# Species specific debromination of PBDEs and relationships to deiodinase

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## Introduction

Polybrominated diphenyl ether (PBDEs) has one of flame retardants and three commercial products (Penta BDE, Octa BDE and Deca BDE product) had been produced. Penta BDE and Octa BDE products have been banned since 2009 by Stockholm Convention, because lower brominated congeners in the two commercial products have toxicity and bioaccumulability. On the other hand, DecaBDE has not been banned until now officially. BDE209 which is dominant congener in DecaBDE product was reported to be debrominated by photodegradation, microbial degradation and metabolism in biological tissues[1-3]. Therefore, BDE209 can be source of lower brominated congeners. Some studies demonstrated metabolic debromination of PBDEs using hepatic microsome. Species-specific and congener specific debromination of PBDEs have been reported by Stapleton et al., Browne et al., Roberts et al. and Mizukawa et al.[3-6]. Some kind of fish, such as common carp and ureogenic goby, were not detected BDE99 in their muscle tissues and high debromination ability was indicated by *in vitro* experiments using their hepatic microsome with BDE99[5,6]. It was expected that *Cyprinidae,* not only common carp, has high debromination ability because some *Cyprinidae* did not accumulate BDE99 in their muscle tissues[7, 8]. Considerable factor of species specific debromination is expression or structure of a catalysis related debromination. It is hypothesized that debromination is caused by deiodinase which is a catalysis for activation of thyroid hormone through removing iodine from Thyroxine (T4) to Triiodothyronine (T3). However the relationships with deiodinase and species specific debromination have not been revealed yet. Therefore the object of this study is investigation of the relationships of them.

## Materials and methods

*Field observations*

***Sample information***

Fish samples were collected from Tamagawa River and Tokyo Bay on 2014-2016 (Table1).

**Table1: Sample list for the field observations**

| Order | Suborder | Superfamily | Famiry | Subfamily | Species | English name |
|---|---|---|---|---|---|---|
| *Cypriniformes* | | | *Cyprinidae* | *Cyprininae* | *Nipponocypris temminckii* | Dark chub |
| | | | | | *Opsariichthys platypus* | common minnow |
| | | | | | *Cyprinus carpio* | common carp |
| | | | | | *Carassius auratus* | silver crucian carp |
| | | | | | *Carassius cuvieri* | Japanese crucian carp |
| | | | | *Gobioninae* | *Hemibarbus barbus* | Japanese barbel |
| | | | | | *Pungtungia herzi* | striped shiner |
| | | | | | *Gnathopogon elongatus* | Field gudgeon |
| | | | | | *Pseudorasbora parva* | Topmouth minnow |
| | | | | *Leuciscinae* | *Tribolodon hakonensis* | Japanese dace |
| | | | | | *Tribolodon brandtii* | Pacific redfin |
| | | *Cobitoidea* | *Cobitidae* | *Cobitinae* | *Misgurnus anguillicaudatus* | weather loach |
| | | | *Balitoridae* | *Nemacheilinae* | *Lefua echigonia* | Japanese eight barbel loach |
| *Osmeriformes* | *Osmeroidei* | *Osmeroidea* | *Osmeridae* | *Plecoglossinae* | *Plecoglossus altivelis* | ayu |
| *Siluriformes* | | | *Siluridae* | | *Silurus asotus* | Japanese catfish |
| *Perciformes* | *Gobioidei* | | *Gobiidae* | *Gobionellinae* | *Rhinogobius flumineus* | Lizard goby |
| | | | | | *Tridentiger obscurus* | Dusky tripletooth goby |
| | | | | | *Tridentiger trigonocephalus* | Redstriped tripletooth goby |
| | | | | | *Chaenogobius gulosus* | Forktongue goby |
| | *Scombroidei* | | *Scombridae* | *Scombrinae* | *Scomber japonicus* | Chub mackerel |
| *Beloniformes* | *Belonoidei* | | *Exocoetoidea* | *Hemiramphidae* | *Hyporhamphus sajori* | Japanese halfbeak |

***Extraction and cleanup of muscle tissues***

Each sample was homogenized, freeze-dried and ground. These samples were extracted by pressurized fluid extraction in a ASE200 (Thermofisher Scientific, Waltham, MA, US) with dichloromethane /acetone (3:1, v/v). Aliquot of the extract was used to gravimetrically measure lipid contents. Another aliquot was spiked with surrogates as used for the incubated samples and purified by 5% $H_2O$ deactivated silica gel column chromatography, gel permeation chromatography and fully activated silica gel column chromatography according to Mizukawa et al.[6]

***Instrumental analysis***

We determined mono- to hexa-BDEs and BDE181, 183 and 190 by GC-ITMS (Thermofisher Scientific). The other BDEs were analyzed on GC-μECD (Agilent Technology). The method for instrumental analysis is shown in Hirai et al.[9] Compounds were identified and quantified against native standards (Cambridge Isotope Laboratory, Andover, MA, US).

*Laboratory experiments*

***Sample information***

The samples for in vitro experiment were common carp (*Cyprinus carpio*) and killifish (*Fundulus heteroclitus*). Both of them were cultured at pet shop or in aquarium at Marin Biological Laboratory on 2016. The liver of them were cut out from the body and kept in -80 °C until using.

***Microsome preparation and incubation***

The microsome was isolated according to Benedict et al.[10], Mizukawa et al.[6] and Stapleton et al.[3] The livers (150–250 mg wet weight) were homogenized in buffer. The homogenate was centrifuged (700 g, 4 °C, 10 min. and 10000 g, 4 °C, 20 min.). The supernatant was then ultracentrifuged (105000 g, 4 °C, 70 min.). After removing cytosol, the pellet was resuspended in the buffer. The microsome was stored at –80 °C until incubation. Before incubation, the microsome was diluted to 1 mg-protein/mL with incubation buffer. BDE99 standard were diluted to 3 nM in acetone. 200 μL of the microsomal solution was into a 4 mL glass amber incubation tube. The incubation was started by the addition of 1 μL of acetone PBDE solution. The microsomes with BDE99 were incubated at 25 °C with shaking at 50 rpm for 10 min. The reaction was stopped by the addition of 200 μL ice-cold methanol. Heat-inactivated microsomes were used as negative control.

***Extraction and cleanup of incubated samples***

After stopping the incubation, the incubated solutions were spiked with BDE105 as surrogate. Liquid–liquid extraction was conducted with 600 μL of hexane. After shaking, the organic phase was passed through anhydrous sodium sulfate for dehydration. The extraction was repeated three times. The extract was placed on a SPE cartridge (Bond-Elute SI, Agilent Technology, Hanover, CA, US) and first elution of 5mL of hexane was analyzed for PBDEs.

***Instrumental analysis***

BDE47, 49, 99 and 105 were determined by GC-MS (Agilent Technology). The instrumental method was set up as well as the method of field observations. Compounds were identified and quantified against native standards (Accustandard, New Haven, CT, US).

***RNA extraction, making cDNA and PCR***

The RNAs of the livers were extracted by TRIZOL® and DNase treatment was conducted for removing DNA. After extraction of RNA, we made cDNA by reverse transcript. The primers for our target protein: DIO2, which is one of deiodinase was designed based on NCBI Reference Sequence: NM_001309956.1(for killifish), CCD22052.1 (for common carp: DIO2.11) and CCD22053.1 (for common carp: DIO2.2). PCR was conducted using the primers and the cDNA and the products were loaded to electrophoresis with 1% agarose gel.

**Results and discussion**

*Field observations*

The congener profile of 8 PBDEs congeners from tetra to hexa in muscle tissues of each fish was shown in Figure1. From field observations, BDE99 was not detected from almost of the *Cyprinidae*, except for striped shiner which had very slight amount of BDE99. It can be explained that *Cyprinidae* have high debromination ability of BDE99. Though weather loach did not have BDE99, it was detected in Japanese eight barbel loach. They are *Cobitoidea* belonged to *Cypriniformes.* It is hypothesized that the high debromination ability has been diverged between *Cyprinidae* and *Cobitoidea.* Some kind of gobies was detected BDE99 such as red striped

tripletooth goby, another kind of goby was not detected BDE99 such as dusky tripletooth goby. In our previous study, though yellow fin goby had BDE99 in their tissues, ureogenic goby did not have BDE99. It seems that *Gobidae* has more complicated mechanisms of debromination ability than *Cyprinidae*.

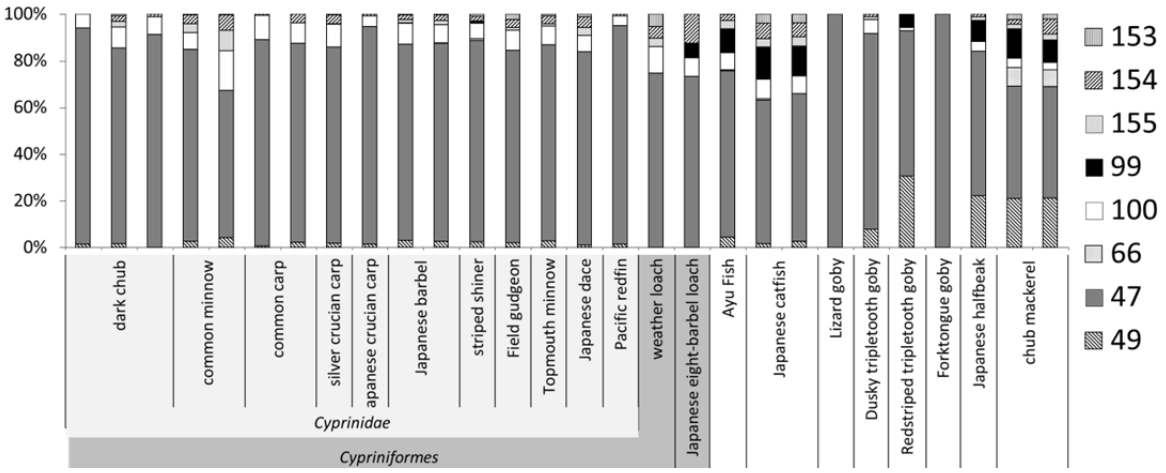


**Figure 1: Σ8 PBDE congener profile in muscle tissues of *Cyprinidae* and some other fish species**

*In vitro experiments*

To know debromination ability of *Cyprinidae* and other kind of fish, we compared debromination of BDE99 by hepatic microsome, deiodinase expression in their liver and deiodinase sequence of them. We used common carp and killifish for these experiments. In common carp, BDE47 was detected and BDE99 was decreased after incubations (Figure 2a). It means that debromination of BDE99 was observed. However killifish did not produce any lower brominated PBDEs and the amount of BDE99 was not changed (Figure 2b).

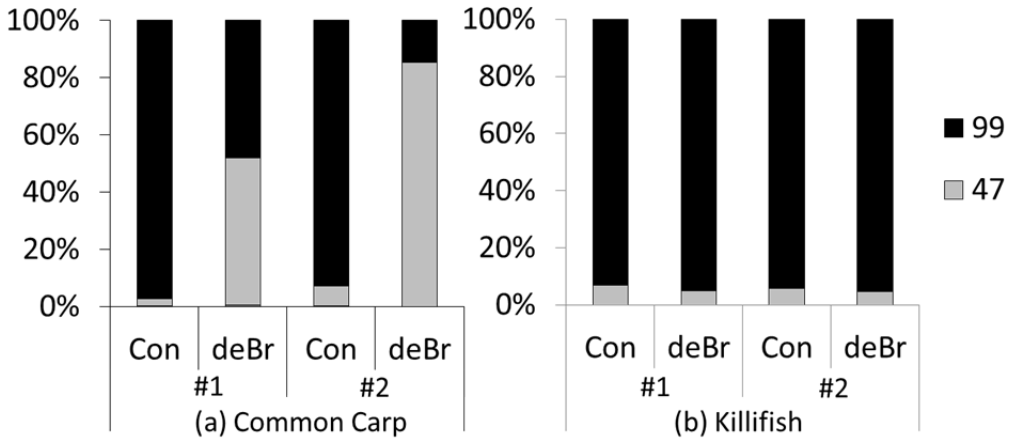


**Figure 2 : Debromination experiments of BDE99 by hepatic microsome of common carp (a) and killifish (b). Con: control section, deBr: debromination section**

There are two hypotheses to explain the difference of debromination ability between common carp and killifish. First is difference of deiodinase expression and second is difference of deiodinase structure. However DIO2 were expressed in both of the fish (Figure 3). Therefore we searched deiodinase sequences of many kinds of fish from NCBI database. Deiodinase has specific insertion which the name is “deiodinase insertion” around 33-61 AA downstream from active center. This insertion of common carp is same sequences among Cypriniformes however different from killifish. The future task is to investigate relationships the difference of crystal structure of DIO2 and debromination activity.

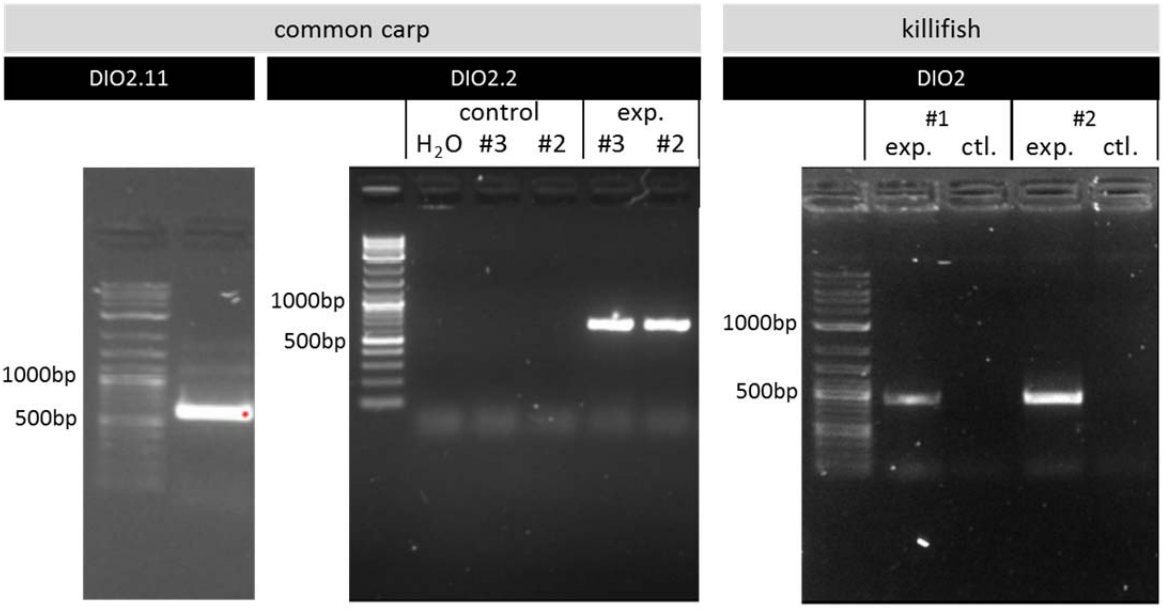


**Figure 3 : Expression of deiodinase in liver of common carp (left) and killifish (right)**

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