The potential for PFHxA to modulate the endocrine system in wildlife: A hypothesis-driven weight-of-evidence analysis across endocrine pathways

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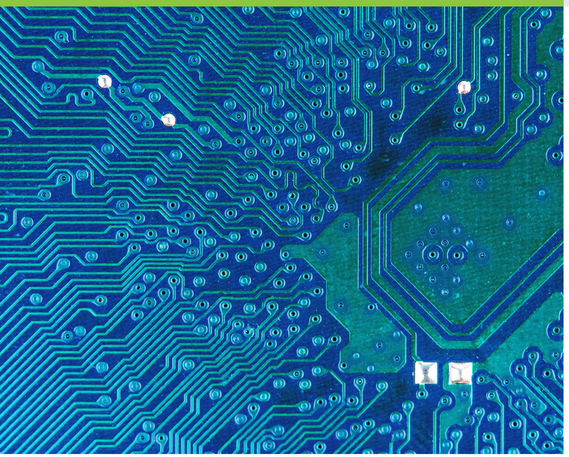
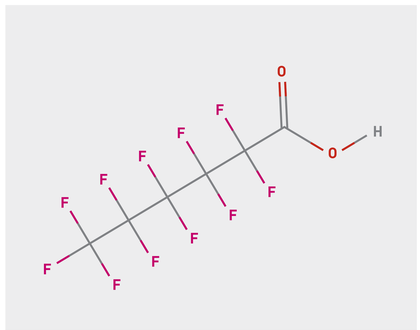


Background

- Perfluorohexanoic acid (PFHxA) (CAS No. 307-24-4) (**Figure 1**) is an environmental degradation product of C6-based fluorotelomer intermediates that are used to produce various kinds of polymers.
- Mixed-chain-length telomers can be metabolized in mammals and transformed in the environment to PFHxA (Rice, 2015). Therefore, levels of PFHxA measured in the environment and in human biomonitoring studies are rarely due to direct exposure to PFHxA itself.
- PFHxA did not cause any reproductive, developmental toxicity in exposed rats or mice or adverse effects in rats following repeat-dose administration.
- Concerns regarding endocrine activity of perfluorinated compounds in general resulted in a need to evaluate the endocrine activity of PFHxA, and determine whether PFHxA has endocrine disruptor (ED) properties according to the World Health Organization's definition of EDs (WHO, 2002):

"Exogenous substances that alter function(s) of the endocrine system and consequently cause adverse health effects in an intact organism or its progeny, or [sub]populations."

Figure 1. Chemical structure of perfluorohexanoic acid (PFHxA)



Study Objective

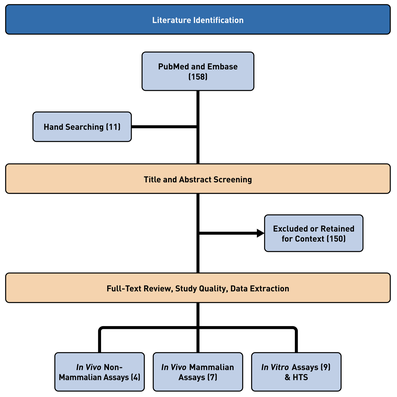
To determine whether PFHxA has endocrine activity in mammalian and or non-mammalian models across the estrogen (E), androgen (A), thyroid (T), or steroidogenesis (S) pathways that would alter the functioning of the endocrine system and cause adverse health effects in an intact organism.

Approach and Findings

Literature search and study report identification

- The endocrine activity of PFHxA was evaluated using a hypothesis-driven weight-of-evidence (WoE) analysis conducted across the E, A, T, and S pathways (Borgert et al., 2014).
- A comprehensive literature search was conducted (via two databases—PubMed and Embase) to capture relevant peer-reviewed literature (primary objective). Search syntaxes unique to each database were reviewed by identified endocrine experts.
- Publications used for contextual information (e.g., toxicokinetics, reviews) were identified for review (secondary objective). Selected hand-searching of full-text articles, along with identification of toxicology study reports, were also captured.
- Those PFHxA studies that were identified as evaluating endocrine activity were categorized as: *in vitro*, *in vivo* mammalian, or *in vivo* non-mammalian.
- Within PubMed and Embase, 158 articles were identified for PFHxA as capturing potentially relevant information (**Figure 2**). Hand-searching of references and secondary literature resulted in an additional eleven publications. Following title and abstract review, 19 publications were identified for full-text review. In some instances, the peer-reviewed articles contained multiple studies within the article (i.e., *in vitro* and *in vivo*), which were reviewed separately.
- Contextual information on PFHxA, along with high-throughput screening (HTS) data, were used to assist in the overall interpretation of the findings and ranking of endpoints, respectively.

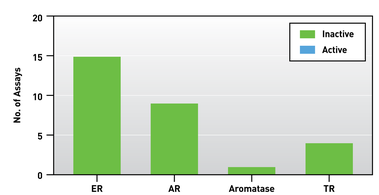
Figure 2. PFHxA studies identified across assay categories (i.e., *in vivo* non-mammalian, *in vivo* mammalian, and *in vitro*) were used to extract endocrine endpoint activity.



High-throughput screening (HTS) Assays

- *In vitro* PFHxA HTS data pertaining to androgen receptor (AR), aromatase, estrogen receptor (ER), and thyroid receptor (TR) activity were extracted from the ToxCast/Tox21 database (invitrodb_v2, released Oct 2015) (U.S. EPA, 2016).
- Chemical-assay bioactivity was characterized by hit calls, represented by values of 1, 0, or –1. A value of 1 indicated an "active" chemical, a value of 0 indicated an "inactive" chemical, and a value of –1 indicated that the activity could not be determined by U.S. EPA (Judson et al., 2016).
  - **Figure 3** summarizes the bioactivity of PFHxA by identifying the number of active assays per total number of assays conducted per pathway. There were no active ER, AR, aromatase, or TR assays reported.

Figure 3. HTS assays reported for ER, AR, TR, and aromatase activity in the ToxCast/Tox21 database. No HTS assays within these categories showed bioactivity.



Study quality and reliability assessment

- The studies identified in **Tables 1 and 2** were reviewed for study quality and relevance to assess endocrine activity, followed by identifying and extracting endocrine-specific endpoints across the E, A, T, and S pathways.
- Peer-reviewed articles and toxicology study reports identified for data extraction were reviewed for study quality using the software-based tool "ToxRTool" (Toxicological data Reliability assessment Tool) (Schneider et al., 2009). Scores of 1, 2, or 3 are assigned: 1, reliable without restrictions; 2, reliable with restrictions; and 3, not reliable (significant methodological or documentation deficiencies).
- As reported in **Table 1**, mixed quality scores were reported for the *in vitro* assays, with all but two mammalian *in vivo* studies having high quality scores (=1 or 2). Three non-mammalian *in vivo* assays received the lowest study quality score (=3), with a high quality score (=1) for one Organisation for Economic Co-operation and Development (OECD) guideline study (**Table 2**).
- Study reliability was considered within the context of the OECD Conceptual Framework for Testing and Assessment of Endocrine Disruptors (OECD, 2012), in which five levels of information are considered:

  Level 1: Existing data and non-test information, including all available eco-toxicological data from standardized or non-standardized tests.

  Level 2: *In vitro* assays providing data about selected endocrine mechanism(s) or pathways(s) (mammalian and non-mammalian methods).

  Level 3: *In vivo* assays providing data about selected endocrine mechanism(s) or pathways(s) for both mammalian and non-mammalian models. Some of these assays may provide evidence of adverse effects.

  Level 4: *In vivo* assays providing data on adverse effects on endocrine-related endpoints, where effects can be sensitive to more than one mechanism and may be due to non-ED mechanisms.

  Level 5: *In vivo* assays providing more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism, where effects can be sensitive to more than one mechanism and may be due to non-ED mechanisms.
- The OECD Conceptual Framework suggests using information collected from Level 1 and 2 methods of the framework primarily for prioritizing and informing mechanisms of action. Level 3 methods are for screening activity *in vivo* and informing possible endocrine mechanisms or toxicological pathways, and methods in Levels 4 and 5 provide information to characterize possible adverse effects at sensitive life stages and through life-cycle exposures. The level of methods used for PFHxA are indicated in Tables 1 and 2.

Table 1. Level 2 assays—*In vitro* assays to assess PFHxA per endocrine pathway; study quality and activity

Pathway	Assay	Activity	Quality Score	Data Source
Thyroid	Inhibition of T4 binding to hTTR	Positive (Weak)	2	Weiss et al., 2009
Thyroid	Thyroid-specific gene expression in exposed CEN/HGEN cells	Positive (weak)/ Negative	2	Vongphachan et al., 2011
Thyroid	Hex and Pax 8 expression in H4IIE rat hepatoma cells	Negative	2	Naile et al., 2012
Thyroid	Binding to hTRa-LBD T-screen cell proliferation	Negative/ Negative	2	Ren et al., 2015
Thyroid	Binding to hTTR/hTBG	Positive (weak)/Negative	3	Ren et al., 2016
HTS -Thyroid	TH receptor binding and transactivation	Negative (4/4 assays)	No Score	ToxCast/Tox21 Database
Estrogen	Trout ER binding	Positive (Weak)	2	Benninghoff et al., 2011
Estrogen	Estrogen nuclear transactivation in human MMV-Luc cells	Negative	2	Wielogorska et al., 2015
Estrogen	Aromatase activity in JEG-3 cells	Negative	2	Gorrochategui et al., 2014
Estrogen/ Androgen/ Steroidogenesis	ERTA (human receptor)/ARTA/ H295R-Steroidogenesis	Negative/ Negative/ Negative	1	Rosenmai et al., 2016
HTS-Estrogen	ER binding and ERTA	Negative (15/15 assays)	No Score	ToxCast/Tox21/EDSP21 Database
HTS-Androgen	AR Binding and ARTA	Negative (9/9 assays)	No Score	ToxCast/Tox21/EDSP21 Database
HTS-Aromatase	Aromatase Inhibition	Negative (1/1)	No Score	ToxCast/Tox21 Database

ERTA — ER Transactivation Assay. ARTA — AR Transactivation Assay.

Table 2. PFHxA *in vivo* mammalian and non-mammalian studies: Study quality and relevance with endpoints assessed per endocrine pathway

Study Type	Endpoints Assessed	Quality Score	Peer-Reviewed Study/ Toxicology Study Report
Mammalian — Level 1 and 4 methods			
Combined repeated-dose toxicity study (28-day) with reproduction/developmental toxicity screening OECD 422 – rats – Level 4	Estrogen, Androgen, Thyroid, Steroidogenesis	1	Kirkpatrick, 2005
Sub-chronic (90-day) toxicity study – rats	Estrogen, Androgen, Thyroid, Steroidogenesis	2	Chengelis et al., 2009
Chronic (2-year) cancer study – rats – Level 4	Estrogen, Androgen, Thyroid	1	Klaunig et al., 2015
Sub-Chronic (90-day) OECD 408; One-generation reproductive study OECD 415; Developmental study OECD 414 – mice (NaPFHx) – Level 4	Estrogen, Androgen, Thyroid, Steroidogenesis	1	Loveless et al., 2009
Combined developmental and perinatal/ postnatal reproduction study – rats (PFHxA ammonium salt) – Level 4	Estrogen, Androgen	1	Iwai and Hoberman, 2014
Epidemiological study – Level 1	Androgen	3	Zhou et al., 2016
Epidemiological study – Level 1	Thyroid	3	Li et al., 2017
Non-mammalian — Methods (Level 1 and 4)			
OECD 206, (quail; 21 weeks) –Level 4	Estrogen, Androgen, Thyroid	1	Frey et al., 2010
FSTRA-like (trout; 7 days) – Level 1	Estrogen, Androgen, Steroidogenesis	3	Benninghoff et al., 2011
OECD 206-like (quail; single dose) – Level 1	Thyroid	3	Cassone et al., 2012
AMA-like (FETAX) (frog; 10 days) – Level 1	Thyroid	3	Kim et al., 2015

FSTRA — Fish Short-Term Reproduction Assay. AMA — Amphibian Metamorphosis Assay.

Hypothesis-Driven WoE Analysis Across Endocrine Pathways

- A hypothesis-driven WoE analysis was conducted for PFHxA based on the ranking of endocrine endpoints extracted from Level 1 to Level 4 studies within eight hypotheses that cover activity across E, A, T, and S pathways (Borgert et al., 2014). The ranked endpoints are described as follows:

  Rank 1: The endpoints are specific and sensitive for the hypothesis, are interpretable without knowing the responses of other endpoints, and are *in vivo* measurements rarely confounded by artifacts or nonspecific activity.

  Rank 2: The endpoints are specific and sensitive for the hypothesis, and are interpretable without knowing the responses of other endpoints, but are less informative than Rank 1, often due to potential confounding influences; includes *in vitro* and *in vivo* endpoints.

  Rank 3: The endpoints are relevant for the hypothesis, but only when they corroborate Ranks 1 and 2 endpoints; includes some *in vitro* and many apical *in vivo* endpoints.
- The eight hypotheses are used to determine whether PFHxA is (1) an estrogen agonist, (2) an estrogen antagonist, (3) an androgen agonist, (4) an androgen antagonist, (5) a thyroid agonist, (6) a thyroid antagonist, (7) an inducer of steroidogenesis, and/or (8) an inhibitor of steroidogenesis.
- Rank 1 endpoints, which would carry the most weight, are identified mainly in Level 3 methods (OECD, 2012). Because Level 3 methods were not used for PFHxA, the majority of the endpoints were either Rank 2 or 3, except in the case of the one non-mammalian study that was similar to the Fish Short-Term Reproduction Assay (FSTRA) and several mammalian *in vivo* studies in which thyroid endpoints were ranked to evaluate PFHxA as a T antagonist.
- Although the vitellogenin (VTG) endpoint is a Rank 1 endpoint for estrogen activity, the quality of this study needs to be considered for this WoE analysis. This was not the case for thyroid Rank 1 endpoints, because these *in vivo* mammalian studies were all of high quality.
  - **Tables 3–5** summarize the endpoints that were ranked for evaluating PFHxA as an estrogen agonist and thyroid antagonist. Because there is no indication of activity across the other six hypotheses to suggest PFHxA activity, these tabular summaries for those hypotheses are not presented.

Table 3. Ranked endpoints to evaluate PFHxA as an estrogen agonist

Rank 1	Rank 2	Rank 3
FSTRA—VTG increase in males[1] FSTRA-like study, VTG was **increased**, but not significantly relative to controls, in plasma of rainbow trout (no distinction between genders) (14-day) at 250 ppm (Benninghoff et al., 2011).	**Ovarian weight decrease**[1] (female rodent studies) No change in rats (Kirkpatrick, 2005; Chengelis et al., 2009) and mice (Loveless et al., 2009).	**Estrous cyclicity** (in rodent studies) No change in rats (Kirkpatrick, 2005) or mice (Loveless et al., 2009).
	Ovarian histopathology (female rodent studies) No abnormalities in rats (Kirkpatrick, 2005; Chengelis et al., 2009; Klaunig et al., 2015) and mice (Loveless et al., 2009).	**Uterus weight** (female rodent studies) No change detected in rats (Chengelis et al., 2009) and mice (Iwai and Hoberman, 2014; Loveless et al., 2009).
	Testis weight decrease[1] (male rodent studies) No change in rats (Kirkpatrick et al., 2005; Chengelis et al., 2009) or mice (Loveless et al., 2009).	**Uterus histopathology** (female rodent studies) No abnormalities detected in rats (Kirkpatrick, 2005; Chengelis et al. 2009; Klaunig et al., 2015) and mice (Loveless et al., 2009).
	Testis histopathology (male rodent studies) No abnormalities in rats (Kirkpatrick, 2005; Chengelis et al., 2009; Klaunig et al., 2015) or mice (Loveless et al., 2009).	**Vaginal histopathology** (female rodent studies) No abnormalities in rats (Chengelis et al., 2009; Klaunig et al., 2015) and mice (Loveless et al., 2009).
	ERTA Negative for ERTA activity in MMV-Luc cells and BGLucE2 (hER) (Wielogorska et al., 2015; Rosenmai et al., 2016)	**Epididymis histopathology** (male rodent studies) No abnormalities in rats (Kirkpatrick, 2005; Chengelis et al., 2009; Klaunig et al., 2015) and mice (Loveless et al., 2009).
	ERTA and ERBA Negative activity in 15/15 assays integrated ER binding and transactivation assays.	**Mammary histopathology** (female rodent studies) No abnormalities in rats (Kirkpatrick, 2005; Chengelis et al., 2009; Klaunig et al., 2015) and mice (Loveless et al., 2009).
No Rank 1 endpoints in any study category.	No Rank 2 endpoints in any study category.	**Prostate histopathology** (male rodent studies) No abnormalities in rats (Kirkpatrick, 2005; Chengelis et al., 2009; Klaunig et al., 2015) and mice (Loveless et al., 2009).
		ERBA Positive (weak) competitive binding to rainbow trout hepatic ER (Benninghoff et al., 2011).
		Steroidogenesis Assay No changes were detected in 178-estradiol levels in exposed NCI-H295R cells (Rosenmai et al., 2016).

[1] Expected direction of endpoint. FSTRA — Fish Short-Term Reproduction Assay. VTG — vitellogenin.

Table 4. Ranked endpoints to evaluate PFHxA as a thyroid agonist

Rank 1	Rank 2	Rank 3
No rank 1 endpoints in mammalian or non-mammalian studies.	**Thyroid weight** (rodent studies) No changes in thyroid weight measured in rats (Kirkpatrick, 2005), increased weights in high-dose-recovery mice (Loveless et al., 2009).	**Thyroid follicular cell histopathology** (rodent studies) No abnormalities detected in rats (Kirkpatrick, 2005; Chengelis et al., 2009; Klaunig et al. 2015), Minimal changes noted in high-dose mice (Loveless et al., 2009).
	Thyroid hormones No change in chicken plasma T4 levels in OECD 206-like study (Cassone et al., 2012).	**AMA-like assay** Snout-vent length: reduced Head-to-tail lengths were reduced (Kim et al., 2015).

Table 5. Ranked endpoints to evaluate PFHxA as a thyroid antagonist

Rank 1	Rank 2	Rank 3
Thyroid weight (rodent studies) No changes in thyroid weight measured in rats (Kirkpatrick, 2005), increased weights in high-dose-recovery mice (Loveless et al., 2009)	**Thyroid hormones** No change in chicken plasma T4 levels in OECD 206-like study (Cassone et al., 2012).	No Rank 3 endpoints in any study category.
Thyroid follicular cell histopathology (rodent studies) No abnormalities detected in rats (Kirkpatrick, 2005; Chengelis et al., 2009; Klaunig et al., 2015), Minimal changes noted in high-dose mice (Loveless et al., 2009).	**Ovarian weight decreased**[1] (female rodent studies) No change (Kirkpatrick, 2005; Chengelis et al., 2009; Loveless et al., 2009).	

[1] Expected direction of endpoint

Study Category Summaries

In vitro assays (Tables 1 and 3; Figure 3)

- PFHxA was not active in any ER, AR, TR, or aromatase HTS assays.
- PFHxA had weak activity in an ER competitive binding assay reported in the literature, (Benninghoff et al., 2011) and was negative in an ERTA assay (Wielogorska et al., 2015).
- PFHxA did not show any activity in an AR transactivation assay or an assay that measures changes in steroidogenesis (Rosenmai et al., 2016).
- PFHxA also did not inhibit aromatase activity *in vitro* (Gorrochategui et al., 2014).
- A number of assays suggested potential T activity; however, in the study described by Vongphachan et al. (2011), although PFHxA increased expression of thyroid-responsive genes, the observed responses were not concentration-related or occurred only at extremely high concentrations. In addition, the same results were not observed in a second bird cell line.
- Weiss et al. (2009) and Naile et al. (2012) suggested evidence of thyroid activity, but these studies did not provide dose-dependent observations that PFHxA is a strong agonist/antagonist (<1% of T4 binding affinity). In a series of *in vitro* assays by Ren et al. (2015, 2016), no relevant binding with the TR was demonstrated.

In vivo mammalian (Tables 2-5)

- The rodent *in vivo* studies evaluated were of high quality (score of 1 or 2) and were relevant for evaluating endpoints across the E, A, T, and S pathways (Kirkpatrick, 2005; Loveless et al. 2009; Chengelis et al. 2009; Iwai and Hoberman, 2014; Klaunig et al. 2015). These studies included reproductive and developmental endpoints, along with repeated exposure up to 2 years without any adverse toxicity indentified.
- There were two human studies that did not provide insight or changes in hormone levels that could be correlated to PFHxA exposure; these studies were considered to be of low quality and used Level 1 methods (Zhou et al., 2016; Li et al., 2017).
- Based on the studies that provided Ranked 1, 2, or 3 endocrine endpoints across the eight hypotheses evaluated, PFHxA did not show any activity across E, A, T, or S pathways.

In vivo non-mammalian (Tables 2–5)

- PFHxA did not alter plasma T4 levels, pipping success, or morphology in exposed chickens, with no change in liver weight at any treatment level (Cassone et al., 2012). More importantly, no toxicological findings were reported in a 21-week OECD 206 bobwhite quail reproductive study (Frey et al., 2010).
- An Amphibian Metamorphosis Assay (AMA)-like study conducted by Kim et al. (2015) showed a reduction in an endpoint that could be considered a Rank 3 endpoint for evaluating a substance as a thyroid agonist (i.e., measurement of head-to-tail length). However, no other measures of thyroid activity were either ranked or suggested an alteration in the T pathway.
- A FSTRA-like study conducted by Benninghoff et al. (2011) showed an increase in trout plasma VTG level, but it was not significant relative to controls (no distinction between genders).

Conclusion

- Based on data available from both *in vitro* and *in vivo* mammalian and non-mammalian studies, PFHxA did not show any evidence of endocrine activity across the E, A, T, or S pathways.
- There was no indication of thyroid activity in the available non-mammalian studies, no evidence for adverse thyroid effects in mammalian studies, and overall no binding of PFHxA to TR with weak binding to TTR.
- Together these lines of evidence show limited concern for PFHxA interfering in the T pathway in either mammals or wildlife.

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