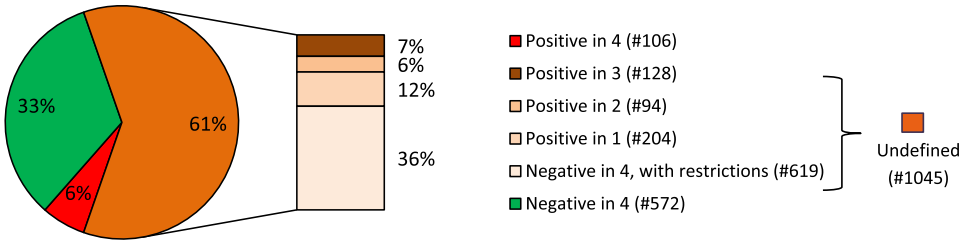

Fig. 3. Distribution of the substances according to overall negative (green), positive (red) or undefined (orange) prediction for Ames mutagenicity when combining 4 (Q)SAR tools (pie). The undefined results are subdivided in substances generating a positive outcome in 1 up to 3 tools or substances negative in all 4 but outside domain (stacked bar). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

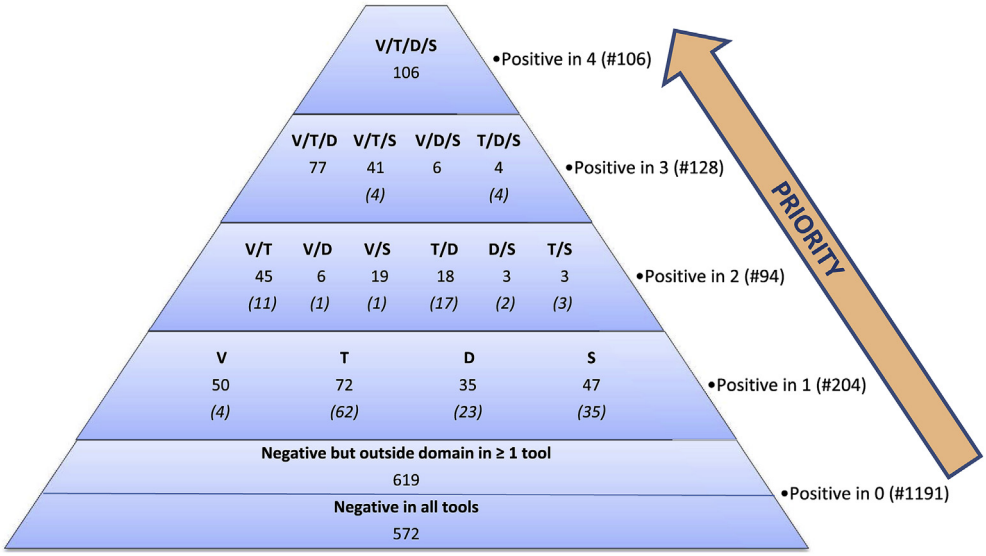

Fig. 4. Priority ranking and distribution of non-evaluated printed paper and board substances according to Ames mutagenicity prediction outcome using 4 (Q)SAR tools; the fraction of substances outside domain in at least 1 remaining tool is mentioned between brackets. V: VEGA, T: Toxtree, D: Derek, S: Sarah.

four tools. However, many of these compounds (619) were outside the AD of at least one tool. Consequently, in the context of this study, 'negative' should be interpreted as 'no positive response reported'. On the other hand, only 8 out of the 128 substances predicted positive in 3 tools are outside the domain of the 4th tool of the *in silico* battery (4 in Derek and 4 in VEGA). It can be debated what significance should be given to results based on outside domain warnings. From a precautionary point of view, it is more appropriate to consider compounds outside the AD as potential mutagens, since this indicates a general lack of knowledge on the (toxicological) properties of the specific chemical class. Hence, after the 106 substances found to be positive in all tools, the 8 compounds found as positive in 3 tools and outside domain in the 4th tool are of second highest concern. Fig. 4 represents the detailed priority ranking following this strategy.

3.3. Priority substances

The 106 substances predicted positive in all 4 (Q)SAR tools are considered of highest priority for further investigation of potential mutagenicity. Fifty-three of these are found in the model training sets, hence they are presumed experimental Ames mutagens (Table 4a). For these 53 compounds, if possible the primary literature should be consulted to verify the positive outcome, and if confirmed, *in vivo* data are required to either endorse or overrule the *in vitro* positive results. In case mutagenicity is confirmed *in vivo* or no reliable negative *in vivo* data are available, they are of highest priority for migration testing. Indeed, the mutagenic potential of a FCM compound is only of concern in case it migrates into the food. Furthermore, migrants need to become bioavailable to be able to cause (mutagenic) effects. Consultation of the combined inventory described in 2.4. shows that migration into food followed by oral bioavailability is very likely for all these 53 compounds (Table 5). The combination of the specific physicochemical parameters considered in the current study has not yet been described elsewhere, nevertheless all are historically known as being indicative for migration and/or oral bioavailability (Van Bossuyt et al., 2016). In line with the precautionary principle, a combination of these parameters is thus highly relevant. Ideally, an elaborate migration and bioavailability model could contribute to a more complete picture, however so far no generally accepted model is available.

Besides the 53 experimental Ames positives, for another 53 substances no experimental data are available in the (Q)SAR systems (Table 4b). Subsequently, their experimental mutagenicity potential should be investigated urgently. All of them are likely to migrate into food due to a molecular weight below 1000 g/mol, and at least 42 out of the 53 meet typical criteria for bioavailability (Table 5).

The majority (99) of the 106 priority compounds are printing ink substances, in many cases (29) pigments or dyes (Table 4a and b). It can be noted that in the context of food contamination with non-

Table 4a
Overview of substances, listed for use in printed paper and board FCM, predicted positive for Ames mutagenicity in 4 (Q)SAR tools and confirmed Ames mutagens according to (Q)SAR model training data.

CAS number	Chemical name	FACET number	Use in FCM
57-14-7	*N*,*N*-Dimethylhydrazine	5914	Monomer in printing ink
74-87-3	Chloromethane	6081	Monomer in printing ink and additive in paper and board
75-00-3	Chloroethane	5491	Monomer in printing ink
75-55-8	2-Methylaziridine	4921	Monomer in printing ink
77-78-1	Dimethyl sulphate	7268	Monomer in printing ink, paper and board and additive in paper and board
78-87-5	1,2-Dichloropropane	/	Additive in paper and board
78-94-4	Butenone	6090	Monomer in printing ink
80-40-0	Ethyl toluene-4-sulphonate	6903	Additive in printing ink
80-48-8	Methyl toluene-4-sulphonate	/	Monomer in paper and board
85-83-6	1-(2-Methyl-4-(2-methylphenylazo)phenylazo)-2-naphthol[a]	/	Dye and pigment (with aromatic azo structure) in printing ink
85-86-9	1-(4-(Phenylazo)phenylazo)-2-naphthol[a]	/	Dye and pigment (with aromatic azo structure) in printing ink
96-23-1	1,3-Dichloropropan-2-ol	/	Monomer in paper and board
98-88-4	Benzoyl chloride	5050	Additive in printing ink
100-44-7	α-Chlorotoluene	7367	Monomer in printing ink, paper and board
101-77-9	4,4′-Methylenedianiline	1079	Monomer in printing ink
101-80-4	4,4′-Oxydianiline	4902	Monomer in printing ink
106-50-3	*p*-Phenylenediamine	6832	Monomer in printing ink
106-87-6	7-Oxa-3-oxiranylbicyclo[4.1.0]heptane	4456	Solvent in printing ink
106-88-7	1,2-Epoxybutane	5094	Monomer in printing ink, paper and board and additive in paper and board
106-90-1	2,3-Epoxypropyl acrylate	2094	Monomer in printing ink, paper and board
106-92-3	Allyl 2,3-epoxypropyl ether	4807	Monomer in printing ink
107-02-8	Acrylaldehyde	4586	Monomer in printing ink, paper and board
107-05-1	3-Chloropropene	6867	Monomer in printing ink, paper and board
107-07-3	2-Chloroethanol	5471	Monomer in printing ink
111-44-4	Bis(2-chloroethyl) ether	5480	Monomer in printing ink and additive in paper and board
111-64-8	Octanoyl chloride	6256	Monomer in printing ink
122-60-1	2,3-Epoxypropyl phenyl ether	4128	Monomer in printing ink
123-73-9	(E)-crotonaldehyde	/	Monomer in paper and board
128-95-0	1,4-Diaminoanthraquinone	/	Dye and pigment in printing ink
130-15-4	1,4-Naphthoquinone	3961	Monomer in printing ink
140-95-4	1,3-Bis(hydroxymethyl)urea	2563	Additive in printing ink, paper and board and monomer in paper and board
286-20-4	1,2-Epoxycyclohexane	4457	Additive in printing ink
302-01-2	Hydrazine	2647	Monomer in printing ink
556-52-5	2,3-Epoxypropan-1-ol	4127	Monomer in printing ink
558-30-5	2,2-Dimethyloxirane	6838	Monomer in printing ink
1854-26-8	4,5-Dihydroxy-1,3-bis(hydroxymethyl)imidazolidin-2-one	/	Additive in paper and board
2210-79-9	2,3-Epoxypropyl *o*-tolyl ether	4129	Monomer in printing ink
2224-15-9	2,2'-[Ethylenebis(oxymethylene)]bisoxirane	5412	Additive in printing ink
2386-87-0	7-Oxabicyclo[4.1.0]hept-3-ylmethyl 7-oxabicyclo[4.1.0]heptane-3-carboxylate	3447	Solvent in printing ink
2426-08-6	Butyl 2,3-epoxypropyl ether	5067	Monomer in printing ink
2451-62-9	1,3,5-Tris(oxiranylmethyl)-1,3,5-triazine-2,4,6(1H,3H,5H)-trione	7407	Monomer in printing ink
2461-15-6	[[(2-Ethylhexyl)oxy]methyl]oxirane	4197	Monomer in printing ink
3101-60-8	*p*-Tert-butylphenyl 1-(2,3-epoxy)propyl ether	6834	Monomer in printing ink
3252-43-5	Dibromoacetonitrile	/	Additive in paper and board
3266-23-7	2,3-Epoxybutane	5097	Monomer in printing ink
4016-14-2	2,3-Epoxypropyl isopropyl ether	6839	Additive in printing ink
4170-30-3	Crotonaldehyde	4171	Monomer in printing ink
6471-49-4	3-Hydroxy-4-[(2-methoxy-5-nitrophenyl)azo]-*N*-(3-nitrophenyl)naphthalene-2-carboxamide[a]	2822	Dye and pigment (with aromatic azo structure) in printing ink
7665-72-7	(Tert-butoxymethyl)oxirane	6293	Monomer in printing ink
17557-23-2	1,3-Bis(2,3-epoxypropoxy)-2,2-dimethylpropane	6840	Monomer in printing ink
21490-63-1	*Trans*-2,3-dimethyloxirane	6297	Monomer in printing ink
26249-20-7	Epoxybutane	5098	Monomer in printing ink
857892-58-1	Oxirane	6299	Additive in printing ink

[a] Aromatic azo compound predicted positive in 2 local QSARs.

plastic FCM, in particular constituents of printing inks are found as a major contamination source. This is among others reflected in a high number of notifications through the Rapid Alert System for Food and Feed (RASFF) (European Commission, 2016a; Lago et al., 2015). Up to now, the latter notifications mainly concern photo-initiators originating from the UV-curing treatment of printing inks.

The 106 compounds positive in the 4 (Q)SAR tools represent a relatively large number of substances requiring experimental (toxicological and/or migration) data. One option to establish a refined priority ranking lies in the investigation of the actual use of these substances. Although FCM manufacturing companies in general do not wish to disseminate detailed information on this matter, a first indication can already be found through consultation of the Flavours, Additives and food Contact materials Exposure Task (FACET) tool (Hearty et al., 2011). In the EU-funded FACET project, a probabilistic modelling tool was developed to estimate consumer exposure to food contact substances (Oldring et al., 2013). Information on substance application and relative use was obtained from a FACET Industry Group (FIG) consisting of 13 European FCM

Table 4b
Overview of substances, listed for use in printed paper and board FCM, predicted positive for Ames mutagenicity in 4 (Q)SAR tools and requiring experimental testing.

CAS number	Chemical name	FACET number	Use in FCM
82-38-2	1-(Methylamino)anthraquinone	/	Dye and pigment in printing ink
136-84-5	1,3-Bis(hydroxymethyl)imidazolidin-2-one	4216	Additive in printing ink
624-65-7	3-Chloropropyne	6897	Monomer in printing ink
938-18-1	2,4,6-Trimethylbenzoyl chloride	5051	Monomer in printing ink
1208-52-2	2,4′-Methylenedianiline	4134	Monomer in printing ink
1606-83-3	1,1'-[But-2-yne-1,4-diylbis(oxy)]bis[3-chloropropan-2-ol]	4252	Additive in printing ink
1719-57-9	Chloro(chloromethyl)dimethylsilane	7055	Monomer in printing ink
1742-95-6	4-Aminonaphthalene-1,8-dicarboximide	6178	Additive in printing ink
2095-03-6	2,2'-[Methylenebis(*p*-phenyleneoxymethylene)]bisoxirane	6296	Additive in printing ink
2238-07-5	2,2'-[Oxybis(methylene)]bisoxirane	5479	Additive in printing ink
2478-20-8	6-Amino-2-(2,4-dimethylphenyl)-1H-benz[de]isoquinoline-1,3(2H)-dione	/	Dye and pigment in printing ink
2530-83-8	[3-(2,3-Epoxypropoxy)propyl]trimethoxysilane	2638	Monomer in printing ink and additive in paper and board
2602-34-8	[3-(2,3-Epoxypropoxy)propyl]triethoxysilane	2893	Monomer and additive in printing ink
2897-60-1	[3-(2,3-Epoxypropoxy)propyl]diethoxymethylsilane	7052	Additive in printing ink
3049-71-6	2,9-Bis[4-(phenylazo)phenyl]anthra[2,1,9-def:6,5,10-d'e'f']diisoquinoline-1,3,8,10(2H,9H)-tetrone[b]	2999	Dye and pigment (with aromatic azo structure) in printing ink
3126-95-2	(Propoxymethyl)oxirane	6292	Monomer in printing ink
3176-79-2	1-[[3-Methyl-4-[(3-methylphenyl)azo]phenyl]azo]-2-naphthol[a]	/	Dye and pigment (with aromatic azo structure) in printing ink
3271-22-5	2,4-Dimethoxy-6-pyren-1-yl-1,3,5-triazine	4135	Additive in printing ink
3454-29-3	1-(2,3-Epoxypropoxy)-2,2-bis[(2,3-epoxypropoxy)methyl]butane	7403	Additive in printing ink
4378-61-4	4,10-Dibromodibenzo[def,mno]chrysene-6,12-dione	5304	Dye and pigment in printing ink
4482-25-1	5,5'-[(4-Methyl-1,3-phenylene)bis(azo)]bis[toluene-2,4-diamine][a]	3907	Additive (with aromatic azo structure) in printing ink
5026-74-4	p-(2,3-Epoxypropoxy)-*N*,*N*-bis(2,3-epoxypropyl)aniline	6358	Monomer in printing ink
6410-38-4	4-[(2,5-Dichlorophenyl)azo]-3-hydroxy-*N*-(2-methoxyphenyl)naphthalene-2-carboxamide[b]	2847	Dye and pigment (with aromatic azo structure) in printing ink
6448-95-9	3-Hydroxy-4-[(2-methyl-5-nitrophenyl)azo]-*N*-phenylnaphthalene-2-carboxamide[b]	2829	Dye and pigment (with aromatic azo structure) in printing ink
6471-50-7	4-[(4-Chloro-2-nitrophenyl)azo]-3-hydroxy-*N*-(2-methylphenyl)naphthalene-2-carboxamide[b]	/	Dye and pigment (with aromatic azo structure) in printing ink
6539-67-9	3-[[2-(Acetylamino)-4-[(4-amino-6-chloro-1,3,5-triazin-2-yl)amino]phenyl]azo] naphthalene-1,5-disulphonic acid	/	Dye and pigment (with aromatic azo structure) in printing ink
6655-84-1	3-Hydroxy-4-[(2-methyl-5-nitrophenyl)azo]-*N*-(*o*-tolyl)naphthalene-2-carboxamide[b]	2995	Dye and pigment (with aromatic azo structure) in printing ink
7328-97-4	2,2′,2″,2‴-[Ethane-1,2-diylidenetetrakis(*p*-phenyleneoxymethylene)]tetraoxirane	5411	Additive in printing ink
12225-06-8	*N*-(2,3-dihydro-2-oxo-1H-benzimidazol-5-yl)-3-hydroxy-4-[[2-methoxy-5-[(phenylamino)carbonyl]phenyl]azo]naphthalene-2-carboxamide	2997	Dye and pigment (with aromatic azo structure) in printing ink
12236-64-5	*N*-[4-(acetylamino)phenyl]-4-[[5-(aminocarbonyl)-2-chlorophenyl]azo]-3-hydroxynaphthalene-2-carboxamide	2979	Dye and pigment (with aromatic azo structure) in printing ink
13236-02-7	1,2,3-Tris(2,3-epoxypropoxy)propane	6836	Additive in printing ink
14228-73-0	1,4-Bis[(2,3-epoxypropoxy)methyl]cyclohexane	5250	Additive in printing ink
16096-30-3	2,2'-[(1-Methylethylene)bis(oxymethylene)]bisoxirane	6294	Additive in printing ink
16096-31-4	1,6-Bis(2,3-epoxypropoxy)hexane	3967	Additive and solvent in printing ink
16403-84-2	4-[(5-Carbamoyl-*o*-tolyl)azo]-3-hydroxynaphth-2-anilide	2828	Dye and pigment (with aromatic azo structure) in printing ink
25188-42-5	7-Benzamido-4-hydroxy-3-[[4-[(4-sulphophenyl)azo]phenyl]azo]naphthalene-2-sulphonic acid	/	Dye and pigment (with aromatic azo structure) in printing ink
28804-47-9	Methyl toluenesulphonate	/	Additive in paper and board
31482-56-1	3-[Ethyl[4-[(4-nitrophenyl)azo]phenyl]amino]propiononitrile[a]	/	Dye and pigment (with aromatic azo structure) in printing ink
36215-07-3	1-Chloro-3-methoxypropane	6841	Monomer in printing ink
36968-27-1	4-[[4-(Aminocarbonyl)phenyl]azo]-3-hydroxy-*N*-(2-methoxyphenyl)naphthalene-2-carboxamide	2827	Dye and pigment (with aromatic azo structure) in printing ink
39817-09-9	2,2'-[Methylenebis(phenyleneoxymethylene)]bisoxirane	2347	Monomer in printing ink
50593-68-5	3-Chloro-6-nitro-1H-indazole	4033	Additive in printing ink

52373-93-0	1-Amino-4-(ethylamino)-9,10-dihydro-9,10-dioxoanthracene-2,3-dicarbonitrile	4125	Additive in printing ink
56396-10-2	4-[[5-(Anilino)carbonyl-2-methoxyphenyl]azo]-3-hydroxynaphthalene-2-carboxamide	/	Dye and pigment (with aromatic azo structure) in printing ink
59487-23-9	4-[[5-[[[4-(aminocarbonyl)phenyl]amino]carbonyl]-2-methoxyphenyl]azo]-*N*-(5-chloro-2,4-dimethoxyphenyl)-3-hydroxynaphthalene-2-carboxamide	2814	Dye and pigment (with aromatic azo structure) in printing ink
61847-48-1	Methyl 4-[[(2,5-dichlorophenyl)amino]carbonyl]-2-[[2-hydroxy-3-[[(2-methoxyphenyl)amino]carbonyl]-1-naphthyl]azo]benzoate	2815	Dye and pigment (with aromatic azo structure) in printing ink
61951-98-2	*N*-(2,3-dihydro-2-oxo-1H-benzimidazol-5-yl)-3-hydroxy-4-[[5-methoxy-2-methyl-4-[(methylamino)sulphonyl]phenyl]azo]naphthalene-2-carboxamide	/	Dye and pigment (with aromatic azo structure) in printing ink
62570-50-7	1-Amino-4-(ethylamino)-9,10-dihydro-9,10-dioxoanthracene-2-carbonitrile	3142	Dye and pigment in printing ink
67990-05-0	*N*-(5-chloro-2-methoxyphenyl)-3-hydroxy-4-[[2-methoxy-5-[(phenylamino)carbonyl]phenyl]azo]naphthalene-2-carboxamide	2829	Dye and pigment (with aromatic azo structure) in printing ink
68227-78-1	*N*-(5-chloro-2-methylphenyl)-3-hydroxy-4-[[2-methoxy-5-[(phenylamino)carbonyl]phenyl]azo]naphthalene-2-carboxamide	2992	Dye and pigment (with aromatic azo structure) in printing ink
68516-75-6	*N,N'*-naphthalene-1,5-diylbis[4-[(2,3-dichlorophenyl)azo]-3-hydroxynaphthalene-2-carboxamide]	/	Dye and pigment (with aromatic azo structure) in printing ink
68818-86-0	9,10-Diethoxyanthracene	4905	Additive in printing ink
74336-59-7	3-[(4-Chloro-2-nitrophenyl)azo]-2-methylpyrazolo[5,1-*b*]quinazolin-9(1H)-one[a]	2984	Dye and pigment (with aromatic azo structure) in printing ink

[a] Aromatic azo compound predicted positive in 2 local QSARs.
[b] Aromatic azo compound predicted positive in 1 local QSAR.

trade associations representing among others the printing ink and paper(board) industry (University College Dublin, 2012). As a result, substances with a FACET number indicate substances for which the FIG has confirmed current usage. Forty-four training set Ames positives and 42 positives without experimental data, have a FACET number, suggesting their priority is higher compared to the 20 substances without a FACET number. One weakness of the FACET tool is its limited coverage, which is restricted to FCM substances applied in primary packaging, whereas for a complete assessment secondary packaging and articles should also be considered. The application of substances without a FACET number cannot be ruled out either, as this information is currently lacking. Despite this limitation and even though this approach does not drastically minimize the number of priority substances to be evaluated in-depth, it is reasonable to consider the substances associated with a FACET number prior to the ones without such number.

Another interesting refinement method is the provisional exclusion of compounds predicted negative by local QSAR models, i.e. specific for a particular group of compounds. Indeed, the prediction capacity of a (Q)SAR model can be increased when the chemical domain is well-defined. For example, it was found that 25 of the 106 substances contain an aromatic azo bond, a chemical structure frequently found in pigments and dyes. Recently, the IRFMN developed 2 QSAR models to estimate Ames mutagenicity of aromatic azo substances, one based on CORAL software and a second one based on a k-NN algorithm (Manganelli et al., 2016). Application of the local QSARs resulted in 13 compounds predicted negative in both models (low priority), 5 contradictory results (medium priority) and 7 positive in both (high priority). Upon combining this extended QSAR evaluation with the abovementioned consideration of the existence of a FACET number, 3 substances of highest concern (CAS# 6471-49-4 in Table 4a, CAS# 4482-25-1 and 74336-59-7 in Table 4b) could be identified. They are positive in the 6 (Q)SAR tools and have in addition been assigned a FACET number, confirming their current usage.

3.4. General remarks

Ideally, a (Q)SAR should meet the OECD principles for the validation of (Q)SAR tools in order to facilitate its consideration for regulatory purposes (OECD, 2014). The principles state that it should be associated with 1) a defined endpoint; 2) an unambiguous algorithm; 3) a defined domain of applicability; 4) appropriate measures of goodness-of-fit, robustness and predictivity and 5) a mechanistic interpretation, if possible. Moreover, a checklist with questions is available to facilitate the evaluation of a (Q)SAR for the abovementioned criteria. The guidance document itself points out that these criteria are very difficult to fulfil in practice, however they should be strived for as much as possible. All tools applied in the current work are linked to a well-defined toxicological endpoint, i.e. Ames mutagenicity. Most of the tools feature a clearly established algorithm. Some of the tools dispose of applicability domain indications and provide a mechanistic interpretation for the prediction results. None of the tools is completely transparent when it comes to providing full details of external validation performance. Although all tools have several shortcomings, their type and degree varies. Combining different tools can therefore prove beneficial, especially for priority setting among large groups of chemical substances, as demonstrated in the current study. Evidently, validation of a (combination of) (Q)SAR model(s) for a group of compounds with a specific application is difficult. Indeed, validation requires a substantial number of evaluated compounds with reliable experimental Ames test data. In the case of FCM substances, validation is not only complicated by the

Table 5
Distribution of non-evaluated printed paper and board FCM priority substances according to physicochemical parameters related to migration and bioavailability. Substances with results below the cut-off are likely to migrate (in the case of low molecular weight) or become bioavailable (other cases).

Parameter	Cut-off	≤cut-off		>cut-off		≤cut-off		>cut-off	
		53 Experimental Ames positives				53 Experimental unknown			
		#	%	#	%	#	%	#	%
Molecular weight	1000 g/mol	53	100	0	0	53	100	0	0
Lipinski rule of 5 violations	1	52	98	1	2	42	79	11	21
Polar surface area	140 Angström^2	52	98	1	2	45	85	8	15
Rotating bonds	10	53	100	0	0	46	87	7	13

limited number of evaluated compounds, but also by the variety of chemical classes to which they belong. Due to the current lack of knowledge as to which model is most capable of generating trustworthy predictions for printed paper and board FCM substances, it is thus deemed appropriate to use a screening battery of complementary systems (i.e. SARs and QSARs).

4. Conclusion

In this study, the beneficial role of *in silico* tools in prioritization strategies was demonstrated using non-evaluated printed paper and board FCM substances as an example. However, a much wider range of application domains can be anticipated. For instance, the strategy could be useful in prominent issues among which the prioritization of long-standing industrial chemicals lacking a (recent) safety evaluation, and of secondary substances – found in most chemical formulations – such as impurities or degradation products. In the current work, the selection of model(s) had an impact on the number of positives, as this was substantially lower when using Derek Nexus™ or Sarah Nexus™ compared to using Toxtree or VEGA. One hundred and six substances were consistently predicted positive in a battery of 4 (Q)SAR Ames mutagenicity tools. Subsequent priority ranking to determine the urgency for an in-depth safety evaluation was established by investigating the availability (and quality) of experimental toxicological data within the (Q)SAR tools. Furthermore, local QSAR systems also proved useful for refining the prioritization of well-defined structurally similar molecules. To conclude, the prioritized printed paper and board FCM substances will be subjected to a more extensive investigation of their potential genotoxicity consisting of literature study and, if necessary, *in vitro* testing.

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