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Are the results of customary methods for analyzing dioxin and dioxin-like compound congener profiles court-proof?

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ABSTRACT

The congener profile of samples contaminated with dioxin and dioxin-like compounds allows identifying sources of contamination. This article studies the statistical methods of congener profile analysis reported in the literature with respect to the reliability of obtained results. The performance of customary analysis methods regarding raw data transformation and applied TEF (toxic equivalency factor) values is discussed. In particular, the method of principal component analysis and *k*-means cluster is taken as an example and examined in detail. Reasons for occurring inconsistencies such as the dependence of results on raw data transformation and the disregard of measurement uncertainty are described, and it is shown that they also explain inconsistencies in other methods of cluster analysis such as hierarchical cluster analysis and neural networks. It is concluded that these methods cannot be employed to reach court-proof decisions, i.e. decisions which meet court evidentiary standards. An alternative approach to analyzing congener profiles based on mathematical statistics is briefly presented, allowing reliable, court-proof decisions.

1. Introduction

Dioxin and dioxin-like compounds (DLC) represent a severe risk to human health even at very low concentrations due to their high ability to accumulate in the organism and generate persistent effects such as carcinogenicity or teratogenicity. The majority of human DLC intake is from food of animal origin, e.g. meat, dairy products and fish. Contamination of food is the result of contaminated feed and, in particular for wildlife fish, from contaminated waters. Hence, food quality assurance necessitates the control of waters [1–3], soil [4], food [5–7] and feed [6,8].

DLC contaminated samples contain a mixture of dioxin and dioxin-like congeners, exhibiting a broad range of toxicity and bioaccumulation. To assess the risk to human health originating from a contaminated sample, the sum of toxic equivalents (TEQ) of 17 hazardous dioxin and furan congeners is determined [9], as first proposed by Eadon et al. [10]. Analyzed contaminated samples may vary in the concentration ratios of the congeners, i.e. they exhibit different congener profiles. The congener profile allows to identify sources of contamination and to draw conclusions about human exposure. However, the customary methods to analyze congener profiles based on multivariate statistics and neural networks, such as principal component analysis (PCA) and diverse clustering methods can only offer hints which cannot be verified due to lack of proof or evidence. Therefore the question arises whether results from clustering methods allow valid and court-proof decisions. In order to provide valid, court-proof conclusions, the basis of a method to analyze congener profiles should incorporate analytical uncertainty prior to the application of statistical inference.

2. Results and discussion

2.1. The effect of raw data transformation on the results of congener profile analysis

In the literature congener profiles are compared using PCA [11,12] and clustering algorithms such as hierarchical cluster analysis [4], *k*-means cluster or neural networks (e.g. Kohonen maps) [2,3,13,14]. All these methods include an initial transformation of raw data, i.e. the concentration of each congener from each sample is transformed before analysis. For example, the original congener concentration can be transformed into the ratio of the congener TEQ to the overall TEQ of the sample. Upon closer examination, it becomes clear that commonly used transformations are not uniformly used throughout the literature (e.g. compare [14] and [13]) and can only be justified phenomenologically.

In the present study we observe widely varying results from clustering analysis, which are due to different data transformations and to variations in the applied toxic equivalency factors (TEFs). This feature is examined in detail for PCA and *k*-means cluster.

Fig. 1 and Table 1 summarize the raw data of 16 samples from data of a study on sediment of a river. Using methods based on

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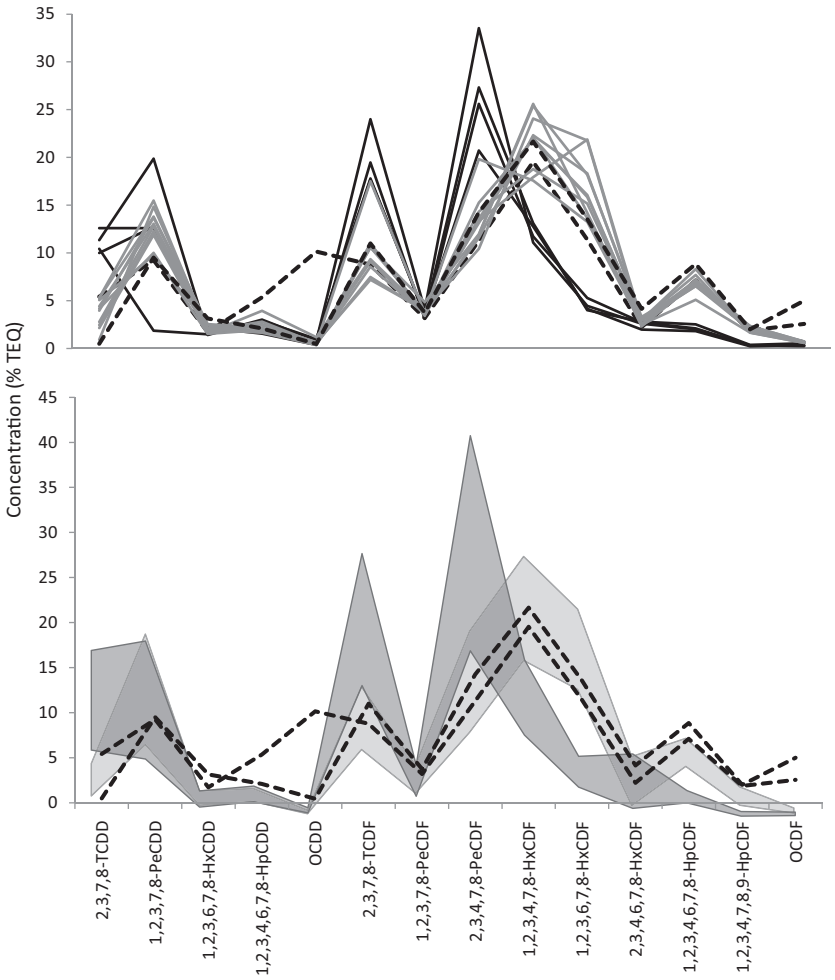

Fig. 1. *Upper panel*: Congener profiles (data transformed into relative TEQ applying the actual WHO TEF from 2005 [23]) of 16 samples from a study on sediment of a river. The statistically significant allocation of each sample to one of the two clusters is indicated by different lines (black solid, gray solid), the two outlier samples are depicted as black dashed lines. *Lower panel*: For each cluster the congener profile (relative TEQ) and the respective uncertainty range is shown, outlier samples are depicted as black dashed lines.

statistical inference we were able to allocate the 16 samples into two sub-populations regarding their congener profile and to identify two outlier samples (combined in a third sub-population). The obtained sample allocation is quite reasonable as it separates samples of downstream and upstream parts of the river into different sub-populations. The two outlier samples can be explained by the differing method of sampling (out of an helicopter) for the sample 324/I and by the geographical position of sample 678/II at the estuary mouth. By calculating the uncertainty range of the respective congener profile for each subpopulation and by determining the homogeneity within each sub-population, we are able to ensure a statistically significant sample allocation (see [15]). This approach was conducted by applying the web service hosted at [16].

In contrast to our congener profile analysis based on statistical inference, Table 2 presents the results of congener profile analysis by means of PCA and clustering using *k*-means cluster exemplary illustrated for one transformation in Fig. 2. PCA and *k*-means clustering were performed using the statistical computing language and environment R (version 2.12.2). PCA itself is not a clustering method but it provides plots (e.g. loading plots) which serve as a survey of the data and can possibly exhibit cluster structures. The allocation of samples into sub-populations is achieved by *k*-means cluster (number of clusters = 3) after reducing the dimensions of the dataset by PCA and the respective sample allocation is indicated in Table 2. Different results of these methods are obtained through application of different raw data transformations and of different TEFs. Moreover, it should be noted, that repeated application of the *k*-means cluster algorithm does not necessarily result in equal sample allocation to sub-populations due to the initial random choice of the cluster centers as a first step of the algorithm. The method of congener profile analysis based on statistical inference allows statistically verified allocation of samples to sub-populations and identification of outliers, whereas the congener profile analysis based on PCA and *k*-means cluster involves a distance determination disregarding any underlying uncertainty. The decision about the assignment of two samples, e.g. two points in a loading-plot obtained by PCA (similar to Fig. 2), to the same sub-population is based on the distance of the corresponding points in the plot. If their distance is small, it means that their transformed raw data is very similar and in consequence they are regarded as belonging to the same sub-population. Hence it immediately follows that the reason for different appearances of plots obtained by different raw data transformations is the fact that actually the transformed data is compared and not the raw data. However, for evaluation of one plot the same "ruler" is used to measure the distances between all points, implying that transformed data of all samples exhibit equal absolute errors. This assumption does not hold true for real data irrespective of the applied transformation, a fact reflected by the shape of the uncertainty ranges in Fig. 1 which

Table 1
Raw data of 16 sediment samples of a river and TEF values [23,25,26]: figures labeled with * were replaced by the half of the respective detection limit as measured figures were not available (below detection limit). The congeners 1,2,3,4,7,8-HxCDD, 1,2,3,7,8,9-HxCDD and 1,2,3,7,8,9-HxCDF were not considered as too many measurements were below the detection limit. Abbreviations are: TCDD, tetrachlorodibenzodioxin; PeCDD, pentachlorodibenzodioxin; HxCDD, hexachlorodibenzodioxin; HpCDD, heptachlorodibenzodioxin; OCDD, octachlorodibenzodioxin; TCDF, tetrachlorodibenzofurans; PeCDF, pentachlorodibenzofurans; HxCDF, hexachlorodibenzofurans; HpCDF, heptachlorodibenzofurans; OCDF, octachlorodibenzofurans.

	Congener												
Sample (kilometer marker/No.)	2,3,7,8-TCDD	1,2,3,7,8-PeCDD	1,2,3,6,7,8-HxCDD	1,2,3,4,6,7,8-HpCDD	OCDD	2,3,7,8-TCDF	1,2,3,7,8-PeCDF	2,3,4,7,8-PeCDF	1,2,3,4,7,8-HxCDF	1,2,3,6,7,8-HxCDF	2,3,4,6,7,8-HxCDF	1,2,3,4,6,7,8-HpCDF	1,2,3,4,7,8,9-HpCDF	OCDF
1/I	1.1	1.4	2	27.3	347	19.4	13.1	10	14.4	4.4	3.1	27.8	4.2	201
1/II	3	3	5.2	37.3	310	46.3	24.8	20.3	27.9	12.6	6.5	49.8	7.2	221
172/I	1.2	2.1	1.5	30.6	354	18.8	12.6	7.3	13.5	4.4	2.1	19.2	2.5	129
172/II	1.4	0.25*	2	40.7	364	32.2	18.3	15	14.9	6	3.5	26.9	4.4	119
324/I	1.7	2.9	5.4	168	10600	27.5	33.4	11.8	61.2	35.7	7	223	59.3	2680
324/II	1.8	4.3	6.6	83.6	751	75.2	53.9	28.4	75.5	57	10.8	219	72.4	841
465/I	1.2	5.5	7	170	1690	40.2	55.7	16.6	96	78.8	12.8	281	83.5	1050
465/II	2	6.9	10.4	98.4	678	43.1	60.6	25.6	110	80.9	14.6	329	119	1210
475/I	1.2	6.6	9	139	1240	50.6	66.7	22	141	101	18.3	373	124	1290
475/II	3.6	6.9	17.3	125	876	77.6	84.5	31.5	127	155	20.9	502	157	1610
605/I	1.6	8	11.8	164	1380	46.9	87.1	22.7	157	142	16.7	469	153	1600
605/II	2.5	7.4	11.3	94.4	786	60.2	73.3	25.7	125	90	17.6	376	119	1330
629/I	0.1*	1.6	2.3	26.4	216	11.4	17.5	4.3	27.6	15.3	2.5	74.2	20.7	248
629/II	1	2.6	4.8	41	295	20.7	21.5	9	35.4	28.6	5.7	145	31.1	377
678/I	1.2	3.5	4.1	63.8	406	16.9	29.4	9.9	50	30.9	6.8	188	38.3	540
678/II	0.1*	1.9	6.3	42.2	314	22	25.1	9.5	43.3	27.1	8.3	177	40	3340
TEF EPA (1987)	1	0.5	0.04	0.001	0	0.1	0.1	0.1	0.01	0.01	0.01	0.001	0.001	0
NATO I-TEF (1988)	1	0.5	0.1	0.01	0.001	0.1	0.05	0.5	0.1	0.1	0.1	0.01	0.01	0.001
TEF WHO (2005)	1	1	0.1	0.01	0.0003	0.1	0.03	0.3	0.1	0.1	0.1	0.01	0.01	0.0003

are neither equally wide for each congener of one sub-population profile nor equally wide for a certain congener throughout all sub-populations. Hence the absolute errors in the transformed data are inconsistent among congeners of one sample and between samples. Consequently the two samples cannot be evaluated in one and the same plot by the same "ruler".

As a second example illustrating the high performance of congener profile analysis based on statistical inference, a much larger dataset of food fish from three different regions was analyzed and clustered. In this example, the second region is geographically located between region 1 and 3. The results of the sample allocation using statistical inference are juxtaposed to sample allocations resulting from PCA and *k*-means cluster in Table 3 by giving the fraction of fish samples from a certain region in each cluster. Obviously, sample allocation based on statistical inference results in a self-explanatory and reasonable regional clustering separating the regions 1 (cluster 1 and 5) and region 3 (cluster 2) and allocating fish samples from the region in between those two throughout clusters containing a small amount of samples from region 1 and 3 as well. In contrast to that, regional affiliation in clusters obtained by PCA and *k*-means cluster appears rather random, i.e. the congener profile clustering does not reflect the geographical origin of the sample.

The conclusions of the different methods of congener profile analysis are considerably different as summarized in Tables 2 and 3. The method based on statistical inference identifies outliers and sub-populations, whereas none of the PCA and *k*-means cluster analyses allow equal conclusions as obtained by statistical inference. We conclude that although PCA and *k*-means cluster is

Fig. 2. Exemplary results of PCA and *k*-means clustering: The PCA is applied to the 16 samples resulting in a loading plot. The statistical significant allocation of a sample to a cluster is indicated by the respective marker (black circles and gray circles for the two clusters, black open squares for outliers). The sample allocation to sub-populations obtained by *k*-means cluster is indicated by surrounding ellipses (cluster 1: gray dashed line, cluster 2: gray dotted line, cluster three: gray solid line). The plot presents the data transformation according to Hagenmaier et al. [24] calculating the congener TEQ relative to the sum of TEQ of the corresponding homologous group in a sample. This transformation is highly dependent on the applied TEF values due to the non-uniform treatment of congeners. Data is shown for the current TEF values according to the WHO from 2005 [23]. Depending on the TEF values applied, different appearances of the loading plots and different clustering by *k*-means cluster are obtained (not shown). Especially for the often applied transformation shown in the plot a decision of division in gray circle and black circle sub-population is not possible. On the contrary, a pseudo-cluster containing 14 out of 16 samples is formed.

Table 2
Sample allocation of 16 samples of river sediment into sub-populations obtained by different methods of congener profile analysis: sub-populations are indicated by numbers, outliers are indicated by "O". Raw data transformation was applied as follows: for sample allocation based on statistical inference the raw data was transformed into congener TEQ relative to the overall TEQ of the sample, the same transformation applies for A, whereas for B the absolute congener TEQ was used. C, D, and E present data transformations according to Hagenmaier et al. [24] calculating the congener TEQ relative to the sum of TEQ of the corresponding homologous group in a sample. This transformation is highly dependent on the applied TEF values due to the non-uniform treatment of congeners. The applied TEF values are: current TEF values according to WHO from 2005 [23] (C), NATO I-TEF from 1988 [25] (D) and values according to US EPA (1987) [26] (E).

	Sample (kilometer marker/No.)															
	1/I	1/II	172/I	172/II	324/I	324/II	465/I	465/II	475/I	475/II	605/I	605/II	629/I	629/II	678/I	678/II
Statistical inference	1	1	1	1	O	2	2	2	2	2	2	2	2	2	2	O
***k*-means cluster algorithm**																
A	1	1	1	1	3	3	2	3	2	3	2	3	2	3	3	3
B	3	1	3	1	3	1	2	2	2	2	2	2	3	3	3	3
C	1	1	1	2	3	1	1	1	1	1	1	1	1	1	1	1
D	1	1	1	2	3	1	1	1	1	1	1	1	1	1	1	1
E	1	1	1	1	3	3	2	2	2	3	2	3	2	3	2	2

based on the same set of raw data, the data transformation leads to considerable differences in the resulting assignation of sub-populations. Furthermore pseudo clusters for samples exhibiting the same analytical systematic error can occur as can be seen in Fig. 2 and Table 2 (transformation C and D) where only one narrow cluster including 14 out of 16 samples and two outliers were found.

Similar effects as described for PCA and *k*-means cluster were observed for other clustering algorithms as well. Every cluster analysis algorithm requires a distance measure to evaluate the similarity of objects with respect to the feature of interest (in our case the congener profile). A prominent example for distance measures is the Euclidian distance which, again, implies certain properties regarding the absolute errors of transformed data. Thereby the choice of the distance measure in combination with the choice of raw data transformation notably affects the results of the clustering algorithms, which even for the same set of transformed data does not necessarily yield in equal results.

2.2. The relation between measurement uncertainty and validity of decisions

The term validity used with respect to a decision reached on the basis of data analysis describes the confidence, i.e. the probability of the correctness of the decision. The contrary probability of error is closely related to the measurement uncertainty of the underlying data as illustrated in Fig. 3 for five hypothetical samples. The black points may represent the measured concentrations of a certain congener. The upper half in Fig. 3 displays a situation of low measurement uncertainty where the true value of each measurement is expected to lie in a narrow range around the measured value (dashed and dotted black lines). The solid gray line is the sum of all these five ranges and represents the probability of finding any true value. The division of data into two sub-populations (dashed and dotted) is statistically significant as there exists an area in between the two sub-populations where the probability to find any true value is very low (solid gray line). In that case the decision to define two sub-populations is valid. In the situation of high measurement uncertainty (lower half) the ranges of the expected true values of each measurement are broad resulting in a considerable overlap of dashed and dotted ranges. The same decision to divide the samples into the two sub-populations (dashed and dotted) is statistically not significant because there is no area in between the two sub-populations where a true value is expected with only a low probability (solid gray line). This demonstrates that a statistically significant division of a population into sub-population requires considering the underlying analytical uncertainty of the data.

Table 3
Sample allocation into sub-populations obtained by different methods of congener profile analysis for food fish data from three different regions. Raw data transformation was applied according to descriptions for Table 2. The sample allocation is given as fraction of all fish samples originating from a region (1, 2 or 3), e.g. for clustering using statistical inference exclusively 100% of all fish from region 3 are allocated to cluster 2 and more than 90% of all fish from region 1 are exclusively allocated to cluster 1 and 5. A comparable result dividing geographically separated samples into clusters was not achieved by any of the PCA and *k*-means clustering approaches.

		Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5
Statistical Inference						
	Region 1	77%	5%	0%	3%	15%
	Region 2	0%	40%	20%	40%	0%
	Region 3	0%	100%	0%	0%	0%
PCA and *k*-means algorithm						
	Region 1	0%	44%	0%	0%	56%
A	Region 2	20%	30%	0%	30%	20%
	Region 3	29%	6%	6%	41%	18%
	Region 1	0%	5%	15%	46%	33%
B	Region 2	20%	30%	0%	10%	40%
	Region 3	12%	65%	0%	0%	24%
	Region 1	5%	15%	46%	33%	0%
C	Region 2	30%	0%	10%	40%	20%
	Region 3	65%	0%	0%	24%	12%
	Region 1	0%	5%	33%	46%	15%
D	Region 2	20%	30%	40%	10%	0%
	Region 3	12%	65%	24%	0%	0%
	Region 1	0%	8%	41%	28%	23%
E	Region 2	30%	30%	0%	30%	10%
	Region 3	29%	41%	0%	18%	12%

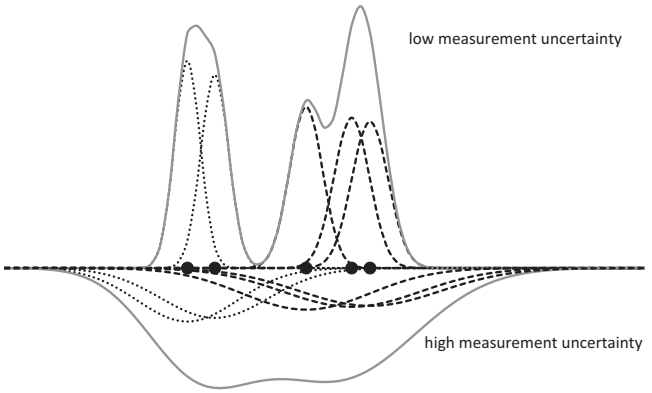

Fig. 3. Illustration of the relation between validity of a cluster analysis and measurement uncertainty. The black points represent measured values, the dashed and dotted black lines illustrate the ranges where the true value is expected to lie: in case of low measurement uncertainty these ranges are narrow (upper half), in case of high measurement uncertainty these range are broad resulting in a considerable overlap of dashed and dotted ranges. The solid gray line is the sum of all five ranges and represents the probability of finding any true value. The division of data into two sub-populations (dashed and dotted) is statistically significant for the case presented in the upper half as there exists an area in between the two sub-populations where the probability to find any true value is very low (solid gray line), whereas it is not statistically significant in the situation of high measurement uncertainty (lower half) because there is no area in between the two sub-populations where a true value is expected with only a low probability (solid gray line).

Customary methods of analysis of congener profiles do not take any data related to measurement uncertainty into account. In terms of the example in Fig. 3 this means that only transformed data represented as black points are obtained, but not the corresponding ranges of uncertainty (dashed and dotted black lines) which would allow evaluation of the validity of the decision to divide the samples into two sub-populations by means of the solid gray line. These analysis methods can therefore be termed exploratory. They are useful to develop and formulate hypotheses, they can describe relations and may lead to first explanations but they are not suitable to test or confirm a hypothesis in contrast to methods of statistical inference. Methods of statistical inference provide information about the probability of error, the measurement uncertainty and the significance. Thus they allow verification of conclusions and thereby court-proof decisions.

We want to point out that there are a number of clustering algorithms yielding probabilities of affiliation to a cluster for each object instead of the assignment of each object to exactly one cluster. (An excellent review on a number of customary methods used for food pattern recognition can be found in [17].) These probabilities are based on the transformed data and the applied distance measure whereas the actual measurement uncertainty is again disregarded. Thus, the obtained affiliation probabilities must not be confused with the probability of error based on the measurement uncertainty.

The analysis of congener profile can only lead to an impartial allocation of the samples to sub-populations if the analytical measurement uncertainty is considered, i.e. only samples exhibiting congener profiles whose differences are statistically significant with regard to the analytical measurement uncertainty are assigned to different sub-populations. This connection between validity of a decision and measurement uncertainty is actually the reason why determination of the measurement uncertainty of a certain analysis method is an essential requirement for accredited test laboratories [18].

3. Conclusions

The analysis of congener profiles of DLC contaminated samples and the subsequent assignation of samples into sub-populations is used to identify sources of contamination and human exposure origins. The sample allocations resulting from analysis methods based on multivariate statistics or neural networks are highly dependent on the applied raw data transformation and TEF values. Moreover, none of the reviewed analysis methods takes the measurement uncertainty into consideration and hence the probability of error and the significance cannot be determined. Accordingly it is not possible to evaluate the validity of cluster assignation. Resulting decisions regarding sample allocation to different clusters should therefore be seen as assumptions and not as valid, court-proof results.

We want to emphasize the fact that the reviewed methods do not meet the requirements stated in the international standard ISO/IEC 17025 [18] for they do not consider measurement uncertainty.

In order to provide valid, court-proof conclusions, the basis of a method to analyze congener profiles has to be statistical inference and include an analytically sound model of uncertainty. The method described in [15] fulfills this requirement and allows statistically significant statements regarding the sources or origins of congeners, e.g. the maximal percentage of contamination in the sediment at a certain location which originates from a defined area.

The application of methods based on statistical inference is not only meaningful to analyze congener profiles of DLC contaminated samples, but these mathematical methods should also be applied to similar profile or pattern analysis methods. For example, rather than using PCA, methods according to Stachel et al. [15] should be used in the investigation of human exposure to polybrominated diphenyl ether (PBDE) congeners according to She et al. [11], for fly ash analysis [19], and for food analysis [17,20–22].

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