DANISH MINISTRY
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The Advisory list for self-classification of dangerous substances

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Preface

The current report describes the work underlying the advisory list for self-classification of chemicals that the Danish EPA has prepared in collaboration with researchers from the National Food Institute – Technical University of Denmark. The advisory list for self-classification of chemicals is available as a database via www.mst.dk.

This report provides the following background material to the database:

Chapter 1: A general regulatory background on classification of chemicals and how (Q)SAR based assessments can be used in this context.

Chapter 2: Description of the general methodology applied to make the advisory self-classifications.

Chapter 3: Description of the (Q)SAR models and the (Q)SAR based assessments in relation to the classification criteria for individual endpoints.

Chapter 4: Discussion and conclusions regarding comparisons with the previous advisory list.

Summary

All chemical substances marketed in the EU must be classified and labelled according to the regulation on classification and labelling of dangerous substances (7). Substances with harmonised classifications adopted in the EU are to be found on the List of harmonised classification and labelling of hazardous substances (Annex VI of 1272/2008/EU). This list covers around 7000 substances which have been classified for their hazardous properties. However, this also means that about 93,000 of the 100,204 existing substances in the EU (EINECS list), are not formally classified. With these substances, it is the manufacturer's or importer's responsibility to carry out an appropriate classification of the dangerous intrinsic properties ("self-classification"). In most cases, no test data (from animal testing, etc.) is available on their hazardous properties in relation to human health or the environment.

To address this issue, the Danish Environmental Protection Agency published the Advisory Self-classification List /5/. This report describes the updating of this list. The Advisory Self-Classification list is created by the use of (Q)SARs ((Quantitative) Structure-Activity Relationships) to predict the effects of chemicals. The updated Advisory Self-Classification list contains the results of a systematic assessment of 49,292 discrete organic EINECS substances in relation to the following endpoints for which new and/or improved (Q)SAR model predictions were available:

- o   Mutagenicity
- o   Carcinogenicity
- o   Reproductive toxicity (possible harm to the unborn child)
- o   Danger to the aquatic environment

The advisory classifications for mutagenicity, carcinogenicity and danger to the aquatic environment are updates of the advisory classifications on the previous self-classification list. Reproductive toxicity is a new endpoint on the self-classification list.

Advisory classifications from the previous advisory list for two endpoints have not been updated in the current project. For these endpoints the advisory classifications from the previous list still apply (and are not discussed further in this report):

- o   Acute oral toxicity
- o   Skin sensitisation

The updated advisory list is available as an Excel file for download from DK-EPA's website and as an online searchable database. This includes the 23,922 chemicals with new advisory classifications resulting from this project, making in all, a total of 30,179 chemicals with advisory classifications, either from this project, or with advisory classifications for acute oral toxicity or skin sensitisation from the previous advisory list.

The advisory classifications are made by using combinations of (Q)SAR models relevant for each classification endpoint . This report describes the methodology and the models applied.

Further updates of the advisory list are also under consideration. One relevant update would be to modify it to meet the criteria set out in the new CLP-regulation for the classification and labelling of chemicals. The list could also be updated based on new or updated (Q)SAR model predictions for other endpoints.

Dansk sammenfatning

Alle kemiske stoffer, der markedsføres i EU, skal klassificeres og mærkes efter reglerne i klassificeringsbekendtgørelsen (Bek nr. 329 af 16/5 2002) og listen over farlige stoffer (1272/2008/EU, bilag VI, tabel 3.2). Listen over farlige stoffer dækker i dag ca. 7.000 stoffer og stofgrupper, som er blevet klassificeret for deres farlige egenskaber af EU's kompetente myndigheder. Det vil sige, at omkring 93.000 af de 100.204 eksisterende stoffer i EU (EINECS-fortegnelsen) endnu ikke har undergået formel klassificering. For disse stoffer er det producentens eller importørens ansvar at påføre en korrekt klassificering for stoffernes iboende farlige egenskaber ("selvklassificeringer"). Imidlertid er der for de fleste af disse stoffer kun få eller ingen test resultater (fra dyreforsøg m.m.) om stoffernes farlighed overfor mennesker eller miljø.

Som bidrag til at afhjælpe denne problemstilling, har Miljøstyrelsen tidligere offentliggjort den såkaldte selvklassificeringsliste og nærværende rapport beskriver opdateringen af denne liste. Selvklassificeringslisten er lavet ved brug af (Q)SAR modeller ((kvantitative) struktur-aktivitets sammenhænge) som er blevet brugt til at forudsige farlige virkninger af kemiske stoffer. Modellerne er med henblik på denne liste blevet anvendt til en systematisk vurdering af 49.292 organiske enkeltstoffer fra EINECS-fortegnelsen for effekter, hvor der findes pålidelige modeller:

- o Skader på arveanlæggene
- o Kræftfremkaldende effekt
- o Reproduktionstoksicitet (skader på afkommet)
- o Farlighed for vandmiljøet

De vejledende klassificeringer for skader på arveanlæggene, kræftfremkaldende effekt og farlighed for vandmiljøet er opdateringer af de vejledende klassificeringer på den foregående selvklassificeringsliste. Reproduktionstoksicitet var ikke med på den foregående liste.

Vejledende klassificeringer for følgende to effekter som var inkluderet på den foregående selvklassificeringsliste er ikke blevet opdateret. For disse effekter er de vejledende klassificeringer fra den foregående liste stadig gældende (og vil ikke blive beskrevet yderligere i denne rapport):

- o Akut dødelig virkning ved indtagelse
- o Sensibiliserende virkning ved hudkontakt

Den opdaterede liste med selvklassificeringer er tilgængelig via Miljøstyrelsen hjemmeside som både Excel fil til download og som en søgbar online database. Inkluderet er de 23.922 kemiske stoffer hvor der er lavet nye vejledende klassificeringer samt stoffer med vejledende klassificeringer for akut toksicitet og sensibiliserende virkning ved hudkontakt fra den foregående liste, i alt 30.179 stoffer med vejledende klassificeringer.

De vejledende klassificeringer er lavet ved hjælp af kombinationer af (Q)SARs som er relevante for hver enkelt klassificering. Rapporten beskriver det

principielle grundlag for at anvende sådanne modeller samt hvordan modellerne konkret er blevet anvendt i dette projekt.

Der er overvejelser om at den vejledende liste skal opdateres yderligere. En relevant opdatering kunne være at sikre at den vejledende liste også kan anvendes ift. de kriterier som er opstillet i den nye CLP-forordning for klassificering og mærkning af kemiske stoffer. Endvidere vil det være relevant at opdatere listen med klassificeringer for andre farlige egenskaber.

1 Introduction to classification and (Q)SAR

1.1 Background

When chemical substances are classified in terms of the danger they represent, their inherent properties are assessed on the basis of the knowledge and information available /2, 60/. Such assessments are often carried out on the basis of laboratory test results because the hazard classification criteria to a large extent refer to such results. Assessment must be carried out individually for each property, which means that often extensive animal testing may be required for a single substance. Thus, complete identification of all the properties for which classification criteria exist, at present requires results of up to 30 animal studies for just one substance.

Given the extensive need for data from animal studies, it is not surprising that lack of test data represents a major problem in the assessment of dangerous properties of chemicals. It is a well-known fact that there are currently few or no test data for a very large fraction of the 100,204 chemical substances on the European INventory of Existing Commercial chemical Substances (EINECS) /3, 4, e.g. 36/. This means that many chemical substances within the European market may have unknown dangerous properties even though they have been used for many years.

With the new chemicals legislation in EU, REACH, new information demands for chemicals have been imposed in the EU. However, especially for chemicals produced in volumes below 10 tpa per manufacturer or importer in the EU it is unlikely that test data on a broad spectrum of dangerous properties will be available within the foreseeable future.

With the aid of mathematical modelling, so-called (Quantitative) Structure-Activity Relationships, (Q)SAR, methods for prediction of the properties of chemicals can be established. Classifications based on use of (Q)SARs to predict dangerous properties can save time and money if used as an alternative to animal testing, as well as increase the level of information for chemicals that will not undergo testing. The Danish EPA in 2001 published the first version of the advisory list for self-classification of dangerous substances (denoted AL2001 in the current report) /5/ where 20.624 substances were assigned advisory classifications according to the following dangerous properties: Acute oral toxicity, sensitisation by skin contact, mutagenicity, carcinogenicity, and danger to the aquatic environment.

1.2 Classification of chemicals

Criteria for classification, packaging and labelling of dangerous substances and preparations is harmonised in order to protect public health and the environment and ensure the free movement of such products /6, 7, 60 /. Harmonised hazard labelling allows consumers to recognize dangerous

substances and preparations easily and to take adequate measures as regards risk avoidance and safe handling and disposal.

Existing regulation

The present regulation for classification and labelling involves an evaluation of the hazard of a substance or preparation in accordance to Council Reg. 1272/2008/EU /7/ and a communication of that hazard via the label.

Classification of a substance or preparation is considered relative to several endpoints concerning physical-chemical properties, health effects or environmental properties. This evaluation must be made for any substance or preparation manufactured within or imported into the EU and placed on the EU market. Classification and labelling is therefore an essential element of risk management measures of chemicals.

All marketed substances and preparations must be evaluated for hazard classification and labelling, irrespective of the quantity placed on the market. The labelling is the first and often the only information on the hazards of a chemical that reaches the user, which could be a consumer or a worker. In addition the classification has a large number of downstream consequences within the EU legislation.

New regulation

By January 2009 the new CLP regulation on classification, labelling and packaging of substances and mixtures has had legal effect in the EU /7/. This regulation will gradually replace the present regulation for classification and labelling. The new regulation will come into force for single substances December 1st 2010 and for mixtures June 1st 2015 /7/. Until December 1st 2010 substances and mixtures shall be classified labelled and packaged in accordance with the present legislation *or* they can be classified according to the CLP regulation.

The CLP regulation is based on the Globally Harmonised System of Classification and Labelling of Chemicals (GHS, UN 2007) /61/. The GHS classification criteria are in certain cases slightly different than those of the current legislation /7/.

It will be considered whether a future update of the advisory self-classification list based on the new criteria will be made.

1.3 (Q)SARs and their use in chemical assessment

Structure-activity relationships (SARs) and quantitative structure-activity relationships (QSARs), collectively referred to as (Q)SARs, are theoretical models that can be used to predict the physic-chemical, biological (e.g. toxicological) and environmental fate properties of molecules based on the chemical structure.

(Q)SARs tools are used more and more by authorities in the US and the EU, as well as by industry, to assess physico-chemical, (eco-)toxicological, and fate properties of substances.

REACH

In the new EU chemicals legislation, REACH, all other options, including use of (Q)SARs, should be considered before performing (or requiring)

vertebrate testing /1/. Annex XI of REACH contains the following wording regarding (Q)SARs:

Results obtained from valid qualitative or quantitative structure-activity relationship models ((Q)SARs) may indicated the presence or absence of a certain dangerous property. Results of (Q)SARs may be used instead of testing when the following conditions are met:

- ***Results are derived from a (Q)SAR model whose scientific validity has been established,***
- ***The substance falls within the applicability domain of the (Q)SAR model,***
- ***Results are adequate for the purpose of classification and labelling and/or risk assessment, and,***
- ***Adequate and reliable documentation of the applied method is provided.***

There will be no formal adoption process for (Q)SARs under REACH. QSAR Model Reporting Formats (QMRF's) to compile information on endpoint, training set, validation results etc. for individual models will be gathered in a JRC QSAR Model Database. There will not be made fixed criteria for how the (Q)SARs should perform to receive regulatory acceptance, but rather a learning-by-doing process to gain experience and common understanding of use of (Q)SARs in chemical assessments /9/.

In the hazard and risk assessment process, (Q)SARs are often used in combination with other sources of information on chemicals, either to prioritise chemicals for further assessment, to supplement or to replace testing.

With the implementation of REACH it is expected that (Q)SARs will be used increasingly for the direct replacement of test data as their use, when available and adequate, is in fact an obligation /9/. The goal of assessing many thousands of chemicals under REACH may not be achievable without the use of (Q)SARs and other non-test methods. Especially for low tonnage chemicals, (Q)SARs and other non-test methods may also give further information beyond the information requirements of REACH.

2 Creation and use of the advisory list for self-classification

Following development of new and/or improved (Q)SAR-models the list of advisory self-classification of dangerous substances has been updated. This chapter of the report presents the methodology applied for this new version of the advisory list for self-classification of dangerous substances.

2.1 The selected dangerous properties

The following endpoints were addressed using (Q)SARs :

- Mutagenicity
- Carcinogenicity
- Reproductive toxicity (possible harm to the unborn child)
- Hazard to the aquatic environment

Two endpoints have not yet been updated in AL2009:

- Acute oral toxicity
- Sensitization by skin contact

(Q)SAR-predictions for these endpoints were used to assign the classifications listed in Table 1.

Dangerous property	Classification	Wording of Classification
Mutagenicity	Mut3;R68	Mutagen, category 3; possible risk of irreversible effects
Carcinogenicity	Carc3;R40	Carcinogen, category 3; possible risk of irreversible effects
Reproductive toxicity	Repr3; R63	Reproductive toxicant, category 3, Possible risk of harm to the unborn child
Danger to the aquatic environment	N;R50	Dangerous for the environment; very toxic to aquatic organisms
	N;R50-53	Dangerous for the environment; very toxic to aquatic organisms, may cause long-terms adverse effects in the aquatic environment
	N;R51-53	Dangerous for the environment; toxic to aquatic organisms, may cause long-terms adverse effects in the aquatic environment
	N; R52-53	Harmful to aquatic organisms, may cause long-terms adverse effects in the aquatic environment

Table 1: Advisory classifications in AL2009

2.2 The evaluated chemical substances

The overall purpose of the current project was to evaluate as many chemical substances as possible with relevance to the existing regulation for chemicals within the EU.

Under REACH /1/ all chemicals with tonnages above 1 ton/year were pre-registered between June 1st 2008 and December 1st 2008. It would have been relevant to evaluate all chemicals in this inventory but at the time of preparation of AL2009 no official list existed.

It was therefore decided to base this project on the EINECS list /3,4/. This list consists of 100,204 entries, covering organic and inorganic substances in both single substance entries (mono-constituent substances) and mixtures (multi-constituent substances and UVBCs).

The exercise was limited to cover "discrete organics," meaning that multi-constituent substances and UVCBs (Unknown, Variable Composition and Biologicals) were excluded for practical reasons – "if you don't know what it is, you can't model it".

Inorganic substances have likewise not been evaluated. These are usually better approached by simpler methods of evaluating the availability of the respective an- and cations with well-known hazard profiles. "Organo-metallics" have also been excluded as being poor candidates for modelling. As an error check, only such structural representations, which could be successfully converted to 3D were used /10/.

When it was possible using a CAS number comparison, all substances already classified on the list with formal EU harmonized classifications, Annex I of Directive 67/548/EEC (List of dangerous substances, /2/) were also removed. However, as there is no official overview of the substances covered by the group entries in Annex I, and because a chemical may have more than one CAS number, a few chemicals covered by Annex I may not have been removed from AL2009.

This resulted in a total of 49,292 discrete organic substances, or about half of all EINECS chemicals, which could be subjected to (Q)SAR based assessment.

2.3 Test data

For the vast majority of the assessed chemicals no test data were available. However, if test data were available as part of the (Q)SAR-model, this was generally used in preference to the estimates.

It is important to stress that no attempt was made to search published or unpublished databases for toxicological, ecotoxicological or environmental fate information to determine whether a (Q)SAR was necessary for any endpoint assesssed.

2.4 Reliability of (Q)SAR-predictions

The reliability of (Q)SAR-predictions depend on numerous parameters relating to the mathematical methods used, the number and precision of the underlying data used for developing the model and how suitable the model is for the particular substance.

In general the uncertainty of (Q)SARs is caused predominantly by two different reasons: a) the inherent variability of the input data used to establish the model (training set); and b) the uncertainty resulting from the fact that a model can only be a partial representation of reality (in other words does not model all possible mechanisms concerning a given endpoint and does not cover all types of chemicals). However, as a model averages the uncertainty over al chemicals, it is possible for an individual model estimate to be more accurate than an individual measurement /9/.

The reliability of (Q)SAR predictions can be described in many ways. Usually a range of parameters and concepts are used (see e.g. /9/ for a more extensive review). These concepts may not be known by all readers. Annex 1 contains descriptions of the concepts applied in this report.

2.5 Validation

Validation is a trial of the model performance for a set of substances independent of the training set, but within the domain of the model. The model predictions for these substances are compared with measured endpoints for the substances in order to establish the predictivity of the model.

Ideally all models should be assessed by checking how well they predict the activity of chemicals, which were not used to make them. This is, however, not always simple. In part valuable information may be left out by setting aside chemicals to be used in such an evaluation, and in part it can be extremely difficult to assess how "external" chemicals relate to the model's domain; that is, if they represent a random distribution within this domain and thereby giving a fair picture of the predictivity of the model.

This problem is often addressed by using one or another form of cross-validation, where a number of partial models are "externally validated" by splitting the training set into a reduced training set and a testing set. The reduced training set is used to develop a partial model, while the remaining data are used as a test set to evaluate the model predictivity.

This is repeated a number of times and the results are used to calculate the predictivity measures for the models; for quantitative models in the form of Q^2 and SDEP (standard deviation error of prediction), and for qualitative (yes/no) models in the form of sensitivity, specificity and concordance (se Annex 1 and refs /9/ and /11/ for further details).

While drawbacks of cross-validation exist /14, 15/, much of the criticism is directed towards a particular form of cross validation; the leave-one-out cross-validation /14/. In the validations carried out on the models applied in this project the more stable leave-many-out cross-validation approach by leaving out random pos/neg balanced sets of 50% of the chemicals, repeated ten times, was used /13/. Leaving out 50% of the chemicals in the partial validation models is a large perturbation of the training set, which generally

leads to realistic, and often pessimistic, measures of the predictivity of the model.

The commercial cancer models were validated by external validation /24/.

Concordance will vary depending on both the method used, and the endpoint in question. In general, accuracy of contemporary (Q)SAR systems can often correctly predict the activity of about 70 – 85% of the chemicals examined, provided that the query structures are within the domains of the models. This also applies to the models described in this paper.

QSAR Model Reporting Formats (QMRF's) for all the toxicity models applied in this project, including training sets for the DK models, have been submitted to the EU JRC QSAR Model Database and the OECD QSAR Application Toolbox /35, 37/.

2.6 Applicability domain

When applying (Q)SAR's it is important to assure that an obtained prediction falls within the domain of the models i.e., that there is sufficient similarity (in relevant descriptors) between the query substance and substances in the training set of the model.

There is no single and absolute applicability domain for a given model /9/. Generally, the broader the applicability domain is defined the lower predictivity can be expected. The applicability domain should be clearly defined and the validation results should correspond to this defined domain, which is again used when the model is applied for predictions.

The applicability domains for MultiCASE models as defined by the US Food and Drug Administration (FDA) and implemented in the MultiCASE software were used in this project: No warnings in the predictions were accepted, except warning for one unknown fragment in chemicals where a significant biophore has been detected. Only positive predictions where no significant deactivating fragments were detected were accepted.

For aquatic toxicity endpoints, warning for one unknown fragment in chemicals which were predicted negative was also accepted, as these chemicals underwent prediction by a subsequent model (a log K_{ow} equation) to predict if they exerted aquatic toxicity by non-polar narcosis.

The EPISUITE models for rapid biodegradation /43, 45/ and the bioconcentration factor in fish /42/ do not automatically flag the predictions for domain coverage. No attempts have been made to consider the applicability domain for predictions made by these models.

Depending on the endpoint in question, predictions outside the applicability domain were obtained for between 27 and 58% of the chemicals examined by the individual MultiCASE models.

2.7 Application of the models

It is important to note that the applied models in principle do not predict a "classification" – they predict a biological activity that may lead to a classification.

Because of the large number of chemicals involved, "rules" were used for each endpoint to try and link the biological prediction with a risk phrase. In essence the process is no different than that imposed upon a human expert forced to interpret the information available in order to comply with the duty to make an assessment and self-classification.

The applied models have been used in combinations / batteries within the chosen classification endpoints to reach a final call in an attempt to reach further reliability beyond individual model predictions and to best comply with the classification criteria.

2.8 The result

The result of the computer-based assessment is AL2009 which comprises 23,922 chemical substances with advisory classifications for one or more of the dangerous properties selected.
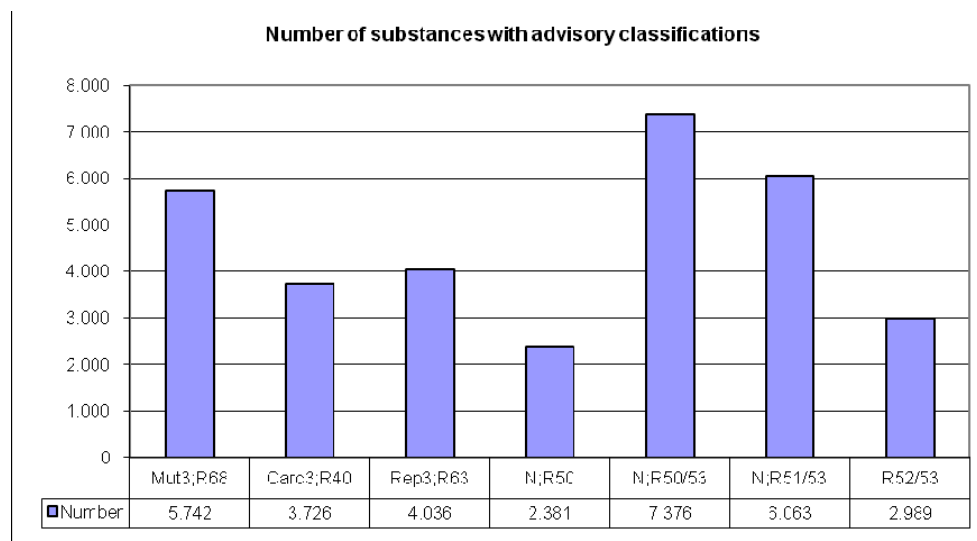
The results only represent POSITIVE predictions (for quantitative models "positive" here means predicted to have the effect or property as determined in relation to a cut-off point). No distinction has been made between a negative prediction for an endpoint, and an unreliable prediction (prediction outside the applicability domain of the model), which was simply discarded.

Evaluated substances which are not on the list, or substances which are on the list but without advisory classifications for one or more of the selected dangerous properties, may have been predicted as not having this / these dangerous properties, or the models may not have been valid for this substance (i.e. predictions were outside the applicability domain for these models).

Therefore the advisory list cannot be used to conclude that these substances do not possess dangerous properties.

Another important point is that AL2009 represent (Q)SAR based identifications of possible **hazardous** properties of the included chemicals; no attempt has been made to evaluate the **risk** that these chemicals constitute in their current use in the EU.

All results are available on the website of the Danish EPA (www.mst.dk) where searches can be made on substance name (in Danish), CAS-number, EINECS-number, EINECS-name, CAS-name and chemical formula. The whole list can also be downloaded as an Excel file.

Number of substances with advisory classifications

	Mut3;R68	Carc3;R40	Rep3;R63	N;R50	N;R50/53	N;R51/53	R52/53
Number	5.742	3.726	4.036	2.381	7.376	6.063	2.989

2.9 How the self-classification list can help manufacturers and importers to comply with the classification duties

By making the advisory list for self-classification of dangerous substances available to the public, the Danish EPA wishes to offer manufacturers and importers a tool which can be used when carrying out self-classification of chemical substances for those dangerous properties which are included in the list.

If available, reliable test data or predictions using other non-test methods on specific substances should always be considered in parallel to computer predictions and expert judgements in a weight of evidence (WoE) approach to decide on the appropriate classification for a given endpoint.

It is recommended that the list is used in the following way in the classifications of chemicals:

1. Examine if the substance is on Annex VI, table 2 of the EU regulation for classification, labelling and packaging of dangerous substances /6/. If so it should be classified accordingly. For non-classified endpoints no classification can be recommended, unless new information becomes available.
2. If the substance is not in Annex VI, table 2, it should be classified according to the criteria in the Regulation for classification, packaging and labelling of dangerous substances /6/ using all available test and non-test data, including AL2009.

3 Technical description of the self-classifications

The current chapter gives the detailed description of how the advisory classifications were assigned to the chemicals in the advisory list for self-classification. This includes description of the classification rules and the (Q)SARs used for predicting the dangerous properties of the chemicals. It must be noted that the advisory classifications for acute oral toxicity and sensitisation by skin contact have not been updated. The advisory classifications for these two endpoints are still recommended for use with the documentation as given in the previous version of self-classification list /5/.

3.1 Mutagenicity

The criteria for classification for mutagenicity are divided into 3 different categories:

Classification as mutagen, category 1 (Mut1;R46, May cause heritable genetic damage) is based on evidence of a causal association between human exposure to the substance and heritable genetic damage.

Classification as mutagen, category 2 (Mut2;R46, May cause heritable genetic damage) is based on animal studies showing mutagenity to germ cells either in assays on germ cells or by demonstrating mutagenic effects in somatic cells *in vivo* or *in vitro* as well as metabolic proof that the substances reaches the germ cells.

The criteria for classification as mutagen, category 3 (Mut3;R68, Possible risks of irreversible effects) is based either on *in vivo* mutagenicity tests or on cellular interactions with *in vitro* tests acting as supportive evidence. For this classification, it is not necessary to demonstrate germ cell mutations.

(Q)SAR based evaluation
Five models predicting genotoxicity *in vivo* endpoints were applied in the screening. Data for the training sets were obtained from the literature. The technical specifications for the models are given in Table 2.

Drosophila melanogaster Sex-Linked Recessive Lethal (SLRL) (in vivo)
The training set consists of data from Lee et al. /16/. In the experimental method, Drosophila melanogaster males and females are used. Males are treated with the test substance and mated individually to virgin females. The test detects the occurrence of mutations, point mutations and small deletions, in the germ line of the insect. The mutations are phenotypically expressed in males carrying the mutant gene. When the mutation is lethal in the hemizygous condition, its presence is inferred from the absence of one class of male offspring out of the two that are normally produced by a heterozygous female. The assay has a low sensitivity for genotoxins other than direct-acting agents and simple promutagens, but a very high specificity, which means that

in general a positive result has considerable value for prediction of potential genotoxicity in mammals.

Mutations in mouse micronucleus (in vivo)
The training set includes data from Hayashi et al. /17/, Mavournin et al. /18/, Waters et al. /19/, and Morita et al. /20/. The test detects micronuclei produced by damage to the chromosomes or the mitotic apparatus in red blood cells. Micronuclei are small nuclei produced during cell division. They contain chromosome fragments or whole chromosomes. In the test, mice are exposed to the test substance and young red blood cells (erythrocytes) from the bone marrow are isolated and analysed for micronucleus. The test is especially relevant to assess mutagenic hazard in that it allows consideration of factors of *in vivo* metabolism, pharmacokinetics and DNA-repair processes.

Dominant lethal effect in rodents (in vivo)
The training set is comprised of data from Green et al. /21/ and other references. In the experimental method, mice and rats are used. Treated males are mated to virgin females according to an experimental scheme. Females are sacrificed in the second half of pregnancy and uterine contents are examined to determine the number of implants and live and dead embryos. The category of early embryonic deaths is the most significant index of dominant lethality and as such used as endpoint. The test identifies major genetic damage, mainly the induction of structural and numerical chromosomal anomalies.

Sister chromatid exchange in mouse bone marrow (in vivo)
Data from Tucker et al. /22/ are used in the training set. The sister chromatid exchange (SCE) assay detects interchange of DNA between two sister chromatids of a duplicating chromosome. Mice are exposed to the test chemical. Then a thymidine analog, bromodeoxyuridine (BrdU) is injected. If DNA exchanges occur, BrdU can be identified by use of a fluorescence technique in chromosomes in the metaphase. The test is considered to be a sensitive method for evaluating mutagenicity and may be an indicator of carcinogenicity.

Comet assay in mouse (in vivo)
The training set includes data from Sasaki et al. /23/ plus a number of physiological chemicals theoretically assumed not to have the effect (such as various amino acids, sugar molecules, fatty acids etc.). The latter was included to get a better distribution between positives and negatives in the training set for the model). Included in the training set of the model are results from eight tissue types; stomach, colon, liver, kidney, bladder, lung, brain and bone marrow. The comet assay detects DNA strand break and can be applied to virtually any organ of interest. In the experimental test, a microgel electrophoretic technique is used for detecting DNA damage at cell level. The tested chemical is positive if it produces breaks in DNA-strings, resulting in small strings of DNA that are able to migrate further in a microgel, than intact DNA strings. In the microscope, damaged DNA is seen as a "comet" while not damaged DNA appear as a dot. If appropriately performed, the test has been shown to be reliable with high sensitivity to detect DNA damage in organs that cannot be investigated in other classical mutagenicity assays.

Model	Technical summary
Drosophila melanogaster Sex-Linked Recessive Lethal (*in vivo*)	MultiCASE, DK model Training set: n=377 Cross-validation 10*50% gave Sensitivity: 73.9% Specificity: 88.0% Concordance: 81.6% Domain: 48%
Mutations in mouse micronucleus (*in vivo*)	MultiCASE, DK model Training set: n=358 Cross-validation 10*50% gave Sensitivity: 30.1% Specificity: 84.5% Concordance: 66.1% Domain: 59%
Dominant lethal mutations in rodent (*in vivo*)	MultiCASE, DK model Training set: n=191 Cross-validation 10*50% gave Sensitivity: 41.3% Specificity: 95.2% Concordance: 75.9% Domain: 42
Sister chromatid exchange in mouse bone marrow (*in vivo*)	MultiCASE, DK model Training set: n=265 Cross-validation 10*50% gave Sensitivity: 70.4% Specificity: 86.9% Concordance: 85.5% Domain: 53%
COMET assay in mouse (*in vivo*)	MultiCASE, DK model Training set: n=286 Cross-validation 10*50% gave Sensitivity: 63.3% Specificity: 93.3% Concordance: 83.9% Domain: 45%

Table 2: Technical summary for the mutagenicity models
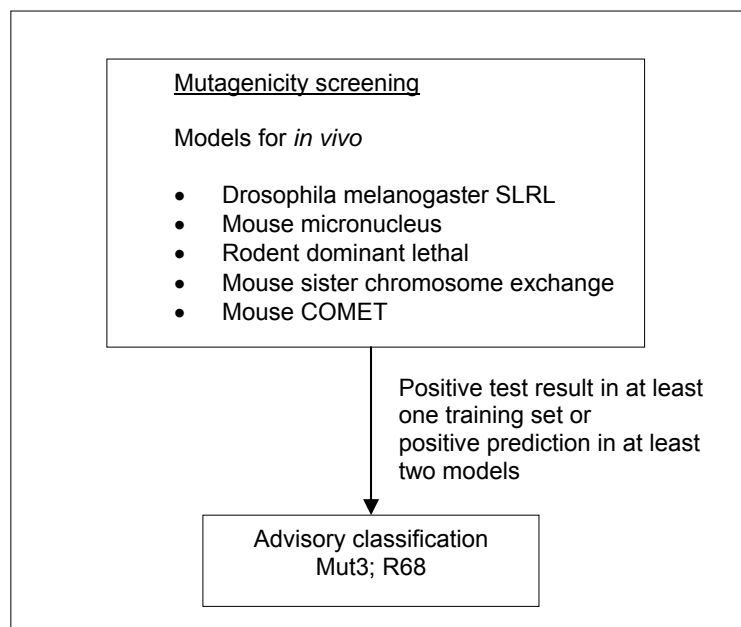

Figure 1: Schematic diagram illustrating the systematic evaluation applied to assign advisory classifications for mutagenicity.

For a substance to be selected as a probable mutagen it was necessary for the following criteria to be fulfilled: Positive prediction in two or more models, accepting only predictions where no significant deactivating fragments were detected. If one or more positive tests could be seen (as part of the training

sets for the models) for any genotoxicity endpoint, this took precedence over model predictions.

When classification is proposed on basis of test data, a positive result in a single *in vivo* test is sufficient evidence on which to base the classification. In contrary to that, positive predictions in at least two models were required here.

5,742 of the chemicals investigated in the current project met the criteria in the systematic evaluation and were assigned advisory classifications Mut3;R68.

3.2 Carcinogenicity

This endpoint can result in classification in 3 different categories:

Classification as carcinogen in category 1 (Carc1;R45, Toxic; May cause cancer, or Carc1;R49, Toxic; May cause cancer by inhalation) is based on a strong causal relationship in humans.

Classification as carcinogen in category 2 (Carc2;R45, Toxic; may cause cancer, or Carc2;R49, Toxic; may cause cancer by inhalation) is based on conclusive animal data from 2 species or 1 species with supportive evidence such as genotoxic effects *in vitro* or *in vivo*.

Classification as carcinogen in category 3 (Carc3;R40, Harmful; Possible risks of irreversible effects") is subdivided into two:

   a) Well-investigated substances with restricted tumorigenic effects. It is
       normally based on clear data of tumour formation in one species.
       Mutagenicity data *in vitro* and *in vivo* can be used as supportive
       evidence.
   b) Substances that are insufficiently investigated, but raising concern for
       man.

(Q)SAR based evaluation
Four models predicting carcinogenicity *in vivo* and models predicting three genotoxicity *in vitro* endpoints were applied in the screening. Commercial MultiCASE training sets constitutes the basis of the carcinogenicity models. The technical specifications for the models are given in Table 3.

Carcinogenicity male and female, rats and mice (in vivo)
The models are the MultiCASE commercial models AG1-4 /24/. The training sets were constructed using the NTP (US National Toxicology Program) rodent carcinogenicity database, the Lois Gold Carcinogen Potency Database, FDA/CDER (US Food and Drug Administration / Center for Drug Evaluation and Research) archives, and the scientific literature. Training sets include both non-proprietary and proprietary data. Proprietary (confidential) data constitute around ten percent of the training sets. The open models based on the non-proprietary data were also available and consulted in the screening process.

In the experimental test, the test substance is administered by an appropriate route to the animals for a major portion of their lifespan. The highest dose level should elicit signs of toxicity, without substantially altering the normal lifespan due to effects other than tumours. During and after exposure, the

animals are observed daily to detect signs of toxicity, particularly the development of tumours.

Reverse mutation test, Ames (in vitro)

The training set is from Kazius et al. /25/. The bacterial reverse mutation test detects point mutations, which involve substitution, addition or deletion of one or a few DNA base pairs. Amino-acid (histidin) requirering strains of Salmonella typhimurium are used. Mutations, which revert mutations present in the test strains and restore the functional capability of the bacteria to synthesise the amino acid (histidin), are detected. These appear by the ability of the bacteria to grow in the absence of histidin required by the parent test strain. The test is a useful tool as an initial screen for potential *in vivo* genotoxic activity, and has become the most extensively used *in vitro* short-term test in the screening for mutagenicity.

Chromosomal aberration CHO/CHL (in vitro)

This model was used by Niemela and Wedebye /28/ to evaluate the OECD principles for development and validation of (Q)SARS /27/. The Chinese Hamster Ovary (CHO) model is the commercial MultiCASE model A61 /26/ and the training set for the Chinese Hamster Lung (CHL) model was taken from Ishidata /28,29/. The *in vitro* mammalian chromosome aberration test identifies agents that cause structural chromosome aberrations in cultured cells. Chromosome damage is expressed as breakage of single or both chromatids, sometimes followed by reunion between chromatids or of both chromatids at an identical site. Many compounds that are positive in this test are mammalian carcinogens causing DNA damage.

Mutations in mouse lymphoma (in vitro)

The training set is comprised of data from Grant et al. /30/. The mouse lymphoma assay detects mutations affecting the heterozygous thymidine kinase (TK) locus. It identifies chemicals acting as clastogens (delete, add, or rearrange chromosome sections) as well as point mutagens. Mutations in genes coding for TK are identified. TK is involved in the phosphorylation of thymidin and subsequently in the formation of DNA. Positive chemicals may give rise to mutations in genes coding for TK. A mutation may result in loss of the ability to phosphorylate the pyrimidin analogs, which is detected by the test. The assay has a reputation for high sensitivity and low specificity of detecting genotoxic agents. However, in this exercise the model is used to give mechanistic information to chemicals already predicted to be carcinogens.

Model	Technical summary
Carcinogenicity in male rat (*in vivo*)	MultiCASE, AG1 Training set: n=1381 External validation (100 chemicals): Sensitivity: 58.6% Specificity: 97.6% Concordance: 75.0% Domain: 70%
Carcinogenicity in female rat (*in vivo*)	MultiCASE, AG2 Training set: n=1376 External validation (100 chemicals): Sensitivity: 58.6% Specificity: 97.6% Concordance: 75.0% Domain: 70%

Carcinogenicity in male mouse (*in vivo*)	MultiCASE, AG3 Training set: n=1252 External validation (100 chemicals): Sensitivity: 58.6% Specificity: 97.6% Concordance: 75.0% Domain: 71%
Carcinogenicity in female mouse (*in vivo*)	MultiCASE, AG4 Training set: n=1263 External validation (100 chemicals): Sensitivity: 58.6% Specificity: 97.6% Concordance: 75.0% Domain: 71%
Reverse mutation test, Ames (*in vitro*)	MultiCASE, DK model Training set: n=4102 Cross-validation 10*50% gave Sensitivity: 84.4% Specificity: 82.5% Concordance: 83.5% Domain: 73%
Chromosomal aberration CHO (*in vitro*)	MultiCASE, A61 Training set: n=233 Cross-validation 10*50% gave Sensitivity: 32.0% Specificity: 91.2% Concordance: 69.9% Domain: 45%
Chromosomal aberration CHL (*in vitro*)	MultiCASE, DK model Training set: n=600 Cross-validation 10*50% gave Sensitivity: 57.8% Specificity: 86.5% Concordance: 74.3% Domain: 64%
Mutations in mouse lymphoma (*in vitro*)	MultiCASE, DK model Training set: n=555 Cross-validation 10*50% gave Sensitivity: 68.5% Specificity: 86.3% Concordance: 79.2% Domain: 64%

Table 3: Technical summary for the carcinogenicity models

Identification of carcinogenic substances

For a substance to be selected as a probable carcinogen it was necessary for the following criteria to be fulfilled: Positive according to the ICSAS methodology /24/, corresponding to two or more positive carcinogenicity predictions, accepting only predictions for chemicals without significant deactivating fragments. If one or more positive tests could was observed (as part of the training sets for the models) for any cancer endpoint, this took precedence over model predictions.

While in most cases this resulted in little change (the models are heavily biased towards making a correct prediction for substances used to make them), it was felt that there was no reason to artificially reduce the quality of the advisory classification by neglecting to use data, which happen to be present. One or more negative tests in the training set of each model also took precedence over predictions of that model, except in cases where positive training set tests were present in other cancer models.

Employing this carcinogenicity identification algorithm resulted in a list of 3,726 positive predictions.
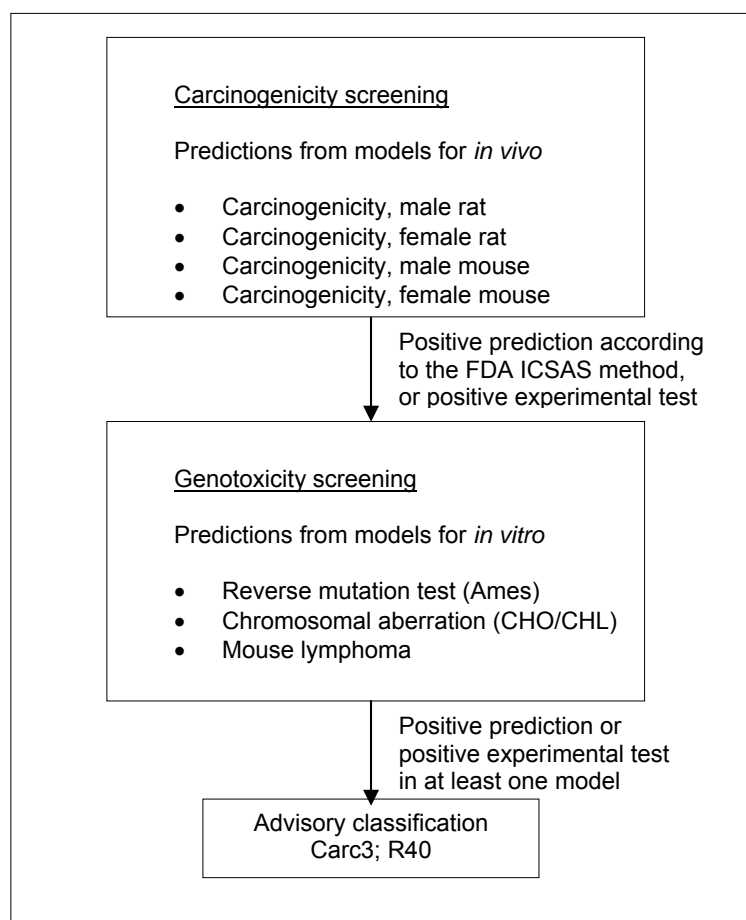
26

Figure 2: Schematic diagram illustrating the systematic evaluation applied to assign advisory classifications for carcinogenicity.

Identification of genotoxic carcinogens

While there are many non-genotoxic carcinogens acting by a wide variety of often-unknown mechanisms, it was chosen to focus here on chemicals likely to cause cancer through a genotoxic mechanism. Therefore, a further selection criterion for genotoxicity was set up.

As opposed to the selection criteria for mutagenicity, not all genotoxic carcinogens are necessarily clastogenic (cause loss, addition or rearrangement of parts of chromosomes). To select the genotoxic chemicals from the chemicals already predicted positive for *in vivo* carcinogenicity, which include genotoxic as well as non-genotoxic carcinogens, a battery of models for sensitive *in vitro* genotoxicity endpoints was used.

The genotoxicity criterion was a positive estimate in one or more of the models for the following *in vitro* genotoxicity endpoints; Reverse mutation test (Ames), chromosomal aberrations (CHO/CHL), or mutations in mouse lymphoma.

A schematic diagram of the systematic evaluation is given in Figure 2. According to these criteria, 3,726 of the chemicals assessed in the current project were identified as genotoxic carcinogens and selected for advisory classification for carcinogenicity. It is not felt that the models employed allow discrimination between classification in the three categories, so the lower classification Carc3;R40 was applied in all cases.

3.3 Reproductive toxicity

This endpoint can result in classification in 3 different categories:

Classification as toxic to reproduction in category 1 (Rep1;R60, Toxic; May impair fertility, or Rep1;R61, Toxic; May cause harm to the unborn child) is based on a strong causal relationship in humans.

Classification as toxic to reproduction in category 2 (Rep2;R60, Toxic; May impair fertility, or Rep2;R61, Toxic; May cause harm to the unborn child) is based primarily on animal data, and secondly on "other relevant information". Data from *in vitro* studies, or studies on avian eggs, are regarded as "supportive evidence" and would only exceptionally lead to classification in the absence of *in vivo* data.

Classification as toxic to reproduction in category 3 (Rep3;R62, Harmful; Possible risks of impaired fertility, or Rep3;R63, Harmful; Possible risk of harm to the unborn child) is based primarily on animal data, and secondly on "other relevant information". Substances in category three are insufficiently investigated, but raising concern for man.

Classification for reproductive toxicity covers a wide range of effects on either fertility or to the developing organism before and after birth (structural or functional damage). The (Q)SAR models applied in the current project only cover certain but far from all types of harm to the unborn child. Hence only certain types of mechanisms causing malformations or death are covered. Furthermore, no (Q)SAR models were used for effects on fertility.

(Q)SAR based evaluation
Three models predicting *in vivo* teratogenicity or fetal lethality related endpoints were applied in the assessment. A commercial MultiCASE training set constitutes the basis of one model. Data for the training sets for the two other models were obtained from the literature. The technical specifications for the models are given in Table 4.

Teratogenic risk (in vivo)
The model is the MultiCASE commercial model A49 /31/. The training set is composed of data taken from the TERIS (Teratogen Information System) and a compilation in which the FDA (US Food and Drug Administration) definitions were used to quantify risk of developmental toxicity from drugs used during pregnancy. The training set consists of clinical and epidemiologicdata. Many biological mechanisms are involved in the effects.

Drosophila melanogaster SLRL effect (in vivo)
The training set consists of data from Lee et al. (1983) /32/. In the experimental method, Drosophila melanogaster males and females are used. Males are treated with the test substance and mated individually to virgin females. The test detects the occurrence of mutations, point mutations and small deletions, in the germ line of the insect. The mutations are phenotypically expressed in males carrying the mutant gene. When the mutation is lethal in the hemizygous condition, its presence is inferred from the absence of one class of male offspring out of the two that are normally produced by a heterozygous female. The assay has a low sensitivity for genotoxins other than direct-acting agents and simple promutagens, but a very high specificity.

Dominant lethal effect in rodents (in vivo)

The training set is comprised of data from Green et al. (1985) /33/ and other references /21/. In the experimental method, mice and rats are used. Treated males are mated to virgin females according to an experimental scheme. Females are sacrificed in the second half of pregnancy and uterine contents are examined to determine the number of implants and live and dead embryos. The category of early embryonic deaths is the most significant index of dominant lethality and as such used as endpoint. The test identifies major genetic damage, mainly the induction of structural and numerical chromosomal anomalies.

Model	Technical summary
Teratogenic risk in humans (*in vivo*)	MultiCASE, A49 Training set: n=323 Cross-validation 10*50% gave Sensitivity: 50.2% Specificity: 91.3% Concordance: 79.3% Domain: 48%
Mutations in Drosophila melanogaster SLRL (*in vivo*)	MultiCASE, DK model Training set: n=377 Cross-validation 10*50% gave Sensitivity: 73.9% Specificity: 88.0% Concordance: 81.6% Domain: 48%
Dominant lethal mutations in rodent (*in vivo*)	MultiCASE, DK model Training set: n=191 Cross-validation 10*50% gave Sensitivity: 41.3% Specificity: 95.2% Concordance: 75.9% Domain: 42%

Table 4: Technical summary for the models for reproductive toxicity.

The dominant lethal test in rodents and the Drosophila SLRL test are initially meant for genotoxicity effects on germ cells, but the resulting effect is early embryonic deaths and lethal effect on offspring, respectively. Therefore, the endpoints are relevant for reproductive toxicity assessment.

In many cases, a toxicological threshold is assumed to exist for reproductive toxicity. With mutagenic chemicals this may not be the case.
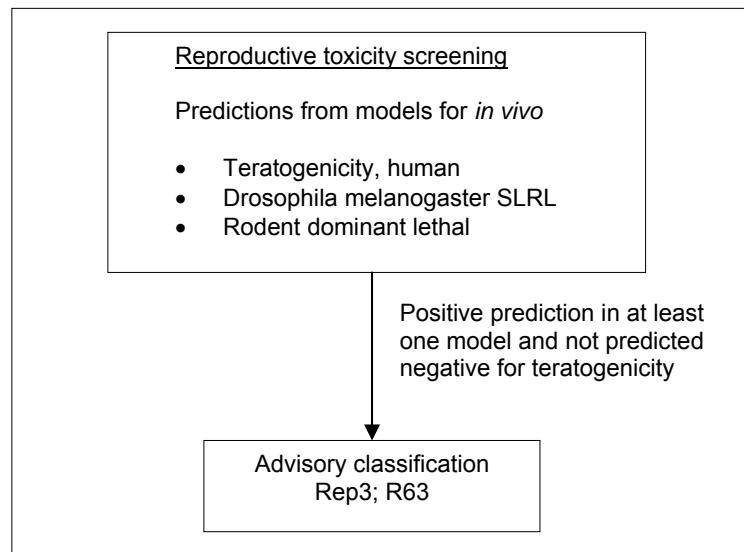

Figure 3: Schematic diagram illustrating the systematic evaluation applied to assign advisory classifications for reproductive toxicity.

For a substance to be selected as probable toxic to reproduction in the assessment, the criterion was a positive prediction in any of the three models and without a negative prediction in the teratogenic risk in humans model (see Figure 3) (see also /34/).

The screening resulted in a list of 4,036 positive predictions. The models employed do not allow discrimination between classification in the three classification categories, so the lower classification Rep3;R63 was applied in all cases.

3.4 Danger to the aquatic environment

The classification criteria are composed of three main elements: 1) potential for rapid degradation, 2) bioconcentration potential in fish, and 3) short-term toxicity to aquatic organisms (fish, daphnia, and algae). Classifications are assigned according to the following scheme:

Classification	Classification criteria*
N;R50 Dangerous for the environment; very toxic to aquatic organisms	Acute toxicity ≤ 1.0 mg/L
N;R50/53 Dangerous for the environment; very toxic to aquatic organisms; may cause long-term adverse effects in the aquatic environment	Acute toxicity ≤ 1.0 mg/L and not readily degradable or BCF** ≥ 100
N;R51/53 Dangerous for the environment; toxic to aquatic organisms; may cause long-term adverse effects in the aquatic environment	Acute toxicity > 1 and ≤ 10 mg/L and not readily degradable or BCF** ≥ 100
R52/53 Harmful to aquatic organisms; may cause long-term adverse effects in the aquatic environment	Acute toxicity > 10 and ≤ 100 mg/L and not readily degradable
R53 Harmful to aquatic organisms	Solubility in water < 1 mg/L and not readily degradable and BCF** ≥ 100

Table 5: EU criteria for classification for danger to the aquatic environment
* The lowest effect concentration, EC_{50}, for fish, daphnia or algae is used
** BCF: Bioconcentration factor

(Q)SAR based evaluation
Advisory classifications were assigned on the basis of combinations of estimates for ready biodegradability, bioconcentration and acute toxicity according to the criteria in Table 5. Classification with risk phrase R53 alone was not done in this exercise, as the strong co-linearity between water solubility and bioconcentration factor made it redundant.
It is noted that compared to the classification criteria according to which abiotic degradation (and assessment of primary degradation products for their environmental hazard classification) can be used, only predictions concerning potential for rapid biodegradation was employed here. Furthermore only predictions for bioconcentration in fish were used even though the classification criteria refers to use of log Kow when reliable measured BCF data in fish are not available.

Biodegradation
Biodegradability was estimated using the Syracuse BIOWIN program /43/.
Only the non-linear equation for rapid/non-rapid biodegradation (BPP2) was applied. Previous validation of this parameter compared with 304 MITI

"ready/not-ready (45:259) results showed that while a relatively high percentage of "not-ready" chemicals were missed (sensitivity result was 53%), 97% of "not ready" predictions were correct (PPV, Positive Predictive Value) in this "chemical universe" of 85% not-ready chemicals /44/. MITI data was also applied by Tunkel et al /41/ who found a sensitivity of 53%, a specificity of 86% and a PPV of 83% for 884 chemicals (385 ready: 499 not-ready). These findings were largely confirmed in a comparison exercise made by the Danish EPA and based on chemicals assessed at OECD (SIAM 11-18), where 128 chemicals (59 ready:69 not-ready), which were not part of the BPP2 training set indicated a sensitivity of 54%, a specificity of 85% and a positive predictive value of 80% /38/. In other words while this model may fail to identify around half of all "non-ready" substances, the number of false predictions for not-ready biodegradability will be very low.

A total of 11,766 chemicals of the 49,292 chemicals studied were found to be "not-readily degradable" according to this criterion.

Bioconcentration

The classification and labelling guidelines prefer measured data for bioconcentration, but as this rarely is available, a Log K_{ow} of greater than three is recommended as an indication that BCF will be 100 or greater, in accordance with the linear equation of Veith /55/. While a good rule-of-thumb, this relation both over- and underestimates BCF for many classes of chemicals, and is only applicable in the Log K_{ow} interval 2-6.

Bioconcentration was therefore predicted using Syracuse BCFWIN /42/, a method based on a combination of Log K_{ow} relations and structural fragment categories. This method was evaluated by its authors as having a statistical accuracy of $R^2 = 0.74$ (n = 694, S.D. 0.65, mean error = 0.47), which is a significant improvement over the standard equation of Veith (log BCF = 0.85 * Log K_{ow} – 0.70) where predictions for the same 694 compounds had a statistical accuracy of $R^2 = 0.32$ (S.D. 1.62 and mean error = 1.12).

No attempt was made to further assess bioaccumulation potential.

For chemicals predicted to have aquatic toxicity concentrations below 10 mg/L and to be readily biodegradable, 4,662 chemicals were predicted to have BCF estimates of equal to or greater than 100.

Acute toxicity

For aquatic toxicity classifications, it is recommended to used $L(E)C_{50}$-values for fish, daphnia and algae. Aquatic toxicity to fish, daphnia and algae were predicted using three models and a theoretical equation.

Fish

For acute aquatic toxicity to fish a DK MultiCASE model using 96h LD_{50} data on 569 chemicals from the Duluth Fathead minnow database was applied /48/. Cross-validation of this model gave a R^2 of 0.735. As there was insufficient test data for very lipofilic substances the MultiCASE model was only applied for chemical substances with Log Kow of 6 or less.

Daphnia

For acute aquatic toxicity to daphnia a DK MultiCASE model using 48h EC_{50} data on 641 chemicals from various sources was applied /49/. Cross-validation of this model gave a R^2 of 0.69. As there was insufficient test data for very

lipofilic substances the MultiCASE model was only applied for chemical substances with Log Kow up to 7.

Algae

For acute aquatic toxicity to daphnia a DK MultiCASE model using EC_{50} data on 531 chemicals (396 tests made at the Danish Technical University for the Danish EPA, plus literature data from various sources) /50/ was applied. Cross-validation of this model gave a R^2 of 0.74.

A regression equation was used on top of MultiCASE predictions to adjust for Log K_{ow} contribution to the toxicity:

Log EC_{50} (μM) = 0.593*Log EC_{50} (MC4PC prediction, μM) – 0.257*Log K_{ow} + 1.076
N = 343, R2 = 0.743, S.E = 0.853
(Log K_{ow} below –1 were set to –1, Log K_{ow} above 7 and less or equal to 8 were set to 5, and Log K_{ow} above 8 were set to 1)

As there was insufficient test data for very lipophilic substances the MultiCASE model was only applied for chemical substances with Log Kow of up to 8.

Non-polar narcosis predictions for highly lipophilic substances

Another relationship was used for chemicals with a Log K_{ow} of greater than six. Here, all substances were assumed to act by non-polar narcosis, and toxicity at equilibrium was estimated according to a relation to the predicted bioconcentration factor in small fish:

LC_{50} (equilibrium) = 8.15 mmol /BCF

The choice of 8.15 mmol corresponds to the theoretical level inducing aquatic lethal effects represented by the non-polar narcosis fish (Q)SAR recommended in the REACH-guidance /51/. Non-polar narcosis Lethal Body Burden's for fish are generally assumed to be within the range of about 2–8 mmol /53/.

While simple Log K_{ow} relationships exist for predicting the non-polar narcotic toxicity for fish, daphnia and algae, these do not distinguish specific toxicity's unique to any of the three taxa, and were not felt to offer any advantage over using the fish models alone, which also adequately predict non-polar narcosis. For all practical purposes, non-polar narcosis induces effects at the same concentration levels in all three taxa at these high Log K_{ow} values.

Aquatic toxicity screening

Using the three Multicase models and the non-polar narcosis equation, 18,809 of the chemicals assessed in the current project had acute aquatic toxicity's of $\leq$ 100 mg/L.

Model	Technical summary
Biodegradation, Syracuse BIOWIN2 non-linear model for rapid/non-rapid aerobic biodegradation probability (BPP2)	Syracuse BIOWIN, US EPA /45/ Training set: n=295 External validation (n=304) gave Sensitivity: 53.3% Specificity: 91.1% Concordance: 58.9% PPV: 97.2%
Bioconcentration (BCF), Syracuse BCFWIN	Syracuse BCFWIN, US EPA /42,46/ Training set: n=694 Cross-validation gave $R^2 = 0.74$ S.D. = 0.65 Mean error = 0.47
Acute toxicity to fish, *Fathead minnow* LC_{50} (96h)	MultiCASE, DK model /48/ Training set: n=569 Cross-validation 3*10% gave $R^2 = 0.74$ Domain: 52%
Acute toxicity to daphnia, *Daphnia magna*, EC_{50} (48h)	MultiCASE, DK model /49/ Training set: n=641 Cross-validation 3*10% gave $R^2 = 0.69$ Domain: 52%
Acute toxicity to algae, *Pseudokirchneriella subcapitata*, EC_{50}	MultiCASE, DK model /50/ plus Log K_{ow} equation Training set: n=531 Cross-validation 10*50% for the two-step model gave $R^2 = 0.74$ Domain: 58%
Non-polar narcosis, LC_{50} (equilibrium) = 8.15 mmol /BCF	Theoretical equation /51-54/

Table 6: Technical summary for the models used for classification of danger to the aquatic environment.

Advisory classifications

A total of 18,809 of the chemicals assessed in the current project were selected according to one of the four classification categories based on the combination of model predictions as indicated in the classification criteria and shown in Figure 4. The classifications for danger to the aquatic environment were assigned to the following number of chemicals:

N;R50	2,381
N;R50/53	7,376
N;R51/53	6,063
N;R52/53	2,989
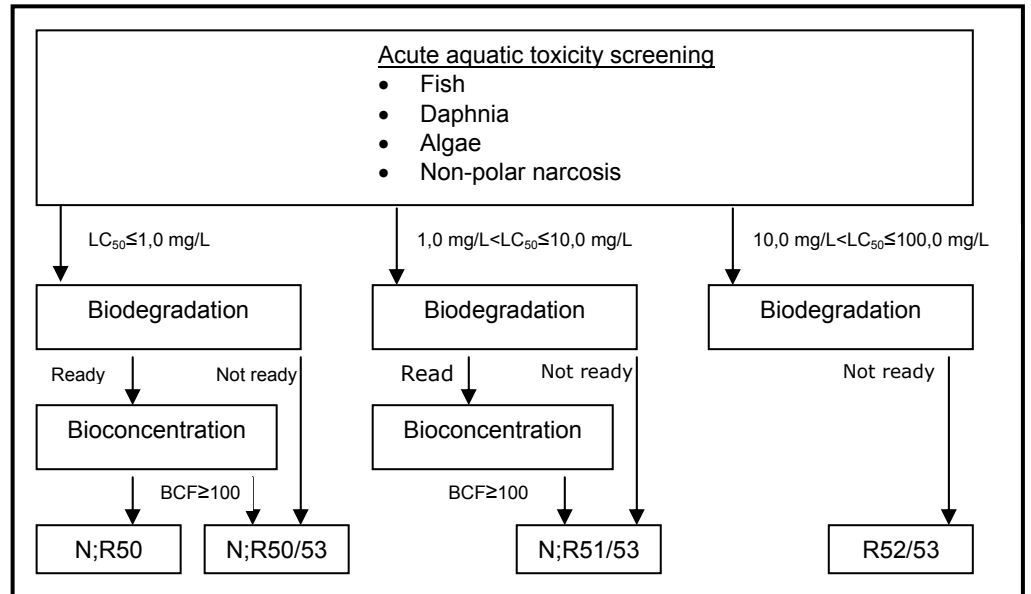
Figure 4: Schematic diagram illustrating the systematic evaluation applied to assign advisory classifications for danger to the aquatic environment.

4 Discussion & Conclusions

The 2009 update of the advisory classification list has been made using entirely new models for the CMR advisory classifications; i.e. none of the models used to make the advisory classifications for mutagenicity and carcinogenicity on AL2001 were used in this update.

Annex 2 contains examples of how further structural analyses of substances belonging to various chemical classes can be made on top of the predicted properties from this project to visualize and gain further insight into relations between sub-structures and, in this case, the carcinogenicity properties of chemicals.

For the environmental advisory classifications some of the models used for AL2001 were used again (BCFWIN and model for aquatic toxicity to Fathead minnow), and new models were applied for biodegradation and aquatic toxicity to Dahpnia and Algae.

The total numbers of chemicals on AL2001 compared to AL2009 cannot be directly compared because AL2001 contained advisory classifications for skin sensitization and acute oral toxicity and these endpoints are not updated in AL2009, whereas advisory classification for reproductive toxicity is included on AL2009 but not on AL2001.

However, comparisons between AL2001 and AL2009 are made in the following for the individual advisory classifications represented in both lists, i.e. advisory classifications for mutagenicity, carcinogenicity and danger to the aquatic environment.

4.1 Chemicals on AL2009 that were not on AL2001

As shown in figure 5 a larger number of chemicals have been assigned advisory classifications for the individual advisory classifications in the current advisory list than in the former. This is due primarily to the application of entirely different models with in many cases larger chemical domains than the models applied for AL2001. Also, a little more substances were included in the start list for AL2009 than for AL2001 (49,292 for AL2009 and approximately 47,000 for AL2001)

For the advisory classifications for danger to the aquatic environment the reasons for the differences more specifically relate to the addition of aquatic toxicity models for Daphnia and Algae, plus the use of the non-linear BIOWIN 2 model instead of the linear BIOWIN 1 model, which was used for AL2001. BIOWIN 1 has a lower sensitivity than BIOWIN 2.

For the carcinogenicity and mutagenicity endpoints the increased number of predictions on AL2009 as compared with AL2001 is generally due to the use of new and improved (Q)SAR-models with larger applicability domains.

Figure 5 presents an overview of the number of advisory classifications for individual endpoints on AL2001 and AL2009. Reproduction is included although this endpoint was not addressed in AL2001.
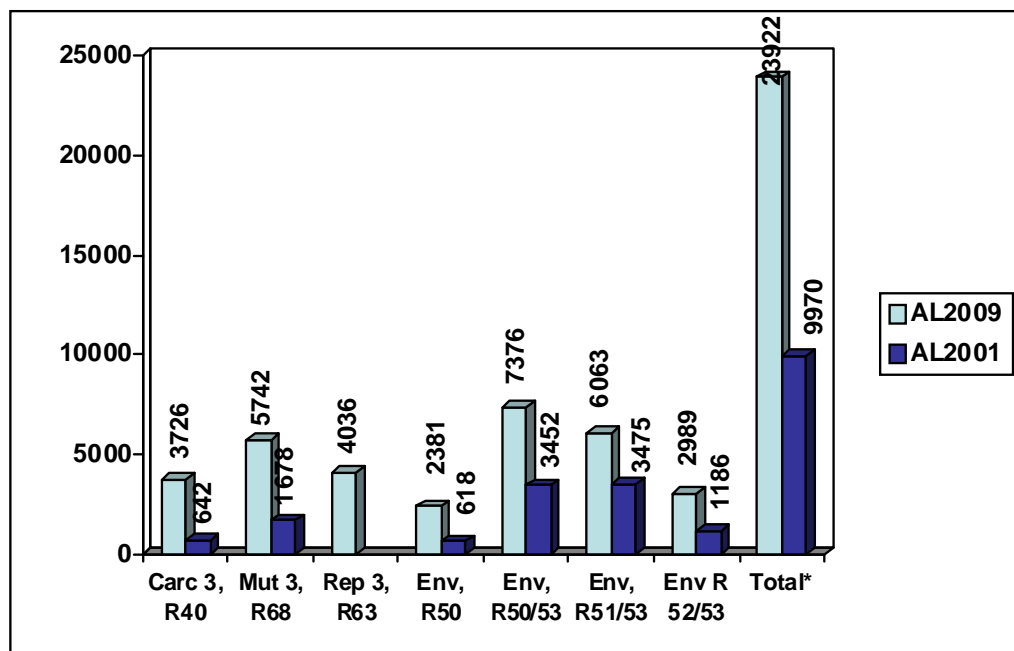

Figure 5: Overview of the number of substances for each advisory classification in the new and the previous version of the advisory self-classification list. The exact number of advisory classifications is shown for each bar. (Note: Reproductive toxicity was not included in AL2001. Also, the advisory classifications for acute oral toxicity Xn, R22 and sensitisation by skin contact, R43 have not been updated in the current list and are therefore not included.)
* AL2001 Total: This number refers only to number of chemicals with advisory classifications for mutagenicity, carcinogenicity and danger to the aquatic environment. The total number of chemicals on AL2001 is 20,624.

4.2 Chemicals on AL2001 that are not on AL2009

There are also substances that were assigned advisory classifications on AL2001 that are not on AL2009. It is for the individual endpoints seen that between 11 and 14% of the advisory classifications from AL2001 are not on AL2009. Again, the differences are primarily due to the use of new models for AL2009.

Chemicals on AL2001 may not have been included in AL2009 for one or more of the following reasons:
- they have been formally classified in the EU
- they were not included on the new starting list for technical reasons (e.g. errors in the structural information or structure information not accepted by the (Q)SAR software)
- they were not within the applicability domain of some or all of the models applied for AL2009
- they do not fulfil the new (Q)SAR model algorithms established for advisory classifications in AL2009

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For the mutagenicity endpoint, for example, where five models were used, many of the chemicals that fell out did not have robust predictions (within applicability domain) in two or three models, but often with flags in one or more of these models showing that a possible active fragment was identified. Additionally, many have positive predictions in models for *in vitro* genotoxicity endpoints (which were not included in the evaluation). In total, the majority of the chemicals that were not identified this time appear to be borderline mutagens.

As there were mixed results (negative / out-of-domain / positive) from the battery of models applied within an endpoint, it is not possible to divide the chemicals strictly into groups of chemicals that were not identified this time because they could not be predicted (i.e. outside domain) or because the models applied in the new selection algorithm for AL09 predict them to be negative for the effect.

A detailed comparison between numbers of chemicals with advisory classifications for carcinogenicity, mutagenicity and danger to the aquatic environment on AL2001 and AL2009 is given in table 7.

Advisory classifi-cation	Substances on AL2001				Substances on AL2009	
	Total no. with this advisory classifi-cation	- also on AL2009 with same advisory classifi-cation	- also on AL2009 but with different advisory classifications	- not on AL2009	Total with this advisory classifi-cation	- not on AL2001 with this advisory classifi-cation
Mut3;R68	1,678	695	742, including - 284 with Carc3;R40 - 80 with Rep3;R63 (total 349)[*]	241 (14%), including - 7 now on Annex 1 - 3 not on start list	5,742	5,047
Carc3;R40	642	287	287, including - 144 with Mut3;R68 - 45 with Rep3;R63 (total 160)[*]	68 (11%), including - 13 now on Annex 1 - 2 not on start list	3,726	3,439
Rep3;R63	-	-	-	-	4,036	-
Danger to the aquatic environ-ment	8,730	7,546[**]	203	981 (11%), including - 22 now on Annex 1 - 3 not on start list	18,809	11,263

[*] Due to overlap; some chemicals have advisory classifications for more than one CMR endpoint
[**] with one of the classifications for danger to the aquatic environment
Table 7. Overview of the occurrence of substances on AL2001 and AL2009

4.3 Conclusion

Due primarily to the application of combinations of new (Q)SAR models, in many cases with larger applicability domains, the number of substances with advisory classifications for carcinogenicity, mutagenicity and danger to the aquatic environment has increased considerably for individual classifications as compared to AL01. Moreover, reproductive toxicity (possible harm to the unborn child) was included for the first time.

5 References

1. Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC.

2. Council Directive 67/548/EEC on the approximation of the laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances.

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Annex 1. Glossary

	Description
Training set	The collection of experimental data on a range of chemicals that have been used to develop the (Q)SAR-model.
Sensitivity	The sensitivity is a measure of how well the model "catches" the substances with positive effect in relation to the endpoint being modelled. A sensitivity of 80% means that 80% of the "true positives" in the validation set were correctly predicted as positives (the remaining 20% were falsely predicted as negatives (false negatives)). The sensitivity is not dependent on the prevalence of positives in the "chemical universe".
Specificity	The specificity is a measure of how well the model predicts substances with lack of effects in relation to the endpoint modelled. A specificity of 80% means that 80% of the "true negatives" in the validation set were correctly predicted as negatives (the remaining 20% of the negatives were falsely predicted as positives (false positives)). The specificity is not dependent on the prevalence of negatives in the "chemical universe".
Concordance	Also referred to as overall accuracy. The concordance is an overall measure of the correctness of the predictions. A concordance of 80% means that 80% of the substances in the validation set were correctly predicted as positives or negatives (the remaining 20% are the false predictions i.e. false negatives and false positives).
Predictive values	Positive and negative predictive values, PPV and NPV are measures of how well the model positive or negative *predictions*, respectively, are correct. A PPV of 80% means that 80% of the positive predictions in the validation set were correct (the remaining 20% were false positives). The predictive values *are* dependent on the split between positives and negatives in the "chemical universe".
Applicability domain	The Applicability Domain (AD) of a (Q)SAR expresses the limits of the training set of the model for which it can give predictions for new compounds with a reliability as determined in the validation. The limits of the training set are expressed by parameters characterising the physico-chemical, structural or biological space of the model. The development of statistical and mathematical methods for defining applicability domains is an active field of current research /9/.
Validation	Validation is a trial of the model performance for a set of substances independent of the training set, but within the domain of the model. The model predictions for these substances are compared with measured endpoints for the substances in order to establish the sensitivity and specificity and overall accuracy of the model.

Annex 2. Analysis of positive predictions of cancer classification

LeadScope is a predictive data-mining tool for exploring and filtering data sets based on both structural features and associated data[1]. This software contains a predefined library of over 27,000 chemical functional groups (medicinal chemistry building blocks), which can be applied in the analysis of structural similarities within data sets. Structural similarities may lead to logical paths linking chemical structures with a biological endpoint.

In this example, structural similarities associated with (Q)SAR predictions used for the advisory classifications for cancer were analysed based on a large data set to try and gain further insight into the predictions.

A random set of 21,000 chemicals from the full set of around 185,000 chemicals in the DK (Q)SAR prediction database was imported into LeadScope. The size of the set, which was chosen for practical and technical reasons, is judged to be representative of the full database. The cancer predictions made in the four Multicase FDA cancer models[2] for carcinogenicity to male and female Mice and Rats, respectively, were entered as the overall call made by the so-called FDA ICSAS methodology[3]. Also entered were predictions from the Multicase Ames mutagenicity model (described in 3.2.2), and an overall prediction of *in vivo* genotoxicity[4] based on five Multicase models for *in vivo* genotoxicity endpoints (Drosophila SLRL, mutations in Mouse micronucleus, dominant lethal mutations in rodents, sister chromatid exchange in mouse bone marrow, and COMET assay in mouse).

The 21,000 chemicals were organized into groups based on structural features according to the LeadScope library of chemical functional groups. This first rough structural grouping in LeadScope is shown in figure 1. The groups are coloured based on the cancer predictions from the FDA cancer models.

Groups with over-representation of positive predictions have red bars, groups with over-representation of equivocal predictions or predictions, which are out of the applicability domain, have grey bars, and groups with over-representation of negative predictions have green bars. Interpretation of colours is indicated in the bottom right corner.

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[3] Positive according to the FDA ICSAS methodology corresponds to two or more positive cancer calls, accepting only predictions for chemicals without significant deactivating fragments. See footnote 2 for reference.

[4] The criteria for the overall call for genotoxicity is the one used for advisory classifications and described in 3.1 Mutagenicity; positive experimental test result in at least one training set or positive predictions in at least two models.

The length of the bars indicates the number of chemicals (plotted on a log scale). For each group there are a number of more narrowly defined subgroups, named clusters, which may have different distributions of positives, negatives and "out-of-domain" chemicals.
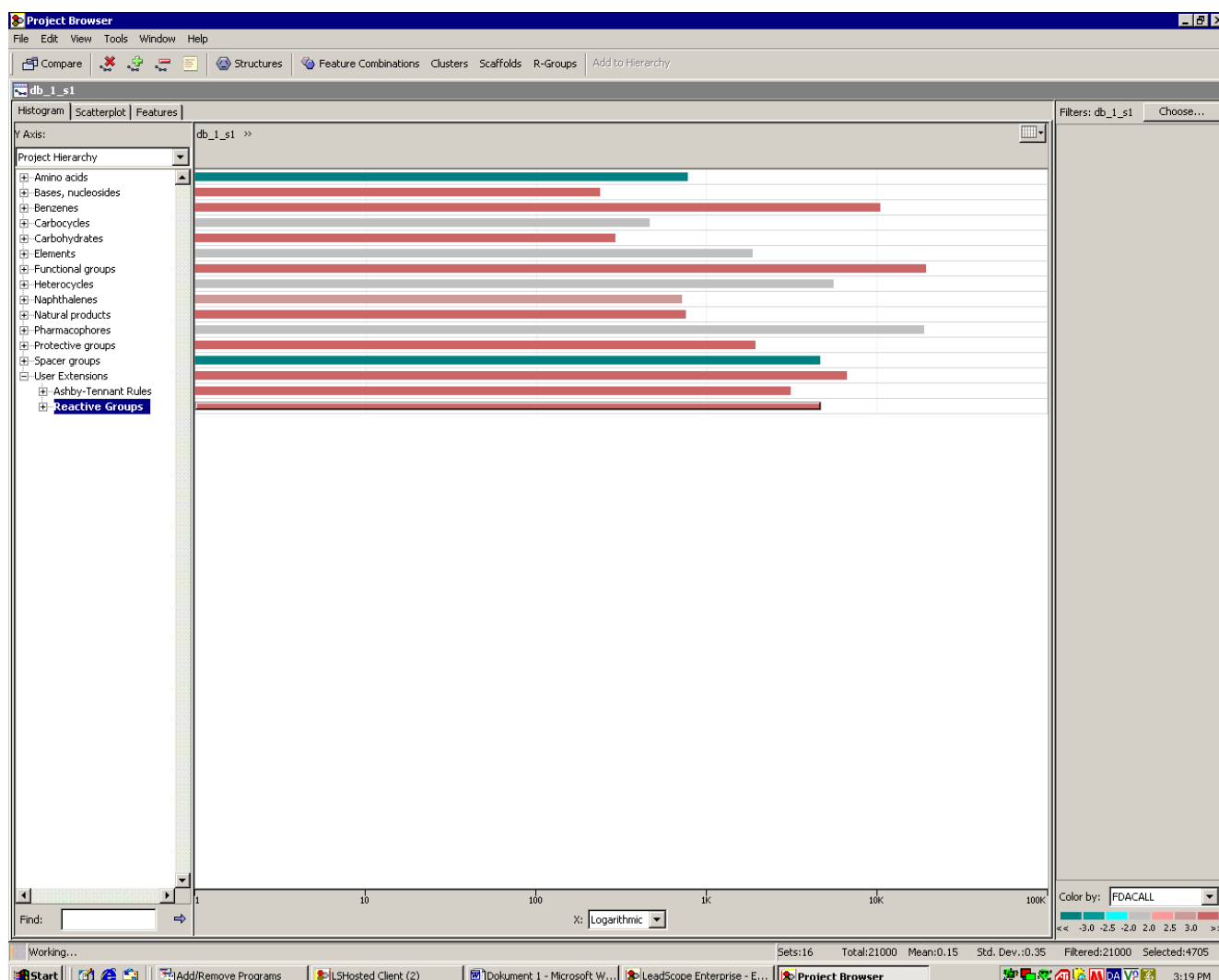

Figure 1. First rough structural grouping in LeadScope of the 21,000 chemicals with FDA ICSAS cancer calls

Out of the 21,000 chemicals, 4,705 chemicals were assigned to the group "reactive groups" by LeadScope. This group is marked with blue in figure 1, and was selected for further analysis in this annex.

Identification of a group of genotoxic carcinogens
Within the "reactive groups" LeadScope made a number of chemical clusters.
Figure 2 gives the first part of a list of these clusters, and again clusters with
over-representation of positive cancer calls are shown in red. Further down
the list are further out-of-domain clusters (grey) and negative clusters (green).

In the leftside of figure 2, the cluster numbered "90" is highlighted in blue.
This cluster is in red colour and contains 24 chemicals.
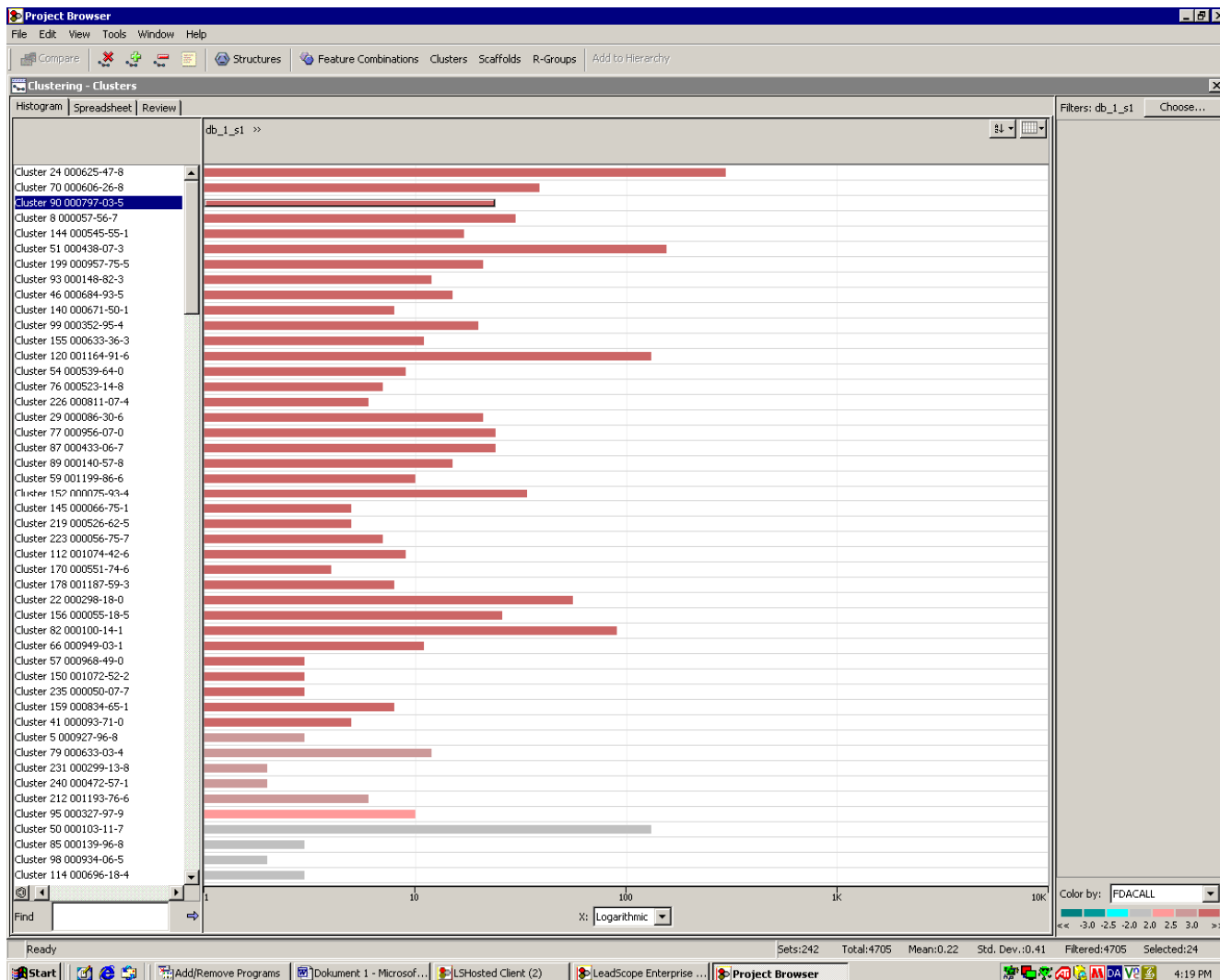
Figure 2. Cluster 90 with positive predictions for cancer

49

The first 20 chemicals in cluster 90 are given in figure 3. The FDA predictions of cancer are given for each chemical in the upper left corner. FDACALL of "1.0" means positive cancer prediction.
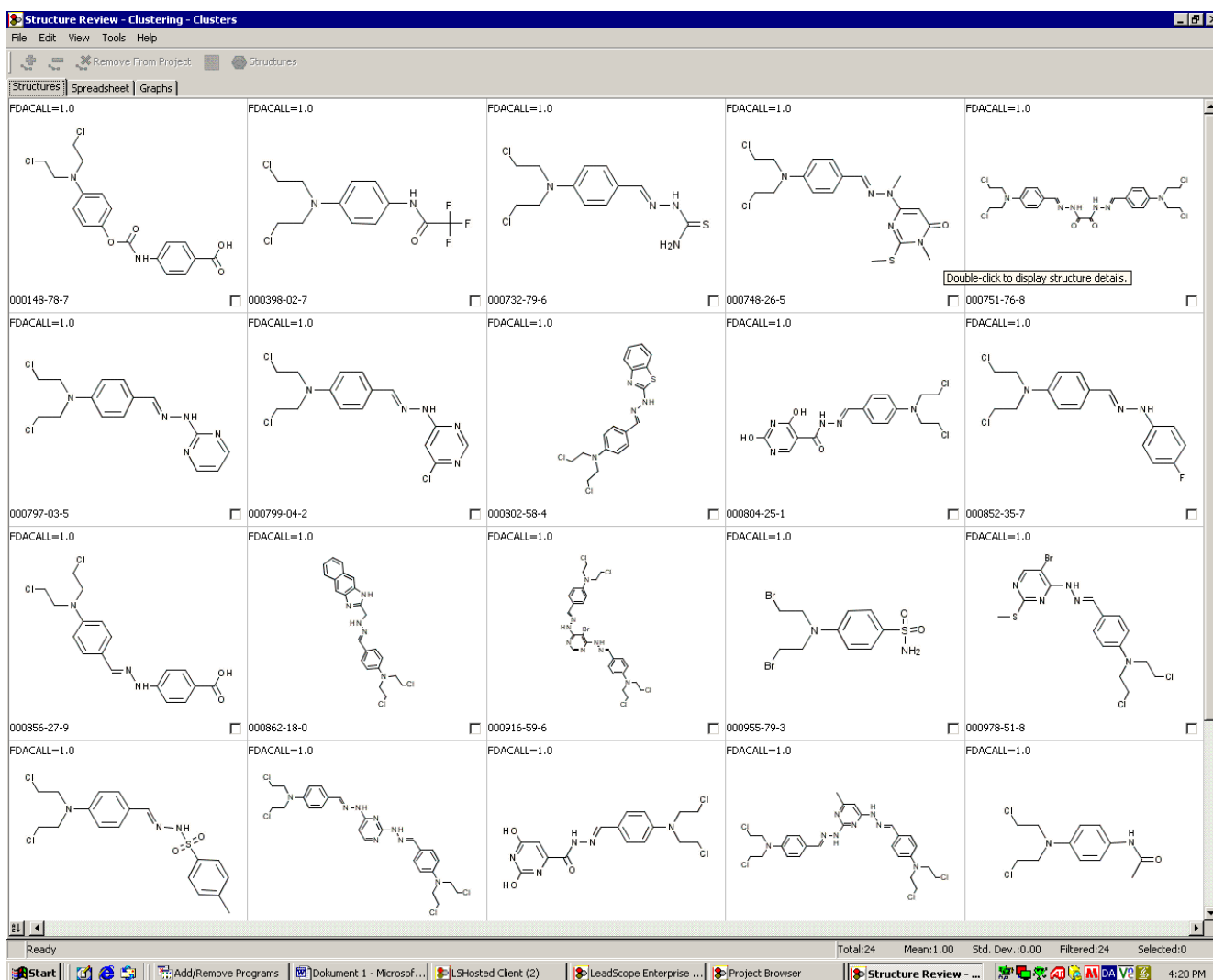
Figure 3. Chemicals in cluster 90

From figure 4 it can be seen that all 24 chemicals in cluster 90 are predicted positive for both cancer (yellow column to the left) and for Ames mutagenicity (yellow column to the right). The chemicals in cluster 90 appear on this basis to be genotoxic carcinogens.
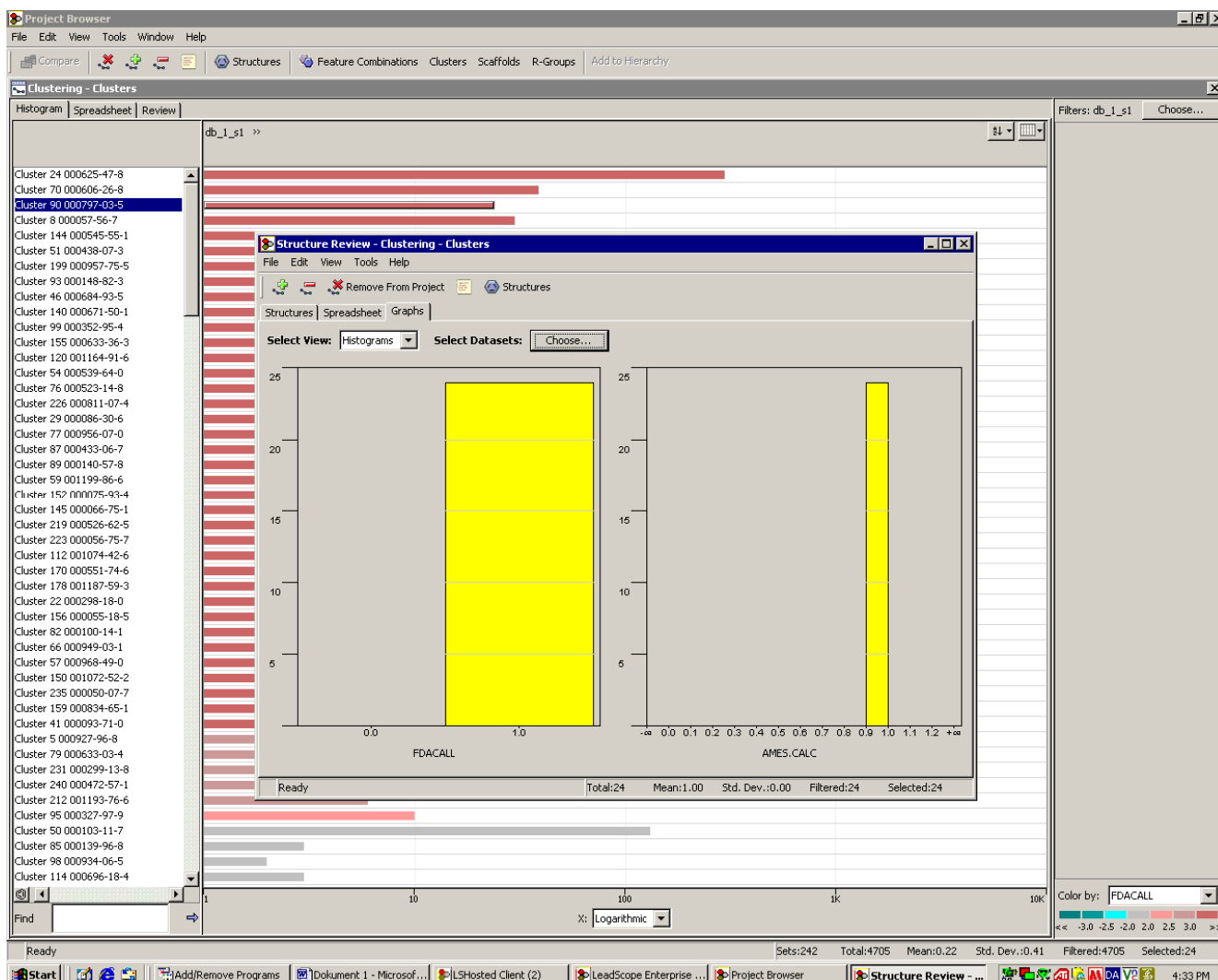
Figure 4. FDA cancer predictions and Ames mutagenicity predictions for chemicals in cluster 90

Identification and mechanistic profile of a group of steroidal carcinogens

If we go back to the clusters within the "reactive groups" and instead of cluster 90, choose cluster 51, we find a very different group of chemicals. In the left side of figure 2, cluster number 51 is highlighted in blue. This cluster contains 156 chemicals, with over-representation of positive cancer predictions as can be seen from the red colour of the bar.
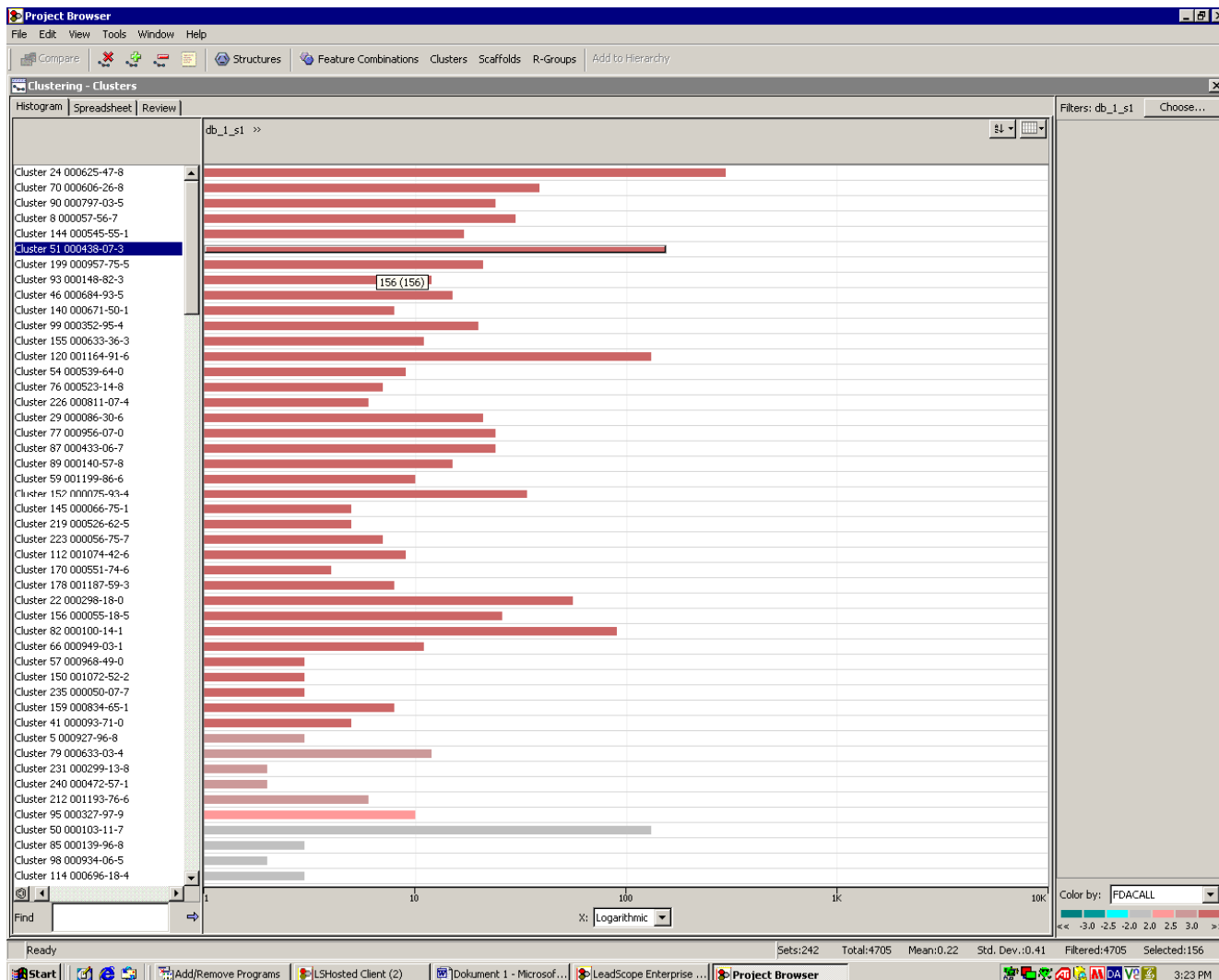
Figure 5. Clusters within the "Reactive groups" with cluster 51 highlighted (left)

Cluster 51 is composed of steroids that are likely to be promoters of cancer. The first of the 156 chemical structures are given in figure 6.
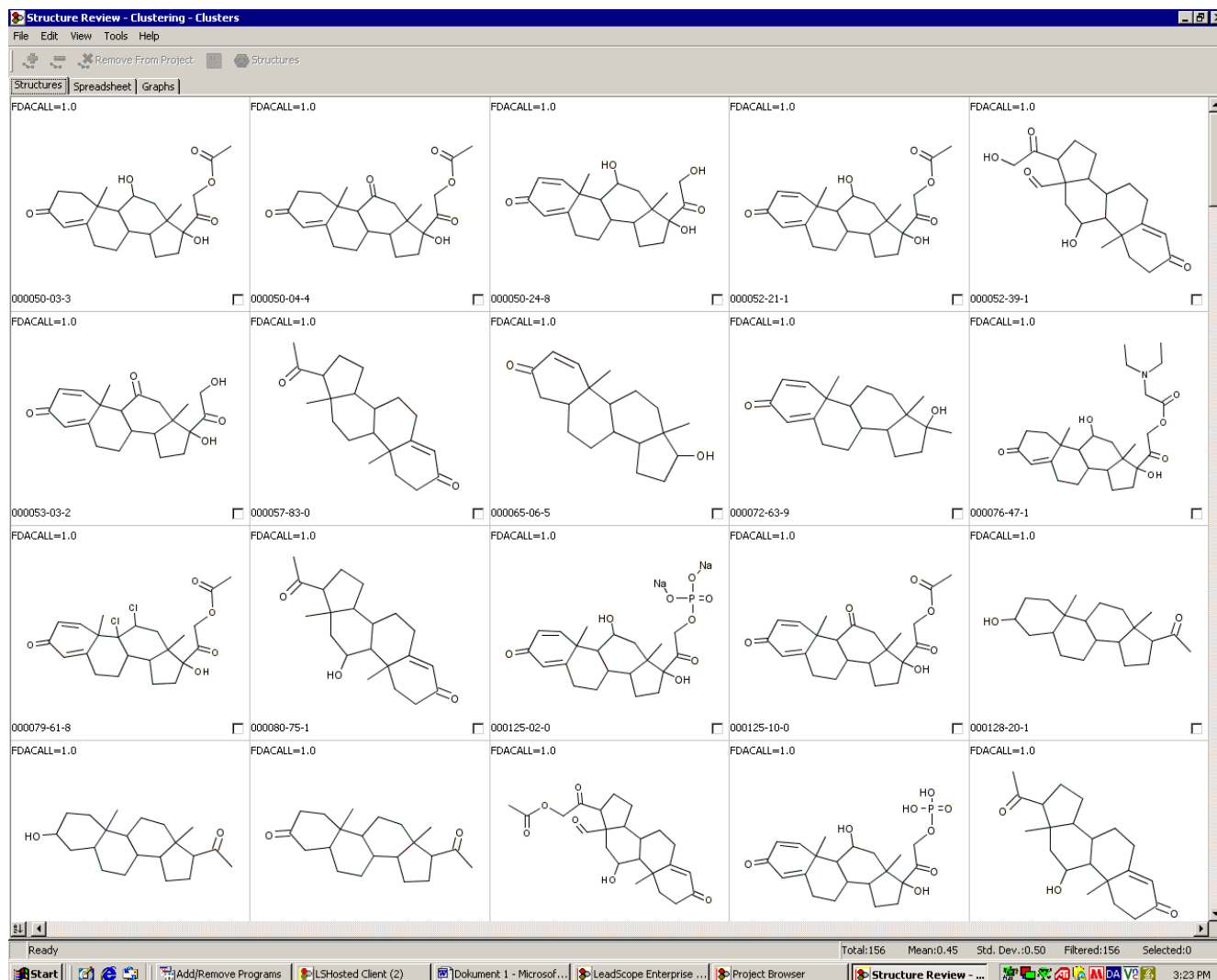
Figure 6. Chemicals in cluster 51; steroids which are likely to be promoters

In figure 7, the distribution of cluster 51 chemicals with positive and negative cancer predictions, Ames mutagenicity predictions and *in vivo* mutagenicity predictions is graphed. "0.0" are the negatives and "1.0" are the positives. Approximately half of the chemicals in cluster 51 are predicted positive for carcinogenicity as can be seen from the graph in the upper left part of figure 6. Almost all chemicals are predicted negative in the Ames model (upper right part), and all chemicals are predicted negative for *in vivo* genotoxicity (lower left part).

I.e. according to the model predictions from models for cancer and genotoxicity, some of the chemicals in this steroid cluster are carcinogens, but probably with a non-genotoxic mechanism. It is well-known that some steroids can cause cancer through a hormonal non-genotoxic mechanism[5].
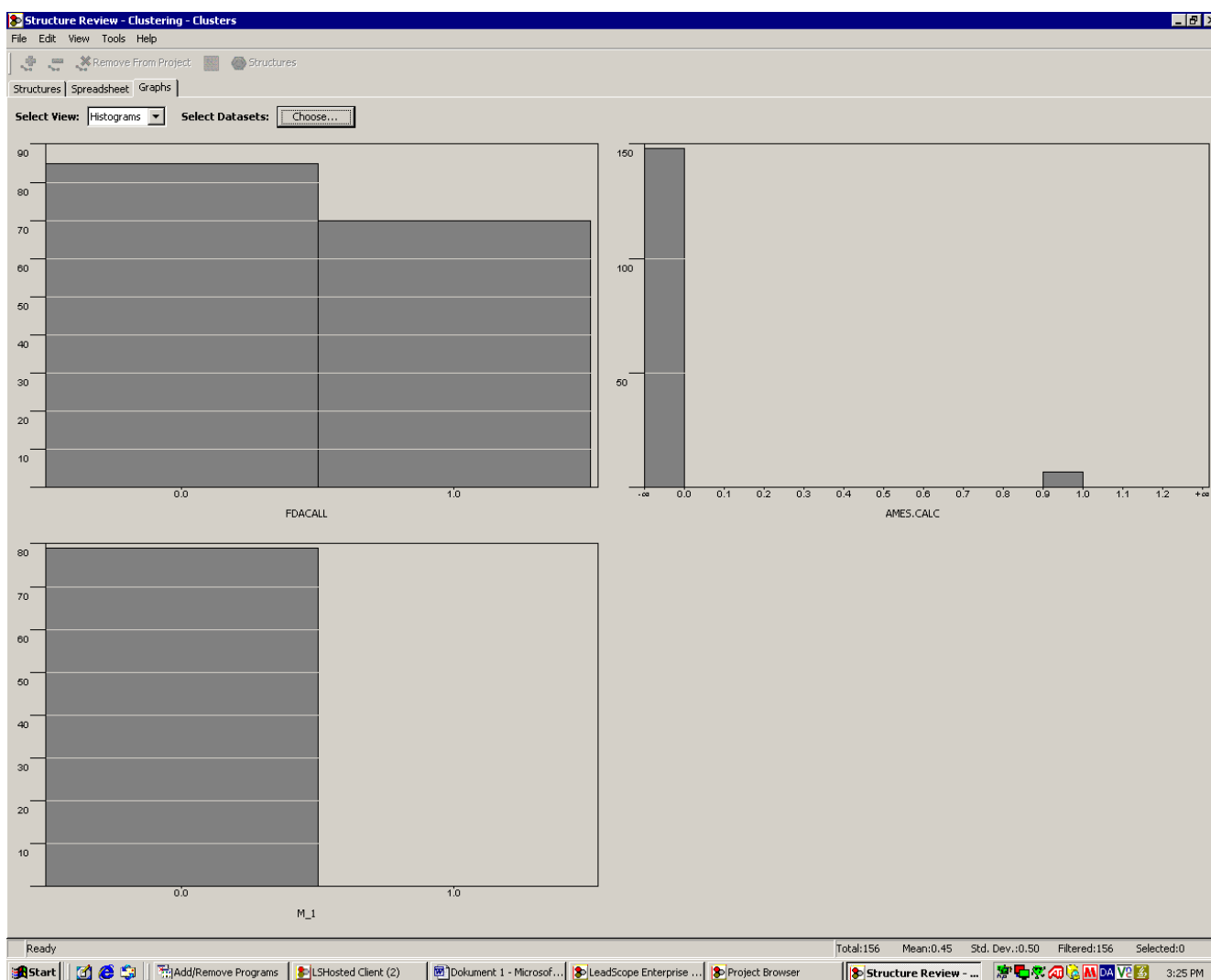
Figure 7. Distribution of cancer predictions (FDACALL), Ames mutagenicity (AMESCALC) predictions and in vivo mutagenicity (M_1) predictions in cluster 51

[5] E.g. Lima, B.S., Van der Laan, J.W.; "Mechanisms of Nongenotoxic Carcinogenesis and Assessment of the Human Hazard", Reg. Tox. and Pharm. (2000) 32, 135-143.

The picture of a non-genotoxic mechanism is confirmed in figure 8 and 9, where the chemicals predicted to be negative (figure 8) and positive (figure 9), respectively, for Ames mutagenicity are highlighted in yellow. Both the predicted Ames positive and negative chemicals are evenly distributed between the chemicals predicted positive and negative for cancer, i.e. there's no significant relation between Ames positive and positive cancer predictions, this confirms that the chemicals in cluster 51 are not likely to be carcinogenic by a genotoxic mechanism.
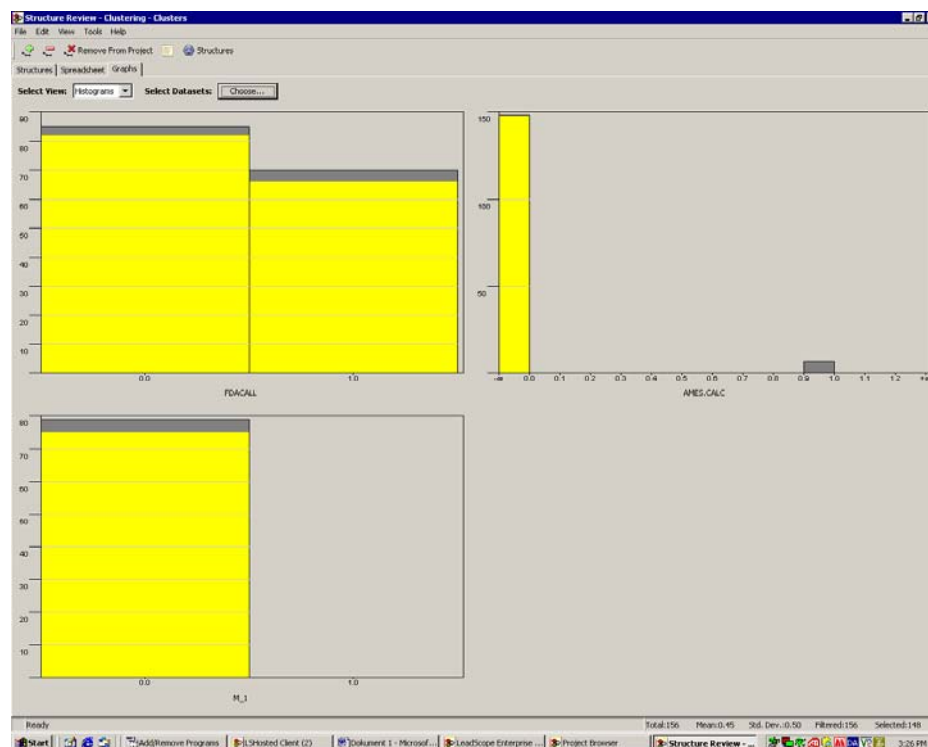
Figure 8. Distribution of Ames negatives among the carcinogenicity and in vivo mutagenicity predictions
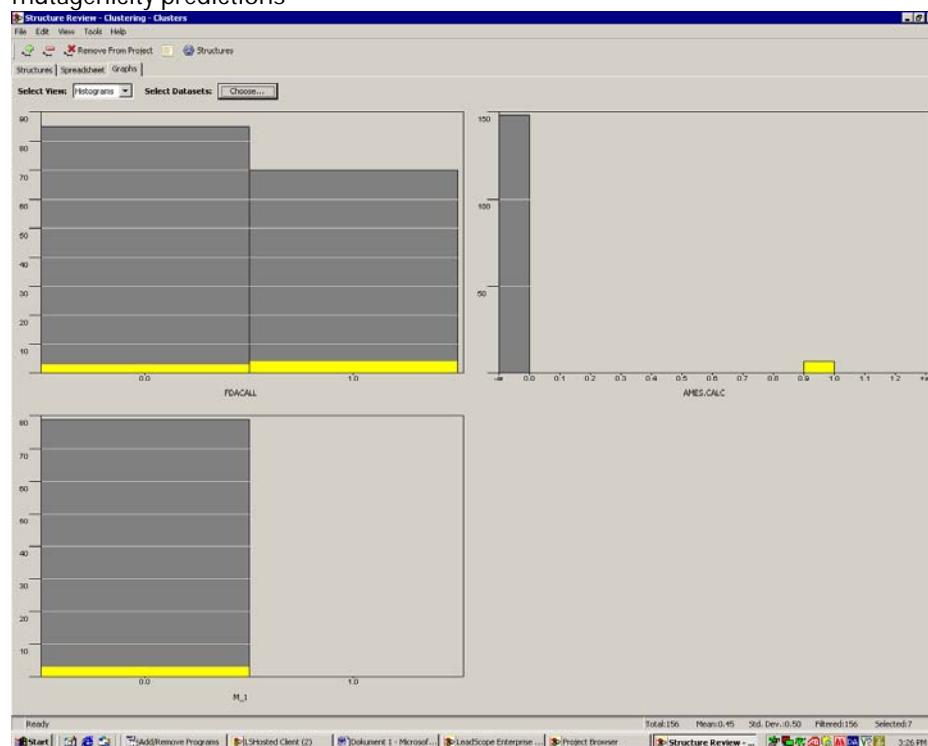
Figure 9. Distribution of Ames positives among the carcinogenicity and in vivo mutagenicity predictions

The seven chemicals predicted to be positive for Ames mutagenicity are shown in figure 10. All of them contain additional reactive fragments such as the diketone, the hydroperoxy group, and the strained 3-member ring (epoxide). By inspection the chemicals look like potential genotoxic compounds by electrophilic mechanisms, not because of the steroid part of the structures but rather because of the additional reactive fragments.
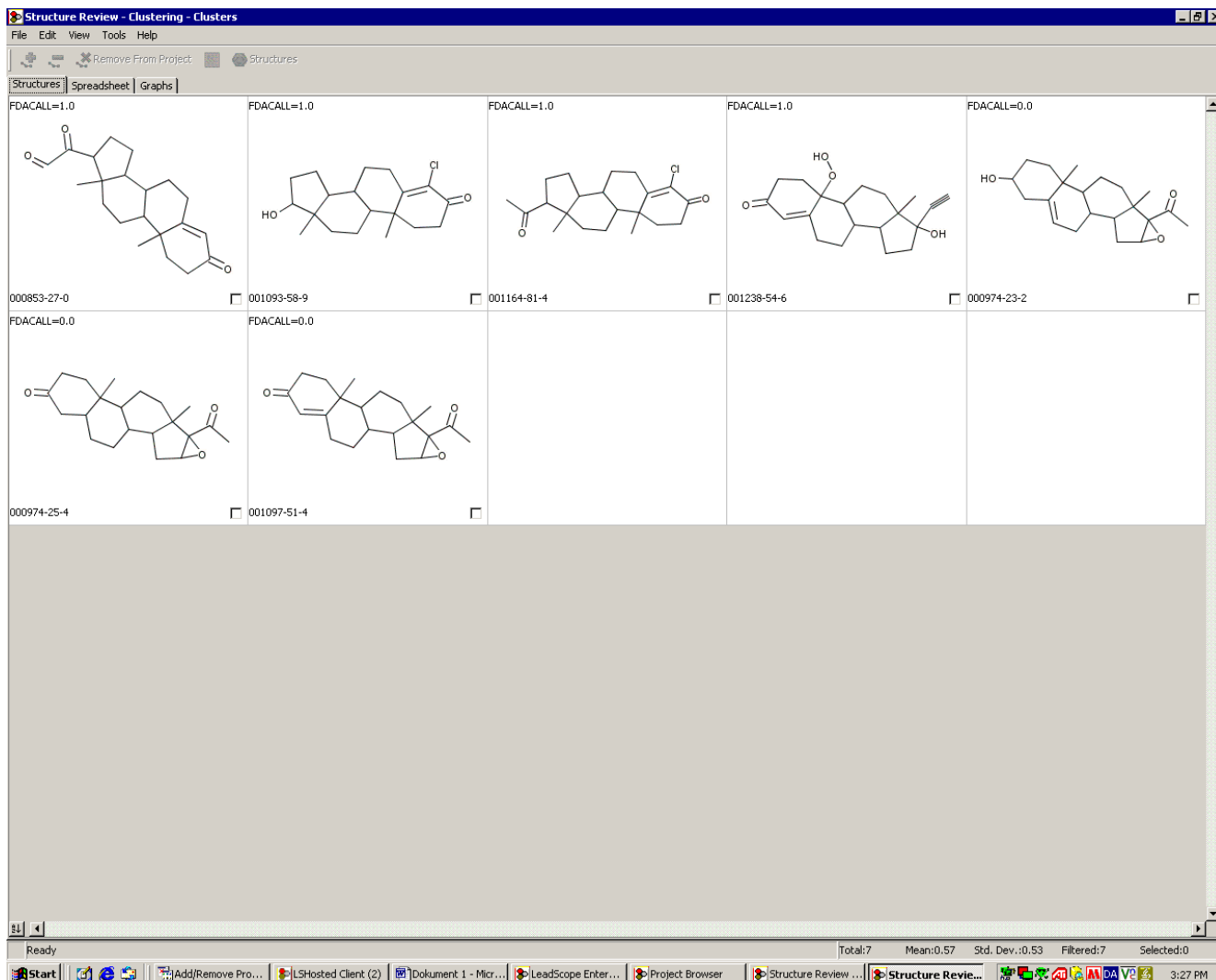
Figure 10. The seven steroid chemicals predicted positive for Ames mutagenicity

Structural identifiers for carcinogenicity of steroids
In the following, LeadScope was asked to find rules about chemical feature combinations that can be used to discriminate between positive and negative cancer predictions within the cluster of 156 steroid chemicals.

Figure 11 shows the generated fragment combination tree. The interpretation of the colours of the boxes is given in the bottom right corner; red box again means over-representation of chemicals with positive cancer predictions, green boxes means over-representation of non-cancer predictions, etc.
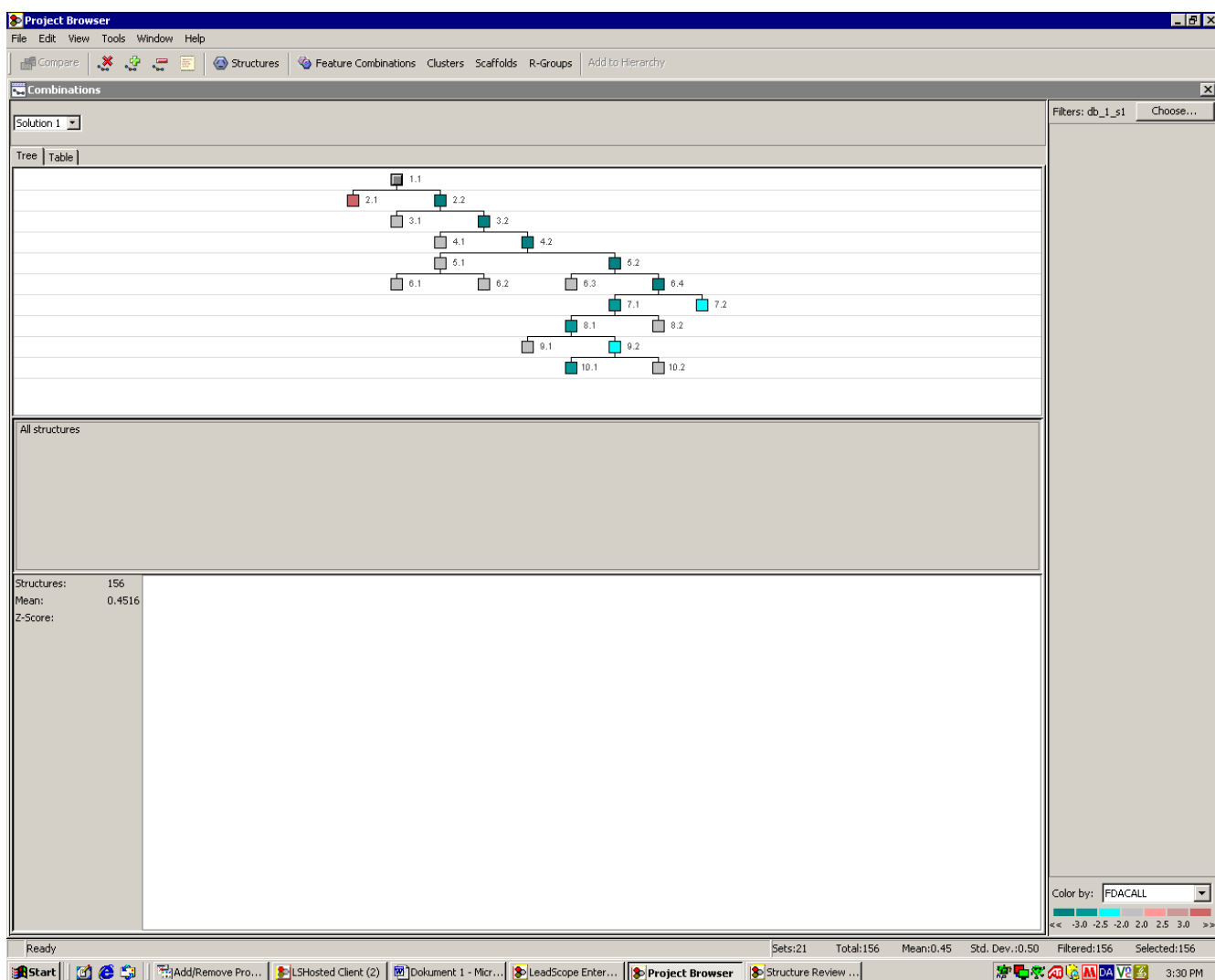
Figure 11. A fragment combination tree within the steroids (red means over-representation of positive cancer predictions)

In figure 12, the red box is marked and the rules leading to classification into this box appears in the bottom windows. As it appears, a positive prediction in the steroid cluster is associated with the 17-hydroxy-steroid skeleton (lower left window) and an unsaturated ketone ring (lower right window). There are 18 chemicals in the selected box.

Figure 12. A positive prediction is associated with the 17-hydroxy-steroid skeleton (left) and an unsaturated ketone ring, cyclohexenone, (right)

The 18 chemicals in the red box are given in figure 13. The highlighted part
of the structure is the combination of the two structural features; the steroid
fragment and the unsaturated ketone ring. The cancer predictions, FDA calls
"1.0", "0.0" or "?", for cancer are shown in the upper left corner for each
chemical. 14 of the 18 chemicals are predicted positive for cancer, 3 are
predicted negative and 1 is equivocal/out-of-domain. In other words, this
simple rule, i.e. a combination of a 17-hydroxy-steroid skeleton and an
unsaturated ketone ring, has a discrimination of 14:3 (not including the out-
of-domain prediction) for predicting whether a chemical is predicted to be
carcinogenic by the Multicase FDA cancer models. In other words, based on
the 17 chemicals with robust cancer predictions, this rule has a Positive
Predictive Value (PPV) of 14*100%/17=82%.
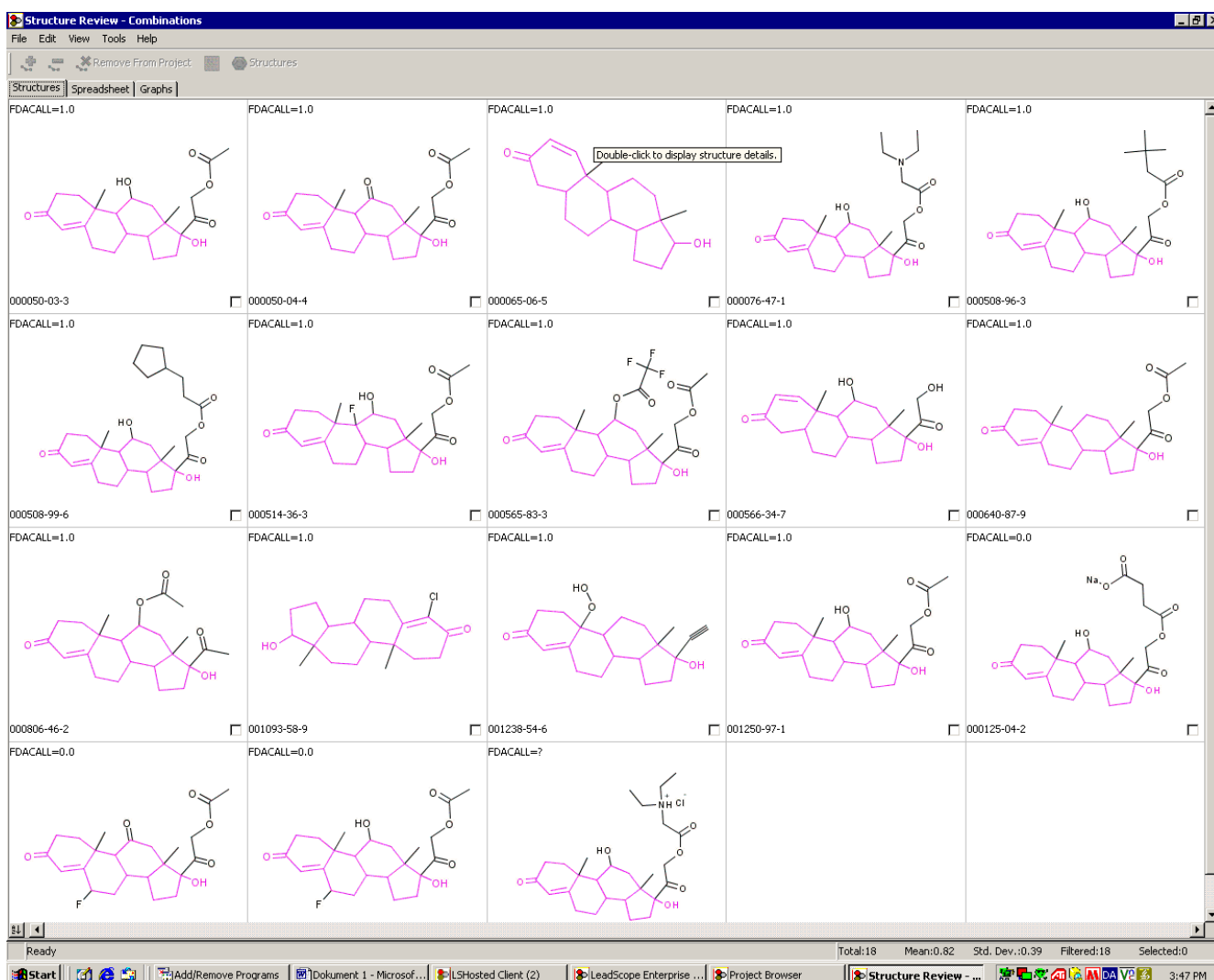
Figure 13. Overlay of steroids containing the two structural combinations

<u>Characterizing non-carcinogenic steroids</u>
Of the remaining 138 chemicals in cluster 51, 132 were predicted negative for cancer. Some of these are shown in figure 11. This gives the rule of structure combinations of steroid skeleton plus cyclohexenone a discrimination of 132:6 for predicting whether a chemical is <u>not</u> predicted to be carcinogenic by the Multicase FDA cancer models. In other words, based on the 138 chemicals, this rule has a Negative Predictive Value (NPV) of 132*100%/138=97%.
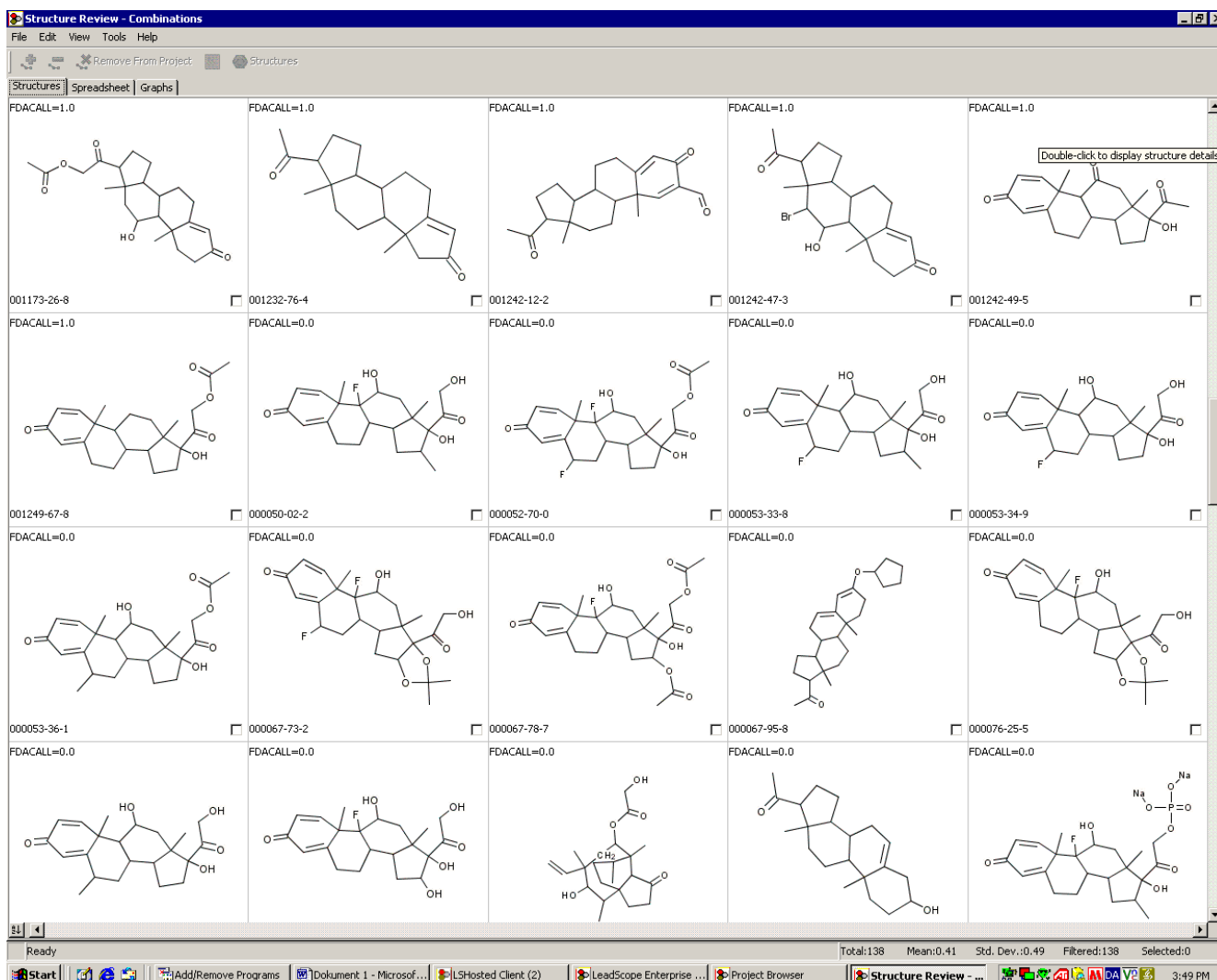
Figure 14. 132 out of the 138 substances are predicted negative for cancer

LeadScope also identified another rule for discrimination between positive and negative FDA cancer predictions as shown in figure 15 (red box). This rule combines a distance between two hydrogen bond acceptors (HBA) and a cyclohexenone fragment. 6 chemicals had this structure combination, of which 4 were predicted positive for cancer according to the Multicase FDA cancer models. This gives a discrimination for positives of 4:2, or in other words, based on the 6 chemicals, this rule has a Positive Predictive Value (PPV) of 4*100%/6=67%.
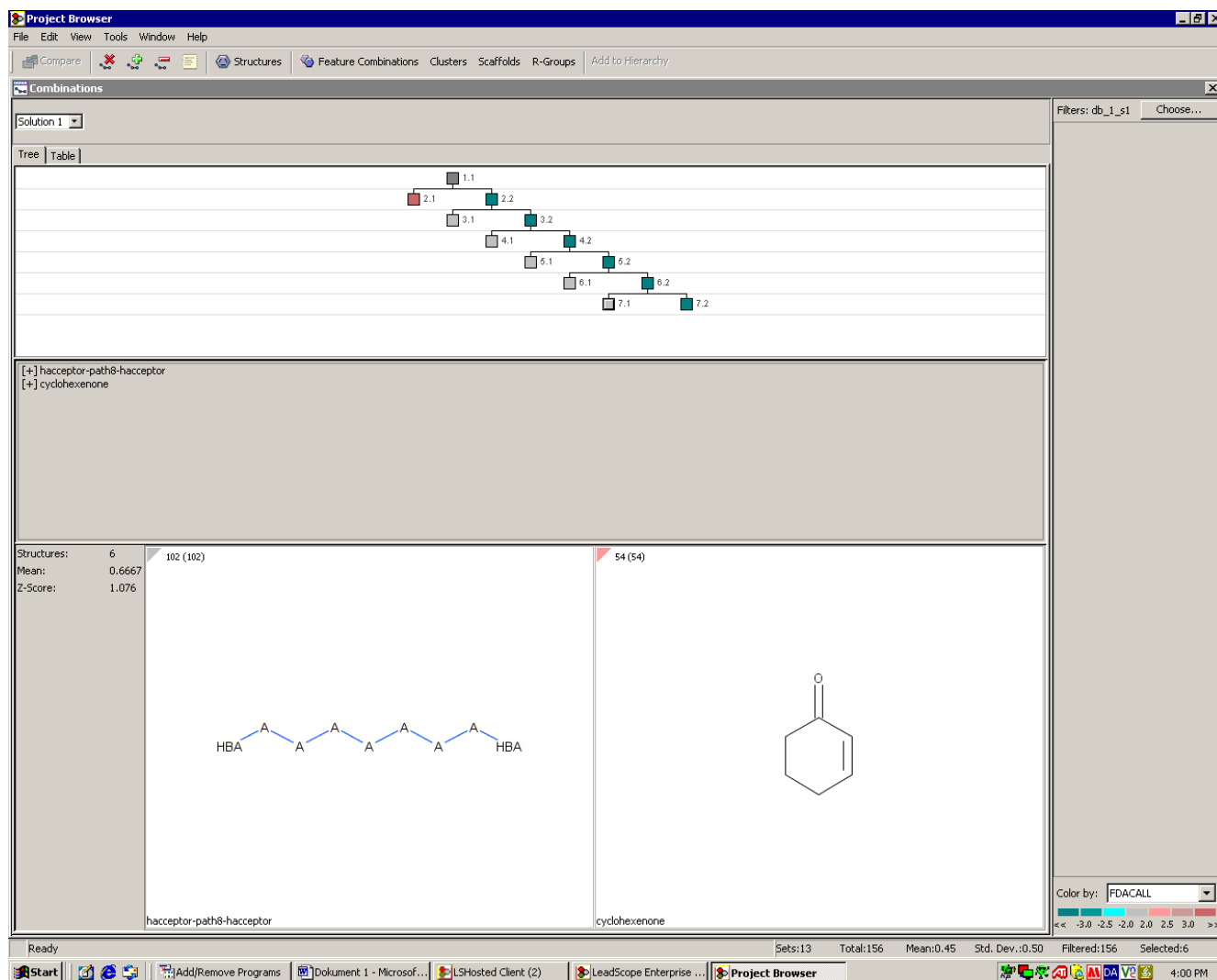
Figure 15. Another feature combination (6 structures) within cluster 51

The 6 chemicals are given in figure 16, with the FDA cancer calls in the upper left corner for each chemical.

Figure 16. 4 of the 6 structures are predicted positive for cancer