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# Studying placental transfer of highly purified non-dioxin-like PCBs in two models of the placental barrier

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ABSTRACT

Currently, toxicology and toxicokinetics of purified non-dioxin-like polychlorinated biphenyls (NDL-PCBs) are poorly characterised. Transplacental kinetics of NDL-PCBs can be studied in a variety of models, but careful validation of each model is crucial. We aimed to develop a standard operating procedure for establishing an *in vitro* model of the human placental barrier. Using this model, we sought to investigate placental transport kinetics of two NDL-PCB congeners. Firstly, we compared the BeWo cell line of the American Type Culture Collection with the BeWo b30 clone and determined parameters for monolayer formation. Secondly, we performed placental perfusions to validate the *in vitro* model. To that end, the transport of radiolabelled PCB52 and 180 was investigated in both models.

We were not able to grow the ATCC cell line to confluency, but determined monolayer formation using BeWo b30. A confluent monolayer is present by day 4 post-seeding, transepithelial electrical resistance being 44.65 ± 11.06 Ω $cm^2$ and sodium fluorescein transport being 4.1% ± 0.18. Both measures can be used as indicators for monolayer formation. Results from kinetic studies *in vitro* and *ex vivo* were in excellent agreement. Both NDL-PCBs crossed the placental barrier within 2.5 h. We found PCB180 to transfer more rapidly and PCB52 to associate more with placental tissue. Since transport and association patterns were similar *in vitro* and *ex vivo*, we conclude that the protocol provided here forms the basis for a good model of the placental barrier using BeWo b30. We hypothesise that the observed differences in transport and association patterns of NDL-PCBs may indicate that toxic effects of PCB52 play a more important role regarding placental function, whereas PCB180 may be of greater importance for fetal toxicity.



## 1. Introduction

The developing fetus represents one of the most vulnerable groups of the human population. Therefore, the placenta plays an important role, serving as a protective barrier as well as facilitating the maternal-fetal exchange of vital compounds. The relative permeability of the placenta is of concern regarding potentially toxic agents consumed by the mother either consciously (e.g., drugs, alcohol) or unconsciously (e.g., environmental pollutants). Polychlorinated biphenyls (PCBs) are amongst the world's most dominant environmental pollutants. PCB levels in humans have decreased within the past 30 years [1] due to a production ban imposed in many countries during the 1970s. However, because of their biological persistence and their tendency to bioaccumulate in the food chain, the human population is still exposed to PCBs mainly via animal based foods such as meat, dairy and fish [2,3]. Of the 209 PCB congeners, the non-dioxin-like PCBs (NDL-PCBs) constitute the major proportion found in the environment and human tissue. Several recent epidemiological studies confirm the presence of NDL-PCBs in pregnant women [4–6]. NDL-PCB concentrations have been reported in maternal and cord blood, suggesting placental transfer of these substances [7,8].

Several models are available to investigate placental transport of PCBs in detail. The use of animal models allows the assessment of uptake through different exposure routes, and biokinetics can be determined for adult and fetal organs. However, careful selection of

*Abbreviations:* NDL-PCBs, non-dioxin-like polychlorinated biphenyls; ATCC, American type culture collection; Na-Flu, sodium fluorescein; DAPI, 4′,6-diamidino-2′-phenylindole; TEER, Transepithelial electrical resistance; ps, post-seeding; CPM, Counts per minute; $P_e/P_{app}$, Permeability coefficient/apparent permeability coefficient; FM ratio, Fetal-maternal ratio; DMSO, Dimethyl sulfoxide.

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the model organism is required due to the variability of placental structure among mammals, as well as the length of gestation. Primate placentae are most similar to human [9], but primates are rarely used as a model organism for placental transfer due to a long gestational period, cost and ethical concerns. Guinea pigs have been used as model organisms due to similarities in placental structure and a convenient gestational period [10,11]. However, species-related differences still remain. The *ex vivo* perfusion of the human placenta can be considered a suitable model of direct human relevance. It has the advantages of maintaining the complexity of the whole organ and being easily available. However, this model is technically challenging and unsuitable as a rapid screening tool due to time consuming preparation and the difficulty of obtaining suitable specimens [12]. In contrast, *in vitro* models of the placental barrier including primary cytotrophoblasts isolated from human placentae and choriocarcinoma cell lines, are easy to handle and can be used for rapid screening of placental transfer of a variety of substances over extended exposure periods. Whilst it is more difficult to grow primary placental cells to confluent monolayers [13], choriocarcinoma cell lines like JEG-3, JAr and BeWo can form monolayers when grown on permeable membranes. The BeWo cell line is an established model of the placental barrier [14], which resembles the normal, undifferentiated cytotrophoblast [13]. Although a variety of authors have used BeWo cells in transport studies, the experimental design and conditions vary considerably from study to study [15]. It has been highlighted that a number of factors need to be taken into account when using cell lines for transport experiments, including culture conditions, seeding density and choice of permeable membrane type [16]. Therefore, it is of great importance to establish a standard protocol to guarantee the quality, reproducibility and comparability of results obtained across laboratories. This will ultimately improve the relevance of the obtained kinetic data and make them more meaningful for human exposure assessments.

The b30 clone of BeWo is a single cell derived clone of the ATCC parent BeWo line, developed by Prof. Alan Schwartz and co-workers in the 1980s. In the first place, this was done to ensure that work was carried out on a uniform cell line. In later studies however, it has been observed that the b30 clone has better monolayer formation capacity than the parent cell line [17].

In this study, we developed an *in vitro* model of the placental barrier using BeWo b30 cells, providing a protocol and parameters for assessing monolayer formation. To support the validity of this model, we compared the transfer of highly purified NDL-PCB52 and 180 *in vitro* and in the *ex vivo* perfused human placenta. These congeners are amongst the 6 most abundant NDL-PCBs present in food and are indicators for different PCB patterns in a variety of sample matrices [18]. We chose PCB52 as a representative for a low chlorinated and PCB180 as a representative for high chlorinated PCBs.

## 2. Materials and methods

### 2.1. Reagents and chemicals

Highly purified (>99.9999%) 2,2′,5,5′-Tetrachlorobiphenyl (PCB52) and 2,2′,3,4,4′,5,5′-Heptachlorobiphenyl (PCB180) were purchased from Chiron AS and radiolabelled with Carbon-14 ($^{14}$C) by Blychem Ltd. $^{14}$C-PCB52 and $^{14}$C-PCB180 were supplied at 0.4 mCi/ml in DMSO (compare Table 1 for physicochemical properties of both NDL-PCBs). Cell culture medium and supplements, trypsin-EDTA, sodium fluorescein solution (Na-Flu), phosphate buffered saline (PBS), paraformaldehyde and Triton-X-100 were purchased from Sigma–Aldrich. Samples from *in vitro* experiments were analysed using Ultima Gold™ scintillation fluid and blank inserts were solubilised with Solvable™, both obtained from PerkinElmer LAS UK Ltd. For sample analysis from *ex vivo* perfusions, Ecosquint scintillation liquid from BN Instruments A/S was used. Primary polyclonal antibodies for tight junction staining of occludin and ZO-1 were rabbit anti-human occludin and rabbit anti-human ZO-1 from Abcam. The secondary antibody was Alexa Fluor® 488 donkey anti-rabbit from Invitrogen. Vectashield mounting medium with DAPI was obtained from Vector Laboratories Ltd.

**Table 1**
Physicochemical properties of PCB52 and PCB180 according to NLM chemical database (http://chem.sis.nlm.nih.gov/chemidplus/) and distribution of PCBs bound to plasma proteins according to [33].

| | PCB52 | PCB180 |
|---|---|---|
| Molecular weight [g/mol] | 291.99 | 395.32 |
| Aqueous solubility [mg/l] | 0.0153 | 0.0039 |
| log *P* (octanol-water) | 6.09 | 8.27 |
| Binding to plasma proteins | ~1% | ~22% |

### 2.2. Cell culture

The placental choriocarcinoma cell line BeWo b30 was obtained from Prof Harry McArdle (Rowett Research Institute, UK) with the kind permission of Dr. Alan Schwartz (Washington University, St. Louis, MO). The b30 cell lineage of the cells had been authenticated by the European Cell and Culture Collection [19]. The ATCC BeWo cell line was purchased from the European Collection of Cell Cultures (cat #86082803). Cells were cultured in Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 Ham (DMEM F-12 Ham) with phenol red, supplemented with 1% L-Glutamine-Penicillin-Streptomycin, 1% Amphotericin B and 10% fetal bovine serum. All experiments were conducted using this supplemented medium, unless otherwise stated. Cells were routinely maintained in polystyrene cell-culture flasks at 37 °C in 5% $CO_2$ humidified atmosphere. At confluence, cells were sub-cultured using a 0.4% trypsin-EDTA solution.

### 2.3. Model optimisation and monolayer confirmation

Transwell® plates (polyester membrane, 0.4 μm pore size) were obtained from Appleton Woods Ltd. Both BeWo cell lines were seeded at $10^5$ cells/cm$^2$ on Transwell® inserts, pre-soaked in medium for 45 min. Medium was changed daily from day 2 post-seeding (ps) until day 13 ps. Transepithelial electrical resistance (TEER) was measured daily from day 2 ps onwards with an Endohm 12 chamber and voltohmmeter (EVOM; World Precision Instruments Inc.). TEER values for the cell layer were obtained by subtracting the intrinsic resistance from the total resistance. TEER values were corrected for surface area and expressed as Ω cm$^2$. Growth of the cell layer was also monitored visually under the light microscope. In the second phase of model optimisation, BeWo b30 cells were seeded as described above. Cells were left to adhere for two days and medium was changed on days 3, 4 and 5 ps. Development of the monolayer was monitored using TEER measurements, Na-Flu transport and transmission electron microscopy (TEM). Na-Flu transport was conducted by adding 5 μM Na-Flu to the apical chamber and supplemented DMEM F-12 Ham medium without phenol red to the basal chamber. Plates were incubated for 3 h at 37 °C. 50 μl samples were removed from the basal chamber into a black 96-well plate and read at excitation 485 nm and emission 520 nm on a microplate reader (BMG Labtech FLUOstar Optima). Values were blank corrected and sample concentrations were determined using a Na-Flu standard curve. For TEM imaging, cells grown on insert membranes were fixed and processed according to Lahtinen et al. [20]. Samples were imaged on days 3–5 ps. To examine the presence of tight junctions in the monolayer, antibody staining was performed on day 4 ps. Cells grown on insert membranes were fixed in 2% paraformaldehyde and permeabilised with 0.3% Triton-X-100. Cells were incubated with primary antibody against ZO-1 or occludin and washed before incubation with the secondary antibody.

### 2.4. $^{14}$C-PCB transfer and recovery in vitro

To estimate the rate of transfer of $^{14}$C-PCB180 and $^{14}$C-PCB52, BeWo b30 cells (passage 34–41) were seeded at $10^5$ cells/cm$^2$ on Transwell® inserts as described above. Transfer studies were conducted on day 4 ps at 37 °C and 5% $CO_2$. Monolayer integrity was evaluated using TEER measurements. TEER values had to fall within a previously determined range in order for the experiment to be carried out. Rate of transfer through the insert membranes alone was also estimated. Solutions of $^{14}$C-PCB180 and $^{14}$C-PCB52, previously dissolved in Dimethyl sulfoxide (DMSO), were made up at 15 μM (0.5 μCi/ml) in medium. The total concentration of DMSO in the medium was 0.125% (v/v). Previous experiments in our lab showed that neither PCB52 nor PCB180 decreased cell viability or disrupted tight junctions of BeWo cells at this concentration and using DMSO as a vehicle. $^{14}$C-PCB solutions were added to the apical and medium to the basal chambers chambers of the Transwell®. Experiments were carried out over two time courses, a short (2 h) and a long time course (24 h). During the short time course, three 50 μL aliquots were taken from the apical and from the basal chamber after 30, 60, 90 and 120 min. The same procedure was performed during the long time course after 2, 4, 6 and 24 h. Three separate wells were used for each time point and were discarded after sampling to avoid alterations to the concentration gradient between apical and basal chambers (no sampling and replacing). The amount of $^{14}$C-PCB180 or $^{14}$C-PCB52, respectively, was determined

by scintillation counting with the addition of Ultima Gold™. Radioactivity was expressed as counts per minute (CPM). Experiments were carried out at least three times for each $^{14}$C-PCB and each time course.

For data analysis, sample values were background corrected. The transfer rate of $^{14}$C-PCB was expressed as permeability coefficient ($P_e$). Firstly, the apparent permeability coefficient ($P_{app}$) was estimated using the following equation [21]:

$$P_{app}(\mathrm{cm/s}) = \frac{k \cdot V_R}{A \cdot 60} \quad (1)$$

Where $k$ is the transfer rate constant, determined by plotting the cumulative amount of test compound permeated in relation to the initial concentration as a function of time and determining the slope via linear regression ($\mathrm{min}^{-1}$). $V_R$ is the volume (ml) in the receiver chamber, and $A$ is the diffusion area. Then, permeability coefficients ($P_e$) were calculated using the following equation [22]:

$$\frac{1}{P_e} = \frac{1}{P_{app}} - \frac{1}{BlankP_{app}} \quad (2)$$

Where $P_{app}$ is the apparent permeability coefficient of the membranes in the presence of BeWo cells, and $BlankP_{app}$ is the mean apparent permeability coefficient of the blank membranes.

Percentage transfer was calculated as follows:

$$\frac{\text{Radioactivity in sample [CPM]}}{\text{Radioactivity in initial solution [CPM]}} \times 100 \quad (3)$$

$^{14}$C-PCB180 and $^{14}$C-PCB52 association studies were conducted in conjunction with transport studies. At 2 h or 24 h, respectively, three inserts were washed with PBS and samples of the wash were taken. The medium was removed and each well, insert and the lid was swabbed with filter paper dipped into 50% ethanol. Membranes were solubilised with Solvable™. Samples were analysed by scintillation counting and percentage association was calculated as described in Eq. (3).

### 2.5. $^{14}$C-PCB transport and recovery ex vivo

Placentae ($n$ = 31) from normal pregnancies and births resulted in nine successful perfusions (success ratio 29%). The placentae were obtained immediately after vaginal birth or elective Caesarean section at the Danish University Hospital, Rigshospitalet. Informed written consent was given before or in relation to birth. The study had been approved by the regional Ethics Committee (KF 01–145/03 + KF(11) 260063) and the Data Protection Agency. The dually perfused recirculating human placental perfusion is described elsewhere in detail [12,23]. Briefly, one vascular unit in the placenta was perfused by cannulation of the fetal circulation and simultaneous supply of maternal perfusion medium to the intervillious space. The tubing consisted of Tygon PVC-free tubing and a 10 cm piece of silicone tubing through the peristaltic pump. The fetal and maternal perfusion media consisted of Krebs Ringer with heparin, penicillin-streptomycin and a physiological concentration of human serum albumin (30 g/l in maternal, 40 g/l in fetal reservoir). The perfusion media were circulated by roller pumps to attain a flow rate of 3 ml/min and 9 ml/min in the fetal and maternal circulation, respectively. A pre-perfusion period of 30 min was used to stabilise the placental tissue before adding antipyrine and 1.5 μM $^{14}$C-PCB180 or $^{14}$C-PCB52, to the maternal reservoir. Background samples were taken from the maternal and fetal circulation prior to addition of test compounds, and from the maternal reservoir immediately after their addition. During the perfusion, samples were taken from the fetal and maternal circulation at 2, 5, 10, 15, 30, 45, 60, 90, 120 and 150 min. Samples were centrifuged at 4000 rpm for 5 min and 2 ml of Ecosquint scintillation liquid was added to the supernatant. The amount of $^{14}$C-PCB was estimated by scintillation counting and percentage transport was calculated as described above. At the end of the perfusion, the perfused cotyledon was separated from the surrounding tissue, and samples of approximately 1 g were extracted with acetonitrile. The amount of $^{14}$C-PCB associated to the tissue was estimated by scintillation counting as described above. A background sample was also taken from the placental tissue before perfusion. To assess association of the PCBs with the perfusion system, a perfusion over 150 min without placenta, but with 3 ml of placental blood added to the medium, was performed for each congener. Samples of the perfusion media were taken from the recirculating system at the time points mentioned above. Again, the amount of $^{14}$C-PCB in the samples was estimated by scintillation counting, using Ecosquint scintillation liquid. A decrease of PCB concentration in the samples over time gives an estimate of the amount adhering to the tubing and to red blood cells, which are spun down before scintillation counting and may represent a source of PCB loss.

Permeability coefficients cannot be calculated in a perfused placenta model due to the unknown size of the total perfused area. In order to make data comparable between the two models, we expressed PCB transport as a percentage of the initial $^{14}$C-PCB concentration (Eq. (3)) and expressed the permeability rate as the initial % appearance of the compound in the fetal compartment per hour. We calculated the fetal-maternal (FM) ratio for both congeners at the end of the experiments by dividing the radioactivity measured in the fetal compartment by the radioactivity measured in the maternal compartment at a given time point.

### 2.6. Statistical analysis

The results herein are expressed as the mean ± standard deviation (SD). Parameters comparing the permeability of congeners (permeability coefficients, permeability rates, FM ratios) were compared by univariate ANOVA. To explore effects and interactions of time, PCB congener and presence or absence of cells on PCB association to the individual compartments of the *in vitro* model, we used a three factorial univariate ANOVA. Values of $p$ exceeding 0.05 were considered insignificant. All statistical tests were performed using SPSS 16.0 for Windows.

## 3. Results

### 3.1. Model optimisation and monolayer confirmation

Previous experiments in our laboratory showed that both BeWo cell lines grew best on Transwell® polyester membranes with 0.4 μm pore size seeded at $10^5$ cells/cm$^2$.Growth of the ATCC and the b30 cell line was monitored over 13 days using TEER and light microscopy. TEER values of the b30 clone increased from 10 Ω cm$^2$ on day 2–60 Ω cm$^2$ on day 13 ps (Fig. 1). The cell layer appeared confluent from day 4 ps onwards. The ATCC cells, however, grew much slower, with TEER values increasing from 10 Ω cm$^2$ on day 2–25 Ω cm$^2$ on day 13 ps. Holes in the cell layer were visible on each day. Therefore, further model development was undertaken with the b30 clone only. As BeWo b30 appeared confluent from day 4 ps onwards, the time frame was restricted to days 3–5 ps in order to determine monolayer formation. TEM images showed holes in the barrier on day 3 and overgrowth on day 5; a confluent monolayer was formed on day 4 ps (Fig. 2). To examine the presence of tight junctions in the monolayer, antibody staining was performed on day 4 ps. Tight junctions were visible using both occludin and ZO-1 stains (Fig. 2), indicating confluent monolayer formation. ZO-1 staining showed less unspecific binding artefacts and resulted in a much clearer image than staining with occludin. On day 4 ps, Na-Flu transport was 0.205 μM ± 0.019 (4.1% ± 0.18) and TEER values were 44.65 ± 11.06 Ω cm$^2$. We used this TEER range to assess monolayer formation before conducting transfer experiments.

### 3.2. $^{14}$C-PCB transfer and recovery in vitro

Fig. 3 shows the transfer of $^{14}$C-PCB52 and $^{14}$C-PCB180 across BeWo cells and blank inserts over a short and long time course.

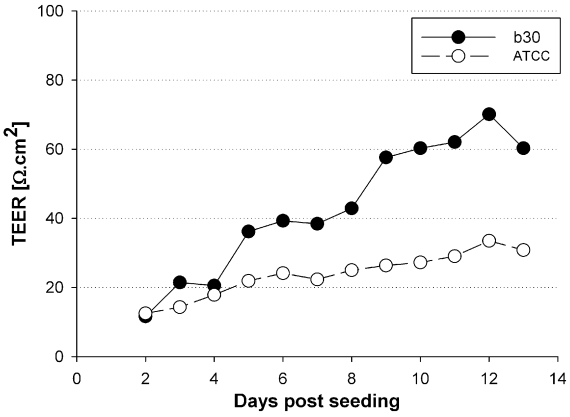


**Fig. 1.** Monitoring the growth of ATCC cell line and b30 clone of BeWo. Transepithelial electrical resistance (TEER) measurements over 13 days post-seeding ($n$ = 1). Cells were seeded at $10^5$ cells/cm$^2$ on polyester Transwells® (0.4 μm pore). Medium was changed daily from day 2 post-seeding until day 13. TEER values for the cell layer were obtained by subtracting the intrinsic resistance from the total resistance. Values were corrected for surface area (1.12 cm$^2$).

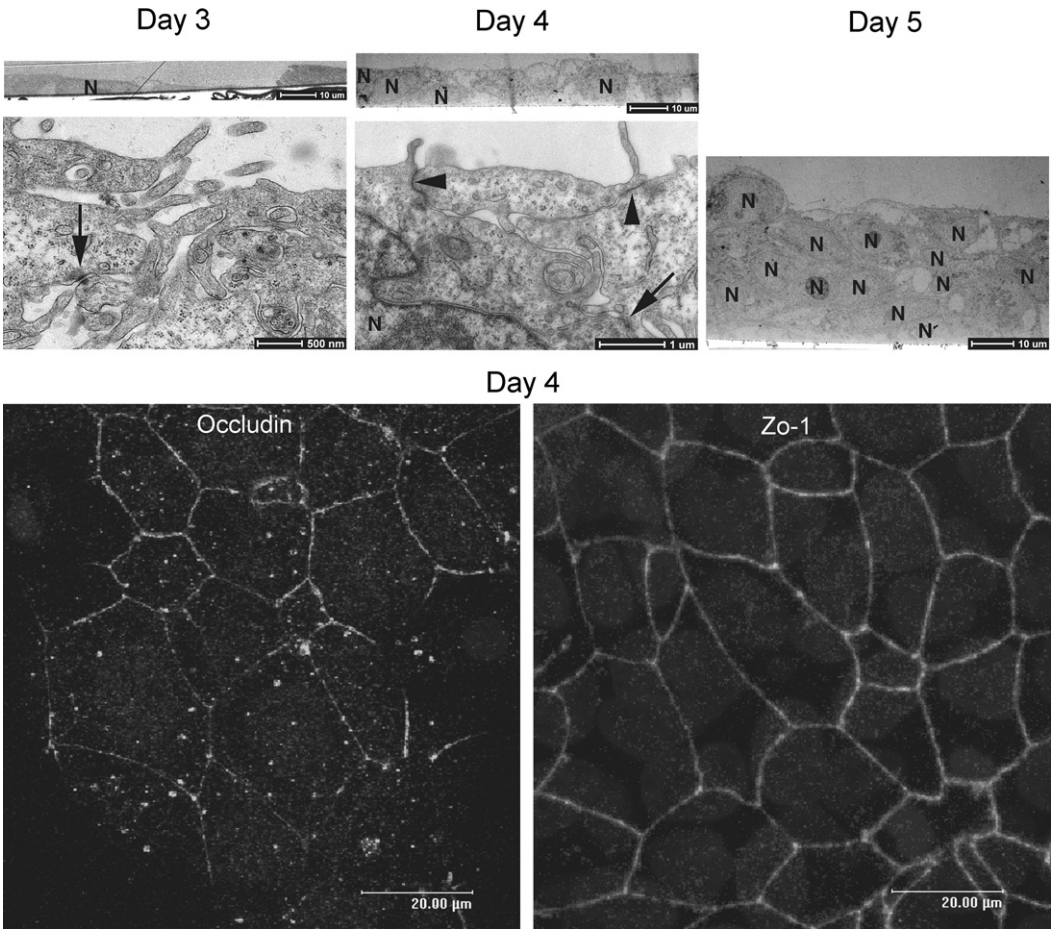


**Fig. 2.** Imaging of BeWo b30. Representative transmission electron microscopy images on days 3, 4 and 5 post-seeding (top) and confocal images of the monolayer on day 4 post-seeding (bottom). Cells were seeded at $10^5$ cells/cm$^2$ on polyester Transwells® (0.4 µm pore), left to settle for 2 days, medium changed on day 3 post-seeding. Top panes: arrows indicate junctional complexes, arrow heads indicate tight junctions, N: Nucleus. Bottom panes: tight junctions stained with antibodies for Occludin (A) and ZO-1 (B) visible as light grey lines, nuclei stained with DAPI appear dark grey.

Both $^{14}$C-PCB transferred as rapidly across the cells as across the blank inserts. $^{14}$C-PCB180 crossed the cell barrier more rapidly than $^{14}$C-PCB52 and reached equilibrium after about 6 h, whereas equilibrium for $^{14}$C-PCB52 was not quite reached after 24 h. Over the long time course, we observed a reversal of the concentration gradient for $^{14}$C-PCB180 (more substance in the basal than in the apical chamber). Permeability coefficients were calculated for both substances from data obtained up to 2 h exposure, as this was the linear part of the graph. We found that $^{14}$C-PCB180 had a higher permeability coefficient and higher FM ratios than $^{14}$C-PCB52 (Table 2).

To assess the amounts of $^{14}$C-PCB associated with cells, inserts, plates and PBS washes, we measured radioactivity in each of these compartments and in the medium of the apical and basal chamber at the end of the experiments (Table 3). Total recovery of both PCB congeners decreased between 2 h and 24 h, $^{14}$C-PCB180 recovery being generally lower (<70%) than $^{14}$C-PCB52 recovery (>70%). Most $^{14}$C-PCB was recovered from the medium, in which the amount of both congeners was reduced after 24 h. Less $^{14}$C-PCB52 was recovered from medium on plates containing cells than from blank plates. $^{14}$C-PCB180 recovery, however, was similar in medium from cell and blank plates. We also found differences in the association patterns of the two congeners to the inserts. $^{14}$C-PCB180 associated more with the blank insert membrane than $^{14}$C-PCB52. In contrast, $^{14}$C-PCB52 associated more with cell inserts. After 24 h, $^{14}$C-PCB180 recovery from blank inserts was higher than after 2 h, but recovery from cell inserts was lower. $^{14}$C-PCB52 recovery from blank or cell covered inserts did not change over time.

### 3.3. $^{14}$C-PCB transfer and recovery ex vivo

A total of 4 and 5 successful perfusions were conducted with $^{14}$C-PCB52 and $^{14}$C-PCB180, respectively. FM ratio of antipyrine at 150 min perfusion was over 0.9, indicating appropriate overlap of the maternal and fetal circulations. Both PCBs transferred rapidly from the maternal into the fetal circulation (Fig. 4), $^{14}$C-PCB180 crossing the placental barrier faster than $^{14}$C-PCB52 as indicated by the higher permeability rate (Table 2).

Recovery rates for both $^{14}$C-PCBs at the end of the perfusion were below 50% (Table 4). $^{14}$C-PCB52 associated more with the tissue of the perfused cotyledon than $^{14}$C-PCB180. Recovery rates from perfusion medium and surrounding placental tissue were similar for both congeners. Both PCBs appeared to have a high affinity for the tubing material of the perfusion system or adhered in some degree to the red blood cells. More $^{14}$C-PCB180 than $^{14}$C-PCB52 was lost from the system during the system adherence test, indicating that $^{14}$C-PCB180 associated with the perfusion system to a greater extent than $^{14}$C-PCB52.

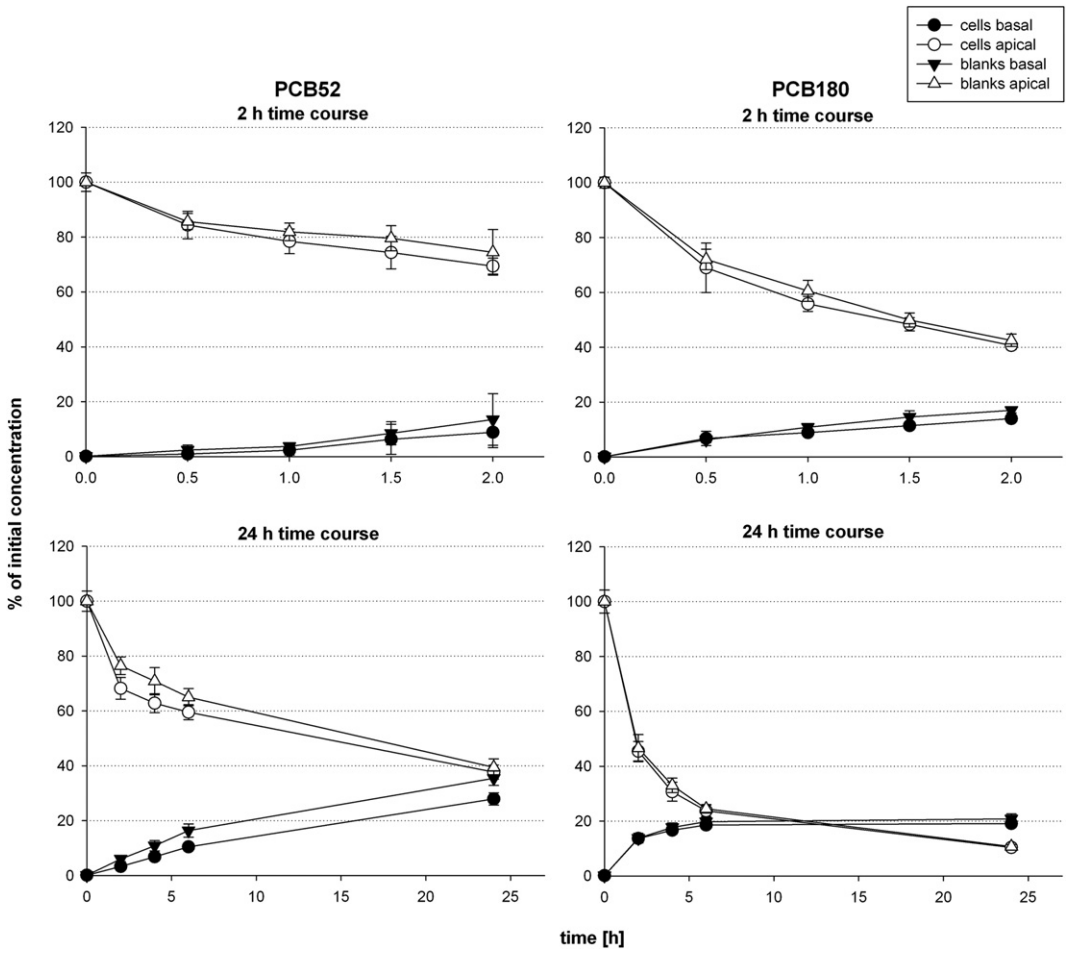


**Fig. 3.** Transport of $^{14}$C-PCB52 and $^{14}$C-PCB180 across BeWo b30 and blank Transwell® inserts *in vitro* over 2 h and 24 h. Cells were seeded at $10^5$ cells/cm$^2$ on polyester Transwells® (0.4 μm pore) and transport studies were conducted on day 4 post-seeding. Samples were taken from the apical and basal chamber at the indicated time points. Separate wells were used per time point (no sampling and replacing). The amount of $^{14}$C-PCB in the samples was estimated by liquid scintillation counting All values are expressed as mean percentage of the concentration initially added to the apical chamber ± SD ($n = 9$ per time point from 3 separate experiments).

### 3.4. Comparison of the two models

In both the *in vitro* and the *ex vivo* model, $^{14}$C-PCB180 crossed the placental barrier faster than $^{14}$C-PCB52. This is reflected in a higher FM ratio and permeability rate of $^{14}$C-PCB180 (Table 2). Both the FM ratio and permeability rate were about three times higher in the perfusion model than in the BeWo model. $^{14}$C-PCB recovery from the *ex vivo* system was lower than from the *in vitro* model. In both models, $^{14}$C-PCB52 was found to associate more with the cells and tissue, respectively, than $^{14}$C-PCB180. $^{14}$C-PCB180 in contrast associated more with the material in both systems (tubing or insert membranes) than $^{14}$C-PCB52.

**Table 2**
Comparison of transport parameters in BeWo and perfusion model.

| | $^{14}$C-PCB52 | $^{14}$C-PCB180 |
|---|---|---|
| BeWo model | | |
| $P_e$ [$\times 10^{-4}$ cm/s]* | 0.92 ± 1.1 | 16.7 ± 8.8 |
| FM ratio after 2 h[a]* | 0.06 ± 0.02 | 0.32 ± 0.04 |
| FM ratio after 24 h* | 0.74 ± 0.09 | 1.85 ± 0.08 |
| Permeability rate [%/h][a]* | 2.31 ± 1.78 | 8.89 ± 0.64 |
| Perfusion model | | |
| FM ratio after 2 h[a]* | 0.23 ± 0.07 | 0.70 ± 0.17 |
| Permeability rate [%/h][a]* | 9.62 ± 2.41 | 26.03 ± 5.43 |

$P_e$: permeability coefficient; FM ratio: fetal-maternal ratio; Permeability rate: appearance of compound in fetal compartment within the first 60 min of *in vitro* experiment and first 15 min of *ex vivo* placental perfusion, respectively.
*significant difference between congeners ($p < 0.05$).
[a] Parameters used to compare the two models.

## 4. Discussion

Studying the transport of environmental pollutants and other potentially harmful substances across the placental barrier allows exposure assessments for the unborn child. Several models are available to investigate these transfer processes. In this study, we have compared two BeWo cell lines regarding their ability to form an intact monolayer barrier model. The *ex vivo* placental perfusion model was used to confirm the validity of the *in vitro* model, and we investigated the placental transfer of highly purified NDL-PCBs, a group of PCBs for which toxicokinetics are poorly characterised. The *ex vivo* perfusion of a placental cotyledon is a well established model [24,25] that has the advantage of representing the complexity of the whole organ, thereby mimicking the *in vivo* situation very accurately. However, there are several constraints to this model that make it particularly unsuitable for rapid screening purposes. The quality of the obtained placentae varies greatly and many do not meet the strict inclusion criteria. Hence, a great number of specimens are required in order to obtain statistically

**Table 3**
Recovery of $^{14}$C-PCB52 and $^{14}$C-PCB180 from *in vitro* system compartments at the end of both time courses.

| | Type | Washes* | Plate | Inserts* | Medium* | Av. Total |
|---|---|---|---|---|---|---|
| Recovery after 2 h | | | | | | |
| $^{14}$C-PCB52 | Cells | 6.91 ± 1.01 | 0.05 ± 0.03 | 8.37 ± 1.11 | 74.90 ± 5.33 | 84.78 ± 5.60 |
| | Blanks | 5.87 ± 1.08 | 0.07 ± 0.05 | 0.60 ± 0.12$^{\dagger}$ | 85.23 ± 3.87$^{\dagger}$ | 87.31 ± 3.93 |
| $^{14}$C-PCB180 | Cells | 5.40 ± 0.71 | 0.07 ± 0.03 | 3.56 ± 1.33 | 56.90 ± 3.34 | 60.30 ± 0.6 |
| | Blanks | 5.80 ± 0.75 | 0.06 ± 0.02 | 1.43 ± 0.34$^{\dagger}$ | 59.97 ± 2.29 | 62.24 ± 2.18 |
| Recovery after 24 h | | | | | | |
| $^{14}$C-PCB52 | Cells | 6.14 ± 0.27 | 0.08 ± 0.01 | 8.73 ± 2.38 | 65.63 ± 2.47$^{\#}$ | 76.43 ± 2.76 |
| | Blanks | 6.66 ± 0.84 | 0.14 ± 0.06 | 0.71 ± 0.09$^{\dagger}$ | 74.90 ± 1.39$^{\#\dagger}$ | 77.92 ± 4.87 |
| $^{14}$C-PCB180 | Cells | 2.97 ± 0.39$^{\#}$ | 0.05 ± 0.01 | 2.41 ± 0.48$^{\#}$ | 29.49 ± 2.14$^{\#}$ | 32.96 ± 0.52 |
| | Blanks | 2.86 ± 0.63$^{\#}$ | 0.07 ± 0.02 | 2.88 ± 0.81$^{\#\dagger}$ | 31.53 ± 1.47$^{\#}$ | 35.01 ± 1.44 |

Values expressed as % of initial concentration ± SD. Note that in columns reporting recovery from inserts, "cells" is the amount measured in insert membranes plus cells, whereas "blanks" indicates the amount recovered from insert membranes alone ($n = 9$ per time point from 3 separate experiments).
*significant difference between congeners ($p < 0.05$).
$^{\#}$significant difference between time points ($p < 0.05$).
$^{\dagger}$significant difference between cells and blanks ($p < 0.05$).

adequate results. Furthermore, the duration of the placental perfusion is rather limited and usually lasts 2–6 h, long perfusions lasting 12–18 h, requiring very controlled conditions [26]. Working with an *in vitro* model is less time consuming, allows high throughput screening, and results may be more reproducible due to more controlled conditions. However, it doesn't represent the complexity of the whole organ and therefore needs careful optimisation and validation.

### 4.1. Model optimisation and monolayer confirmation

During placental development, mononuclear cytotrophoblasts fuse into the multinucleated syncytiotrophoblast which forms the maternal side of the placental barrier. In humans, this barrier consists of a single cell layer. BeWo cells are a useful tool to study transport across the human placental barrier because they are readily available and display many characteristics of the cytotrophoblast *in vivo* [15]. Obtaining a confluent monolayer *in vitro* is crucial for the relevance of the model and requires careful optimisation. Some authors have used multiple layers of BeWo [27,28] or not stated how they ensured monolayer formation [14,29]. Previous findings suggest that monolayer formation may vary depending on clone, insert type, seeding density and frequency of media changes [13,16]. We made similar observations and can confirm the inability of the ATCC clone to form a confluent monolayer, which had been observed by other groups previously [17]. Therefore, it is crucial to develop and observe a standard operating procedure for kinetic studies with BeWo. We used the information obtained from TEM and confocal imaging combined with TEER and Na-Flu transport values to develop a standard operating procedure that assesses monolayer formation on a day to day basis. Using the b30 clone and the protocol described here should enable researchers to grow BeWo b30 as a monolayer. The critical range of TEER combined with Na-Flu transport provided in the results section are reliable indicators for monolayer formation.

### 4.2. Validation of the in vitro model

With the reduction of the placenta to the cytotrophoblast, the complexity of the organ is not represented *in vitro*. Therefore, it is of great importance to confirm results obtained in the BeWo model with a more *in vivo* like model such as the perfused placenta. Here, we used permeability rate and FM ratio as parameters to compare the transfer of two NDL-PCB congeners in both models. These parameters show similar patterns, but different magnitudes *in vitro* and *ex vivo*. FM ratios and permeability rates were three times higher in the perfusions than in the BeWo model. Furthermore, equilibrium of $^{14}$C-PCB180 was almost reached after 2½ h in the perfusion model, whereas it took approximately 6 h to reach equilibrium in the BeWo model. Similar findings have been

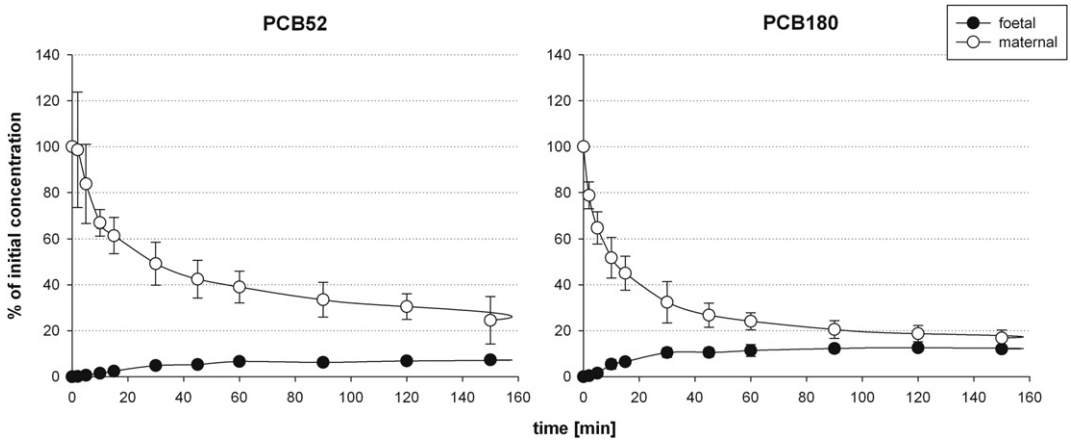


**Fig. 4.** Transport of $^{14}$C-PCB52 and $^{14}$C-PCB180 in the *ex vivo* perfused placenta over 2½ h. Samples were taken from the maternal and fetal reservoir at the indicated time points. The amount of $^{14}$C-PCB in the samples was estimated by liquid scintillation counting. Values are expressed as a mean percentage of the concentration initially added to the maternal reservoir ± SD. $n = 4$ perfusions with $^{14}$C-PCB52, $n = 5$ perfusions with $^{14}$C-PCB180.

**Table 4**
Recovery of $^{14}$C-PCB52 and $^{14}$C-PCB180 at the end of 2½ h perfusions and system adherence test.

| | Perfused cotyledon* | Surrounding tissue | Medium | Av. Total | System adherence test[a]* |
|---|---|---|---|---|---|
| $^{14}$C-PCB52 | 15.08 ± 4.80 | 1.36 ± 1.05 | 33.14 ± 10.08 | 43.57 ± 7.23 | 52.95 |
| $^{14}$C-PCB180 | 6.99 ± 2.77 | 1.69 ± 1.56 | 30.20 ± 4.67 | 41.13 ± 17.15 | 72.72 |

All values expressed as % of initial concentration ± SD from $n = 4$ perfusions with $^{14}$C-PCB52 and $n = 5$ perfusions with $^{14}$C-PCB180.
*significant difference between congeners ($p < 0.05$).
[a] The system adherence test was performed separately for 2½ h in the recirculation system without placenta; figures indicate % of $^{14}$C-PCB lost from system.

reported by Poulsen et al. [30], who investigated the placental transfer of a range of substances in the BeWo model and the perfused placenta. It was proposed that the differences in permeability rate and time to equilibrium are due to the hydrostatic gradient generated by peristaltic pumps in the perfusion system. Adding to that, we observed that in the present study the protein content was higher in the perfusion medium than in the cell-culture medium. Since PCBs associate with proteins [31], they may function as carrier molecules and thus influence transport rate. Apart from differing in magnitude, the transfer and association patterns of the PCBs in both models were in good agreement. Transfer of PCB52 and 180 across and accumulation of these congeners in the placenta have also been observed in epidemiological studies [7,8,32], adding further weight to the validity of the BeWo model.

PCB recovery at the end of the perfusion was much lower than PCB recovery from the *in vitro* system at a similar time point. This may have been due to higher absorption of PCBs to the relatively larger surface area of the tubing in the perfusion system. Furthermore, loss of PCB material may have occurred due to precipitated proteins and red blood cells that are centrifuged down and removed before scintillation counting of the sample. This would be particularly significant for PCB180, which was found to associate to about 22% to plasma proteins, compared to under 5% association of PCB52 [33].

PCB recovery *in vitro* decreased over time, $^{14}$C-PCB52 recovery being generally higher than $^{14}$C-PCB180 recovery. Although the plates were covered with lids, we cannot rule out that the compounds evaporated during sampling from the plate when the lid was removed. PCB180 recovery from the *in vitro* system was particularly poor. This could have been due to incomplete extraction of this congener from plate surfaces and underestimating its content in the PBS washes. Substantial sorption losses of PCB congeners to glass and polymers used in laboratory equipment were observed previously [34]. Furthermore, it has been shown that PCB congeners are not easily desorbed from these materials [35,36]. The low solubility of higher chlorinated PCBs in aqueous media may have contributed further to the low recovery estimates (compare Table 1). The loss of test substance due to adherence is an inherent characteristic of any *in vitro* test system, and depends on the chemical characteristics of the test substance. PCBs and other lipophilic environmental pollutants are known to bind to laboratory equipment and plastic consumables. However, this loss can be controlled for, for example by running system adherence tests as described in this and in previous studies [23,37]. Furthermore, the use of FM ratio as a measure of transfer only considers the dissolved and not the adhered substance. Thus, the FM ratio is a true measure of transfer rate that mitigates the issue of low test substance recovery, and allows to make reasonable predictions for potential *in vivo* scenarios.

### 4.3. Kinetics of $^{14}$C-PCB52 vs. $^{14}$C-PCB180 and suggested implications

*In vitro*, we found that both congeners transferred as rapidly across the BeWo cells as across the blank inserts. This indicates unrestricted diffusion of both compounds across the cells, which is in line with previous findings suggesting passive diffusion as the mechanism by which PCB are absorbed across biological barriers [38]. $^{14}$C-PCB180 in particular diffused very quickly, so that after 24 h we measured a greater concentration in the basal than in the apical chamber. We do not believe that this is due to active transfer as we observed this phenomenon in cell and in blank wells alike. Rather, we suggest that this observation may have been due to a lack of sink conditions. Transport experiments should ideally be performed under sink conditions, i.e. the concentration of the compound in the receiver chamber should not exceed 10% of the concentration in the donor chamber at any time [39]. This condition was maintained for $^{14}$C-PCB52 up to 4 h, but only during the first 30 min for $^{14}$C-PCB180. Therefore, the time course of placental kinetic studies *in vitro* needs to be chosen carefully according to the type of compound (rapidly vs. slowly diffusing). For studies with BeWo it should also be noted that the barrier will overgrow from day 5 ps on, therefore no longer forming a monolayer. The time course for kinetic studies should therefore not exceed 24 h.

The FM ratio of $^{14}$C-PCB52 was relatively low (0.23), which might have been due to high association of $^{14}$C-PCB52 with the placental tissue. A previous study on primary placental cells has shown that lower chlorinated PCBs (Delor 103) accumulated in the placental cells to a greater extent than higher chlorinated ones (Delor 106) [40], which is in line with our findings. We hypothesise that $^{14}$C-PCB52 molecules may have been “trapped” in the lipid vesicles of the cytotrophoblast due to their lipophilicity and small size. $^{14}$C-PCB180, however, is a larger molecule that may not be able to accumulate in the cells to the same extent. Therefore, it was transferred out of the cells along the concentration gradient. This would explain the higher transfer rate of $^{14}$C-PCB180 *in vitro* and *ex vivo*. Recovery rates from medium *in vitro* further reflect that $^{14}$C-PCB52 is mainly contained in BeWo. $^{14}$C-PCB52 recovery from medium was lower in the plates that contained cells, suggesting that $^{14}$C-PCB52 tends to accumulate in the cells when they are present. Notably, $^{14}$C-PCB180 recovery from cell inserts decreased over time but its recovery from blank inserts increased. We hypothesise that $^{14}$C-PCB180 may adhere to the cells in the short term, but partition into the insert membrane and the medium over time. This may be due to its large size which doesn't favour accumulation in the cell and the tendency of higher chlorinated biphenyls to sorb to surfaces [41].

A limitation of our results is the fact that we were not able to determine whether parent PCB or a metabolite was detected by scintillation counting. There is evidence that PCBs may be metabolised in the placenta by cytochrome P450 isozymes [42,43]. BeWo cells also express P450 types [44] and may therefore metabolise PCBs as well. PCB metabolites were found to display different transplacental kinetics than their parent compound [45]. We are also aware that the relatively low PCB recovery rates and the high unspecific binding of the congeners to the perfusion systems present limitations to our data and demand careful interpretation of the results.

Taking into account the different association and transfer patterns observed, we suggest that toxic effects of PCB52 may play a more important role for placental function due to its accumulation

in the tissue, whereas PCB180 may be of greater importance for fetal toxicity as a result of rapid transfer to the fetus.

## 5. Conclusion

This study provides a protocol for establishing a robust model of the human placental barrier using BeWo b30. This *in vitro* model can be used as a rapid screening tool to reliably monitor transport processes along the blood-placenta barrier. As such, the extension of time course experiments to up to 24 h *in vitro* adds to the strength of the model. However, the length of transport experiments should be chosen carefully in order to maintain sink conditions.

Our findings confirm, that the placenta offers the fetus little protection against the NDL-PCBs 52 and 180. The two congeners display different kinetic behaviour, supposedly due to their structural difference. PCB52 tends to accumulate in placental tissue whereas PCB180 is transferred rapidly across the placental barrier. We hypothesise that toxic effects of lower chlorinated PCBs like PCB52 may therefore primarily impact placental function, affecting mother as well as unborn child. Effects of PCB180 or other higher chlorinated congeners, however, may play a more important role regarding fetal toxicity via direct exposure *in utero*. We suggest taking this hypothesis into account in future epidemiological and toxicological studies.

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