Using molecular markers and PCB analysis to infer the origin of the otter (*Lutra lutra*) found on the Knardijk, The Netherlands, in 1998

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Abstract: It is presumed that the otter (*Lutra lutra)* became extinct in the Netherlands in 1988. Since then much effort has been made to restore the freshwater habitat aiming for a return of this top-predator. In 1998 from the central part of the Netherlands an otter was reported as a traffic casualty. It was not known if this individual was a rudiment of the original Dutch population or if it had escaped from the European otter breeding programme. To infer its origin, samples were taken for PCB and DNA analysis. Microsatellite DNA analysis was used to study 81 otter samples from three populations in Europe and from the breeding programme, including this individual. Cluster analysis indicates that it is unlikely that this otter originated from the Dutch population and it was with high probability assigned to the breeding programme. The levels of PCBs found in the otter were in the same range as found in the last Dutch otters, but were also similar to levels found in a two years old otter that was born and died in captivity. This indicates that PCB levels are not always good indicators for discrimination between captive and wild otters.

Keywords: otter, *Lutra lutra*, DNA, PCBs.

Introduction

Shortly after the 'last' otter (*Lutra lutra*) in the Netherlands had been killed on 14 September 1988 on the motorway A6 between Sneek and Joure in the province of Friesland in the northern part of the country, the species was considered extinct in the Netherlands. Congeners eventual still present would not belong to a viable population any longer (Walter 1989, Lina & van Ommering 1994). This view was in line with the decreasing trend of the annual number of road victims with nearly 20 casualties per year in the beginning of the sixties to only one in 1988 (Nolet & Martens 1989). Besides habitat fragmentation, habitat destruction, drowning in fish fykes and traffic mortality, pollution with PCBs was considered as one of the major causes of this extinction.

Of course, traffic death is not the only cause of death. The assumption that the otter found in 1988 was really the last one was not very well founded. Indeed, since then the presence of one or more otters was mentioned from several places. During the period 1990-1998, for instance, footprints from otters were reported from the southern and central part of the southern province of Limburg (Smit 1991, Winter 1993, Jansen 2000, Backbier & Jansen 2002). The tracks were considered to belong to several ani-

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mals living along the river Meuse and its tributaries, belonging to at least two transboundary populations (Buys et al. 1991, Backbier & Jansen 2002). However, until now no otters found dead were reported and because of this no otter could be examined.

In the northern part of the country, otter prints were found in De Oude Venen in the central part of the province of Friesland during the winter 1992/93 (Dulfer et al. 1993). These were ascribed to a single, migrating animal (Dulfer et al. 1993). From the surroundings of the Paterswoldse Meer, also in the northern part of the country, some fishermen stated that here the otter had always been present as they observed the animals repeatedly. This even became an item in newspapers (Anonymous 2003a). Afterwards, however, the supposed otter tracks photographed by a journalist were found to be prints of an American mink (*Mustela vison*) (Anonymous 2003b).

As between 1988 and 1998 no otter-casualties were reported, it was a great surprise when on the 27th of February 1998 an otter was killed by a truck on the Knardijk, where this dike borders the nature reserve 'Harderbroek' in the polder Oostelijk Flevoland from the adjacent lake Wolderwijd (52°22'N, 05°35'E; Dutch atlas grid co-ordinates: 169-486). In this article this animal will further be referred to as 'Knardijk otter'.

The studbook keeper for the European otter breeding programme reported that in the Netherlands during the previous years two otters had escaped from captivity: a juvenile female and an old male (A. Melissen, personal communication). However, post mortem examination of the Knardijk otter determined that this was an 11-year-old female and therefore it couldn't be one of the reported escapees. The questions was whether this otter could be a descendant from the supposedly extinct original population or whether it was just a wandering individual, probably escaped from captivity, and, in case it was an autochthonous Dutch otter, how its burden of PCBs and other contaminants was. A significant decrease of PCB concentrations in the aquatic environment was expected for the last decade (van Mourik et al. 1995, Winter & Smit 1997), to such an extend, it was also expected that the PCB concentration in otters would stay below the critical level. However, field data had to prove whether this expectation was correct.

To answer these questions samples of muscle, liver and subcutaneous fat were collected for DNA and PCB analysis and the results were compared with those obtained from Dutch otters found in the past and some reference populations in Europe.

Materials and methods

The animal

The otter killed on the Knardijk appeared to be an adult female with a body weight of 6.4 kg. Unfortunately the backbone was damaged. Therefore it was impossible to measure the length of the otter in a reliable way. The tail length however, was 41.5 cm long. The physical condition of the animal was good, as the amount of subcutaneous fat was fair. The teeth were moderately worn. Examination of the number of annual layers in the cement of one of the canines by Dr. Hermann Ansorge (Museum of Natural History, Görlitz, Germany) en Dr. Silke Hauer (Zoological Institute, Martin-Luther-University, Halle-Wittenberg, Germany) showed that the animal was 10 or 11 years old. With respect to its age, this otter indeed could have been a descendant from the 'last' Dutch otters.

The female was not pregnant and the uterus, although rather firm, did not give the impression the animal had been pregnant before. Unfortunately all other organs were so damaged that they could not be measured in a proper way.

Sample collection for genetics

Skin and hair samples from 14 mounted museum otter specimens (dried skins) from the period 1956-1965 were collected. Samples were taken by punching out discs, using a 2 mm thick hollow needle. The samples were stored in Eppendorf tubes at -20 °C. Tissue samples from four of

the last otters in the 1980s were added to this group.

The Czech Otter Foundation Fund in Trebon and the Station for Fauna Protection in Pavlov provided muscle samples from 24 dead Czech otters for use as a reference population. All of these otters were collected in the period 1995-1999 in the Czech Republic. Most animals in this sample were from the south-west of the Czech Republic, close to the German and Austrian borders. From the eastern part of Germany, tissue samples of 30 otters collected between 1991-1995 were provided by the Zoological Institute of the Martin-Luther-University (Halle-Wittenberg). In addition, samples from eight otters from the breeding programme were analysed, including a male from the Dutch Otter Station, called 'Makker'. The latter had died in 1989 and tissue samples had been collected to be analysed for contaminants as well. Some of the otters in the breeding programme from which samples were received were relatives. Two of them were brothers and one family with one cub was included.

DNA was extracted from all samples using a silica-gel based extraction kit (DNeasy Tissue Kit, Qiagen Inc.).

Genetic analysis

From the extracted DNA, five different polymorphic microsatellite loci were amplified, using primers Lut701, Lut715, Lut717, Lut832 and Lut833 (Dallas & Piertney 1998). The amplified microsatellites were then visualised with a LI-COR DNA analyzer (GENE READIR 4200), scored and entered in a spreadsheet. From some samples it was not possible to score all the loci, probably due to low quality and quantity of some of the samples.

From the data analysis it was assumed that the dataset contained one single unknown population. Structure was created in this single population by grouping genetic similar individuals in clusters. This was done by two methods. A distance-based method was used measuring the genetic distance of each individual to each other. To visualise this clustering, the distances were plotted in a Principle Co-ordinates Analysis (PCA) using the programme GenAlEx (Peakall & Smouse 2001). For this analysis 17 samples were excluded because of missing values. In addition, a Bayesian clustering procedure (implemented in the software STRUCTURE; Pritchard et al. 2000) was used to infer the number of distinct genetic populations that were included in the sample set. This Bayesian model assumes K (unknown) populations, each being characterised by the allele frequencies at a set of independent loci. The populations resulting from the assignment test were compared to the geographic populations.

Determination of contaminants

The available sample material (liver and subcutaneous fat) was limited. Trace metals were determined in 0.4 g of liver sample. PCBs and chlorinated pesticides were determined in 1.8 g of subcutaneous fat. Samples were freeze-dried and homogenized in a mortar proceeding to further treatment.

Trace metals were analysed in microwave acid-digested samples with atomic absorption spectrometry (flame-, graphite furnace-, and cold vapor-AAS) according to methods described by Van Hattum et al. (1996).

Standard PCBs 28, 52, 101, 118, 138, 153 and 180 (numbers according the International Union of Pure and Applied Chemistry, IUPAC) and chlorinated pesticides were determined with gas chromatography with electron capture detection (GC-ECD) in hexane-acetone soxhlet extracts, after cleanup over deactivated alumina and fractionation over a deactivated silica column according to Leonards et al. (1997).

The non- and mono-ortho substituted PCBs (usually indicated as coplanar PCBs, IUPAC nrs. 77, 126, 169 and 105, 114, 118, 123, 156, 157, 167, 189 respectively) were determined with gas chromatography with mass spectrometric detection (GC-MSD) in dichloromethane-pentane soxhlet extracts, after cleanup over a deactivated alumina, fractionation over a deactivated silica

column and a second fractionation with high performance liquid chromatography (HPLC) with two pyrenyl columns (Nacalai Tesque, Kyoto, Japan, obtained from Promochem, Wesel, Germany). For the quality assurance of the analytical procedure, internal chlorobiphenyl (CB) standards were applied prior to extraction (CB 103, CB 143, ^{13}C-CB 77, ^{13}C-CB 126) as described in detail in Leonards et al. (1994, 1997). The concentrations of the coplanar PCBs were also expressed as equivalent of 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD) concentrations. Toxic equivalent quality (TEQ) concentrations were calculated by multiplying the toxic equivalent factor (TEF) of Ahlborg et al. (1994) for each non- or mono-ortho CB with the corresponding concentration. For the comparison with published PCB otter data the TEF values of Ahlborg et al. (1994) were applied instead of the most recent published TEF values by Van den Berg et al. (1998). Furthermore, the sum of the individual TEQ concentrations was calculated.

Quality control monitoring of analysis of trace metals, PCBs and chlorinated pesticides, further included analysis of procedural blanks, internal control samples, and certified reference materials, including a dog-fish homogenate (DOLT) for the trace metals, distributed by the National Environmental Research Council (Canada) and a herring oil sample with low concentrations of PCBs distributed by the Swedish Environmental Protection Agency (Solna, Sweden).

Results

Genetic analysis

Figure 1 shows the PCA clustering of the different analysed populations. The X- and Y-axes explain 21% and 14% respectively of the total variation. The cluster of the breeding programme seems to fall outside the group. The Czech and East-German clusters almost completely overlap. Regarding genetic distance, the Knardijk otter is closest to the breeding programme and op-

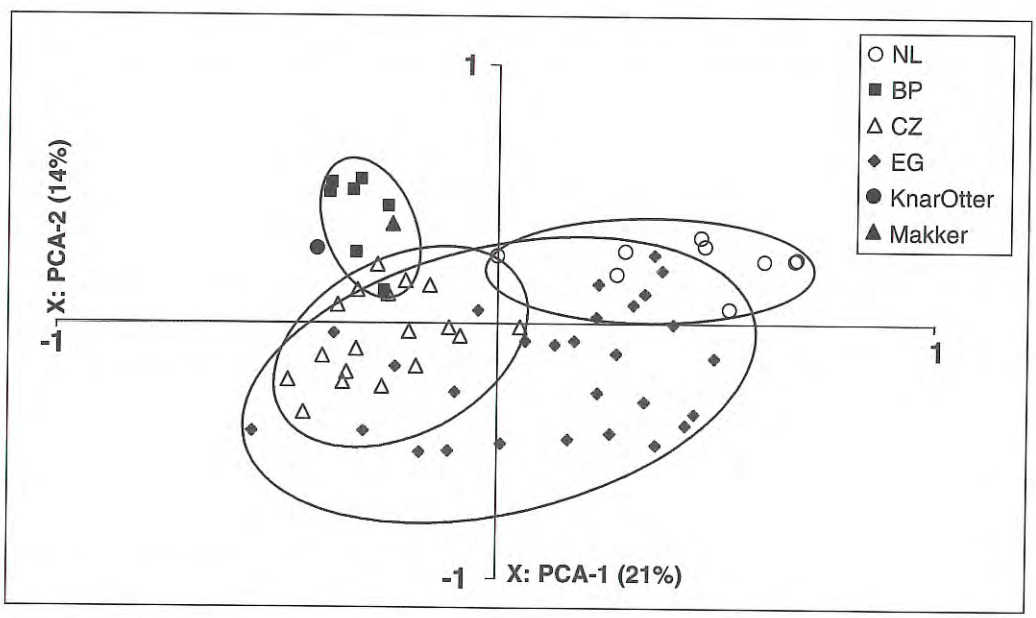

Figure 1. Principle Co-ordinates Analysis (PCA) from the genetic data of the samples, grouped by population. NL = The Netherlands; BP = Breeding Programme; CZ = Czech Republic; EG = East-Germany; KnarOtter = Knardijk otter; Makker = reference breeding programme otter. Ellipses are drawn by hand to facilitate comparison between the populations.

tically least related to the Dutch population cluster. Table 1 (part A) shows that STRUCTURE devised the sample set in three clusters. The Dutch samples are mainly assigned to cluster 2 (88%). Cluster 1 is mainly based on samples from the Czech population (75%) and further a part of the East-German population (36%). Cluster 3 contains mainly samples from the breeding programme (89%). This clustering corresponds with the PCA (figure 1) except for the large fraction of the East German samples that were assigned to the 'Dutch' cluster 2 (62%). Regarding figure 1, the Knardijk otter floats in between the cluster of the breeding programme and the Czech cluster. This slight overlap in clusters can be deduced from table 1 as well; 19% of the Czech samples is assigned to the cluster of the breeding programme. Therefore one might cast doubt on the origin of the Knardijk otter. Fortunately STRUCTURE determines the probability that an individual sample is assigned to one of the clusters (see table 1, part B). For the Knardijk otter this resulted in a probability of 2% assignment to cluster 1, 1% to cluster 2 and 97% to cluster 3. For breeding programme otter 'Makker' these values were 2% assignment to cluster 1, 2% to cluster 2 and 96% to cluster 3. This probability of 97% assignment of the Knardijk otter to the breeding programme indicates its origin with high probability.

Table 1. Cluster analysis from the genetic data in the pooled otter samples. A: percentage of samples originating from a geographic population that were assigned to a cluster. NL = The Netherlands; BP = Breeding Programme; CZ = Czech Republic; EG = East-Germany. B: assignment probabilities of an individual to the distinguished clusters.

	Cluster 1 (%)	Cluster 2 (%)	Cluster 3 (%)
A: Populations			
NL	9	**88**	3
BP	10	1	**89**
CZ	**75**	6	19
EG	**36**	**62**	2
B: Individuals			
Knardijk otter	2	1	**97**
'Makker'	2	2	**96**

PCBs and other contaminants

Measured concentrations of contaminants in liver and fat of the Knardijk otter found in 1998 are indicated in table 2. As the information from the DNA analysis strongly indicated that the animal was from a captive population (see above), we tried to make a comparison with data on the otter 'Makker', held in captivity at the Otter Station, Groningen, that died in 1989. In table 3 a comparison is made with concentrations observed in the last Dutch otters during the 1980s (Broekhuizen 1989, Leonards et al. 1997), the data for the captive otter 'Makker' (E.M. Dijkman, unpublished data), and those of the Knardijk otter. As can be seen concentrations of PCBs, p,p-DDE, Cd and Pb are all in the same range.

Discussion

Genetic relationship

It can be concluded that the Knardijk otter most likely originates from the breeding programme. Statistically there is hardly any reason for assuming it being an individual dispersed from the Czech or East-German population, not to mention it being a rudiment of the Dutch population. Some of the otters in the breeding programme were relatives, resulting in a relative decrease in genetic variability and therefore a smaller cluster in the PCA. Nevertheless, the Knardijk otter was assigned with 97% probability to this cluster, so the more it is likely that this otter originated from the breeding programme. Since the age and sex of the Knardijk otter do not correspond with any *reported* escapee in the Netherlands, the definite origin of this female remains a mystery.

PCBs and other contaminants

The total PCB concentration in the Knardijk otter (7.6 mg/kg wet weight; 13.4 mg/kg lipid

weight) is in the low range of previously reported values for the last Dutch otters, found dead during the 1980s as reported by Broekhuizen & de Ruiter-Dijkman (1988) and Broekhuizen (1989). This female moreover probably never gave birth and by that did not have the opportunity to get rid of part of the PCB burden by means of the young or by lactation. The pattern of the standard PCBs, however, was similar to previous observations for Dutch and Danish otters (Smit et al. 1998). Compared to previously proposed critical levels for total PCBs in the Netherlands of 10-25 mg/kg lipid weight (based on observed levels in European populations, see summary in Smit et al. 1998) the observed value indicates potential risks.

Table 2. Measured concentrations of contaminants in liver (trace metals) and subcutaneous fat (PCBs and chlorinated pesticides) of the Knardijk otter. Dry weight: 27%, lipid weight: 57%.

Contaminants	µg/kg lipid weight	TEQ µg/kg lipid weight
Standard PCBs		
PCB 28	10	
PCB 52	9	
PCB 101	47	
PCB 118	1070	0.11
PCB 153	5439	
PCB 138	2561	
PCB 180	3246	0.03
Mono-ortho PCBs		
PCB 123		<0.04
PCB 114	6	
PCB 105	421	0.04
PCB 167	88	0.001
PCB 156	439	0.22
PCB 157	42	0.02
PCB 189	<0.04	<0.0001
Non-ortho PCBs		
PCB 77	0.1	0.0001
PCB 126	1.9	0.19
PCB 169	0.8	0.01
Sum 7 PCBs	12382	
Sum 17 PCBs	13380	
Sum TEQ		0.62
Chlorinated pesticides		
Hexachlorobenzene	175	
p,p-DDE	561	
Heptachlorepoxide	<5	
Dieldrin	<5	
Trace metals	mg/kg dry weight	
Hg	1.90	
Cd	0.41	
Pb	<0.2	
Cu	18	
Zn	82	
Cr	<0.37	
Ni	<1.9	

Based on dioxin equivalent concentrations the observed level of 0.62 µg TEQ/kg (lipid weight) in the Knardijk otter is below the levels observed in the last Dutch otters during the '80s (Leonards et al. 1997). Because of the equilibrium in concentration between organs and tissues on lipid weight basis (Leonards et al. 1994), concentrations in fat of the Knardijk otter were converted to liver concentrations on lipid weight. Compared to the dose-effect relationship between exposure to planar PCBs (expressed as dioxin equivalents in liver) and hepatic vitamin A deficiency in European otters (Smit et al. 1996, Murk et al. 1998) the observed value in the Knardijk otter is below the safe level (EC_1) of 2 µg TEQ/kg (lipid weight) and the critical level (EC_{50}) of 5 µg TEQ/kg (lipid weight). The pattern of the non-ortho PCBs, which are considered as the most toxic, is similar to the patterns usually observed in liver tissue (Leonards et al. 1997). Especially PCB 126, which usually account for 80% of the total TEQ concentrations in the liver (Leonards et al. 1997), is relatively low in the subcutaneous fat sample of the Knardijk otter (PCB 126/PCB 153 = 0.00035) compared to mean values for Dutch and Danish otters in liver: 0.015 ± 0.007 (n=13). Extrapolating to expected hepatic TEQ concentrations, by corrections of the ratio difference for PCB 126/PCB 153 between liver and fat, this yields a value of 8.7 µg TEQ/kg (lipid weight) in liver, which exceeds the critical level for hepatic vitamin A deficiency. Based on these considerations we assumed that the observed levels were in agreement with expected levels for wild otters in the Netherlands.

Table 3. Comparison of the concentrations PCBs, chlorinated pesticides and heavy metals in the Knardijk otter with those found in previous road casualties during the 1980s and the captive otter 'Makker' [a].

Compound	Units	Knardijk otter (1998)	Road casualties (1984-1988; n=5)	Otter 'Makker'[e] (1989)
PCB 153	mg/kg lipid weight	5	0.8-53	4
Sum 7 PCBs	mg/kg lipid weight	12	2-179	10
Total PCBs	mg/kg lipid weight	13[d]	4-222[b]	21[c]
Sum TEQ	µg/kg lipid weight	0.6	1–35	-
Hexachlorobenzene	mg/kg lipid weight	0.2	0.3-4.7	1.9
p,p-DDE	mg/kg lipid weight	0.6	<0.5-8	0.7
Dieldrin	mg/kg lipid weight	<0.005	<0.5-2	1.1
Trace metals (liver)				
Cd	mg/kg dry weight	0.3	0.4-0.7	0.4
Hg	mg/kg dry weight	–	1.2-2.5	1.9
Pb	mg/kg dry weight	0.2	0.2-0.7	<0.2

[a] Previous measurements in liver, kidney, muscle and fat taken from Broekhuizen (1989) and Leonards et al. (1997); [b] based on 28 congeners; [c] based on 36 congeners; [d] based on 17 congeners; [e] data from E.M. de Ruiter-Dijkman (unpublished).

The implicit hypothesis, that captive otters have much lower concentrations compared to wild otters, clearly is not valid because PCB levels in the captive otter 'Makker' were in the same range as found in wild otters in the Netherlands and in Denmark (Broekhuizen 1989, Leonards et al. 1996, Leonards et al. 1997). Obviously, captive otters can accumulate significant quantities of PCBs from their diet. This is in line with observations of Smit et al. (1996) and Leonards et al. (1997) who analysed biomagnification factors (BMFs) in the food web of the otter. They conclude that the BMFs found for otters (BMF for Sum 7 PCBs for various fish species: 3-209; lipid normalised) belong to the highest values reported in the literature on piscivorous birds or mammals.

Only very scarce data are available on PCB levels in the diet of captive otters. In a feasibility study on the use of caged roach (*Rutilus rutilus*) from a commercial fish culture for active biomonitoring of exposure to PCBs in de Oude Venen, it was found that Sum 7 PCBs levels in 1-3 year old roach (from two companies) ranged from 0.1-4.1 µg/kg lipid weight, which was above levels observed in roach caught in de Oude Venen (0.2–0.6 µg/kg lipid weight) (van Hattum et al. 1992). Using the biomagnification factors reported by Leonards et al. (1997) and Smit et al. (1996) feeding captive otters with fish from commercial fish cultures can easily lead to the levels observed in the captive otter and the otter found dead in 1998.

In summary, the study shows that PCB levels in otters are not always good indicators for discrimination between captive and wild otters.

Conclusions

Molecular techniques have proved to be useful in revealing the origin of an individual animal. Based on the data presented here, we conclude that the Knardijk otter originated from the breeding programme and was not a rudiment of the Dutch population, nor originated from the East-German or Czech otter population.

This study shows again that otters do accumulate PCBs very efficiently. Even otters in captivity fed with supposedly 'clean' fish might contain high levels of PCBs. Therefore it can be concluded that areas with low levels of PCBs are no guarantee for low concentrations in otters.

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Samenvatting

Het gebruik van moleculair genetische techniek en PCB-analyse om de herkomst te ontrafelen van de otter (*Lutra lutra*) die in 1998 op de Knardijk werd gevonden

Op 27 februari 1998 werd op de Knardijk, tussen Oostelijk Flevoland en het Veluwerandmeer Wolderwijd, een doodgereden otter aangetroffen. Het betrof een vrouwelijk exemplaar van circa 11 jaar oud. Omdat er bijna tien jaar verstreken waren nadat de "laatste" otter in Nederland was overreden, rees de vraag of we hier te doen hadden met een nazaat van de oorspronkelijke Nederlandse otterpopulatie, of dat het een uit gevangenschap ontsnapt dier was. Voor zover kon worden nagegaan werd er echter nergens een vrouwelijke otter van deze leeftijd vermist.

Ter vergelijking van het DNA van de Knardijk-otter met dat van oude, deels opgezette otters uit Nederland, van otters uit het voormalige Oost-Duitsland en Tsjechië en van otters die in gevangenschap waren geboren, waren primers voor de amplificatie van vijf verschillende polymorfe microsateliet-loci ter beschikking. Het bleek dat de overeenkomst het grootst was met de in gevangenschap gefokte otters, inclusief de otter 'Makker' uit het fokprogramma van de Stichting Otterstation Nederland, en dat de overeenkomst het kleinst was met de otters afkomstig uit Nederland. Onze conclusie is dat de Knardijk-otter een uit gevangenschap ontsnapt dier is geweest.

Het patroon van de zeven standaard-PCB's in de lever en het onderhuids vet van de Knardijk-otter is gelijk aan dat van zowel vijf Nederlandse otters uit de jaren '80 als van otters uit Denemarken. De concentratie totaal-PCB's in het onderhuidse vet ligt ook binnen de range van waarden van de Nederlandse otters uit de jaren '80, maar is toch relatief laag (13,4 mg/kg vet). Dit geldt temeer daar dit vrouwtje waarschijnlijk nooit jongen had gehad en zodoende een deel van de PCB-belasting kwijt had kunnen raken via jongen en moedermelk. Ook de bijdrage van het zeer giftige PCB-126 is in verhouding laag (PCB 126/PCB 153 = 0,00035), maar toch nog ver boven de voor otters veilig geachte norm.

Doordat zowel de concentraties van het totaal aan PCB's als die van hexachloorbenzeen, p,p-DDE en de zware metalen cadmium, kwik en lood bij zowel 'Makker' als de Knardijk-otter in dezelfde range liggen als bij de eerder onderzochte Nederlandse otters uit de jaren '80, is het niet mogelijk om op grond van die concentraties een idee te krijgen over de herkomst van de Knardijk-otter. Wel geven de concentraties die bij 'Makker' werden gevonden aan dat de veronderstelling, dat een otter in gevangenschap altijd veel lagere concentraties aan contaminanten zouden hebben dan de in het wild levende soortgenoten, niet juist is. Zelfs bij een gevangenschapsdieet kan een otter significante hoeveelheden PCB's accumuleren, die een potentieel risico voor de gezondheid vormen.

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