The potential for 6:2 FTOH to modulate the endocrine system in wildlife: A hypothesis driven weight-of-evidence analysis across endocrine pathways.

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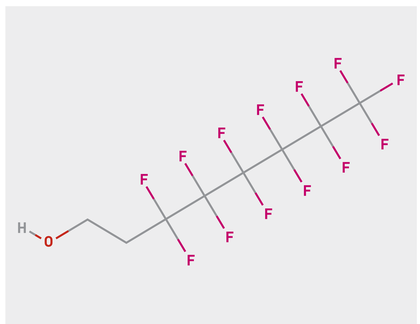
Background

- 6:2 Fluorotelomer alcohol (6:2 FTOH) (CAS No. 647-42-7) (**Figure 1**) is manufactured primarily for use as a raw material in fluorotelomer surfactants and is a product of side-chain fluorotelomer monomers (i.e., acrylates/methacrylate).
- These products are used to impart oil and water repellency and stain release protection to textiles and carpet that cannot be achieved with non-fluorinated alternatives.
- Administration of 6:2 FTOH to rats and mice did not cause any reproductive, developmental, or adverse effects following repeat-dose administration.
- In three aquatic studies, there were signals for weak estrogen-positive activity.
- *In vitro* assays suggested positive, although weak, estrogen receptor (ER) activity.
- Based on a suggestion of weak estrogen activity in published studies reported in the literature, there was a need to evaluate the endocrine activity of 6:2 FTOH and determine whether it has endocrine disruptor (ED) properties according to the World Health Organization's definition of and ED (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 6:2 fluorotelomer alcohol (6:2 FTOH)



Study Objective

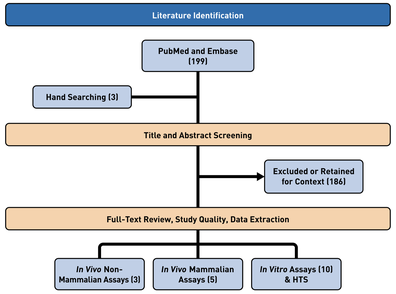
To determine whether 6:2 FTOH has endocrine activity in mammalian and or non-mammalian models across the estrogen (E), androgen (A), thyroid (T), or steroidogenesis (S) pathways that would alter the functioning of the endocrine system and cause adverse health effects in an intact organism.

Approach and Findings

Literature search and study report identification

- The endocrine activity of 6:2 FTOH 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 also identified for review (secondary objective) along with selected hand-searching of full-text articles and identification of toxicology study reports reviewed under REACH registrations.
- Those 6:2 FTOH studies that were identified to evaluate endocrine activity were categorized as: *in vitro*, *in vivo* mammalian, or *in vivo* non-mammalian.
  - A total of 199 articles on 6:2 FTOH were identified within PubMed and Embase to capture endocrine activity (**Figure 2**). Three additional articles were further identified by hand-searching. Following title and abstract review, 18 publications with studies across the three assay categories 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*).
  - Contextual information on the toxicokinetics of 6:2 FTOH along with high throughput screening (HTS) assay data, were used to assist in the overall interpretation of the findings, and ranking of endpoints, respectively.

Figure 2. 6:2 FTOH studies identified across assay categories (i.e., *in vivo* non-mammalian, *in vivo* mammalian, and *in vitro*) used for extracting endocrine endpoint activity.



Evaluation of High-throughput screening (HTS) Assay Data

- *In vitro* 6:2 FTOH HTS data pertaining to androgen receptor (AR), aromatase, estrogen receptor (ER), and thyroid receptor (TR) activity were extracted from the ToxCast/Tox21/EDSP21 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 defined by the U.S. EPA (Judson et al., 2016).
  - **Figure 3** summarizes the bioactivity of 6:2 FTOH by identifying the number of active assays per total number of assays conducted per pathway. 6:2 FTOH showed no bioactivity in the AR, aromatase, and TR assays with only 2/15 ER assays showing bioactivity. However, when the ER assay data were integrated, 6:2 FTOH was identified as inactive for ER agonist and antagonist activity (**Table 1**).

Figure 3. HTS assays reported for ER, AR, TR, and aromatase activity in the ToxCast/Tox21 database. The number of assays showing bioactivity of 6:2 FTOH per number of total HTS assays per category.

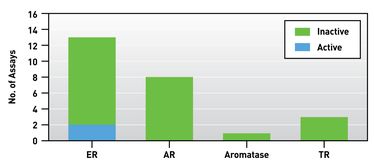


Table 1. Integrated endocrine pathway 6:2 FTOH bioactivity represented by AUC (area under the curve) (EDSP21 dashboard)

Model	Agonist AUC[1]	Antagonist AUC
ER	0.007	0
AR	0	0

[1] AUC values of 0 indicate inactivity, and values of 1 indicate full agonist or antagonist activity. AUC values <0.01 characterize an integrated pathway activity as inactive (Judson et al., 2015).

Study quality and reliability assessment

- The studies identified in **Tables 2 and 3** 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 were assigned: 1, reliable without restrictions; 2, reliable with restrictions; and 3, not reliable (significant methodological or documentation deficiencies).
  - As reported in **Table 2**, mixed quality scores were reported for the *in vitro* assays, with mammalian *in vivo* studies all having the highest quality score (=1), and the three non-mammalian *in vivo* assays receiving the lowest study quality score (=3) (**Table 3**).
- 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.
- Information collected from Level 1 and 2 methods are suggested to be used 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 method levels used for 6:2 FTOH are indicated in **Tables 2 and 3**.

Table 2. 6:2 FTOH; Level 2—*In vitro* assays: Study quality and endocrine pathways

Pathway	Assay	Activity	Study Quality Score	Peer-reviewed study
Thyroid	Competition with T4 for binding hTTR	Negative	2	Weiss et al., 2009
Thyroid	Binding to hTTR and TBG	Negative/ Negative	3	Ren et al., 2016
Thyroid	Binding to TR/ T-screen cell proliferation	Negative/ Negative	2	Ren et al., 2015
HTS, Thyroid	Three TR transactivation assays	Negative	No Score	ToxCast/Tox21
Estrogen	Yeast cells, hER- transactivation	Positive	3	Ishibashi et al., 2007
Estrogen	Yeast cells, Medaka ER, transactivation	Positive	3	Ishibashi et al., 2008
Estrogen	Trout ER binding/ in silico ER (mouse, human, trout) binding	Negative/ Positive (weak)	2	Benninghoff et al., 2011
Estrogen	E-Screen cell proliferation	Positive	3	Vanparys et al., 2006
Estrogen	E-Screen cell proliferation/ Cell cycle analysis/ Gene expression of selected genes	Positive/ Positive/ Positive	2	Maras et al., 2006
Estrogen/ Androgen/ Steroidogenesis	ERTA/ ARTA/ H295R- Steroidogenesis	Positive/ Negative/ Positive	1	Rosenmai et al., 2016
Estrogen	Vitellogenin (VTG) changes in tilapia hepatocytes; estrogen activity/ anti-estrogen activity.	Positive/ Positive	2	Liu et al., 2007
HTS-Estrogen	ER binding and ERTA	Negative	No score	ToxCast/Tox21/EDSP21
HTS- Aromatase	Aromatase inhibition	Negative	No score	ToxCast/Tox21
HTS-Androgen	AR Binding and ARTA	Negative	No score	ToxCast/Tox21/EDSP21

Table 3. 6:2 FTOH; *In vivo* mammalian and non-mammalian studies: Study quality and relevance with endpoints assessed per endocrine pathway

Study Type	Endpoints Ranked per Pathway	Quality Score	Peer-Reviewed Study/ Industry Report
Mammalian — Level 4 studies			
Combined 28-day toxicity study with reproductive and developmental endpoints—rats (OECD 422)	Estrogen, Androgen, Thyroid, Steroidogenesis	1	Kirkpatrick, 2005
Repeated 28-day toxicity study—rats	Estrogen, Androgen, Thyroid	1	Serex et al., 2014
Sub-chronic 90-day toxicity study—rats (OPPTS 870.3100)	Estrogen, Androgen	1	Daikin Industries—Study report, 2007
One-generation reproductive and developmental toxicity—rats (OECD 414/415)	Estrogen, Androgen	1	O'Connor et al., 2014
One-generation reproductive study—mice (OECD 415)	Estrogen Androgen	1	Mukerji et al., 2015
Non-mammalian — Level 1 studies			
FSTRA-like trout (14 days)	Estrogen, Androgen, Steroidogenesis	3	Liu et al., 2009
FSTRA-like medaka (3 days)	Estrogen, Androgen, Steroidogenesis	3	Ishibashi et al., 2008
FSTRA-like zebrafish (7 days)	Estrogen, Androgen, Steroidogenesis	3	Benninghoff et al., 2011

FSTRA — Fish Short-Term Reproduction Assay

Hypothesis-Driven WoE Analysis Across Endocrine Pathways

- A hypothesis-driven WoE analysis was conducted for 6:2 FTOH 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 description of 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 6:2 FTOH 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 in Level 3 methods (OECD, 2012). Because Level 3 methods were not conducted for evaluating 6:2 FTOH, the majority of the endpoints were either Rank 2 or 3, except in the case of the three non-mammalian studies that were similar to the Fish Short-Term Reproduction Assay (FSTRA). Although the vitellogenin (VTG) endpoint measured in these studies is a Rank 1 endpoint, the low quality of these studies was considered in the WoE analysis.
  - **Tables 4 and 5** summarize the endpoints that were ranked for evaluating 6:2 FTOH as an estrogen agonist and inhibitor of steroidogenesis. Because there is no indication of activity across the other six hypotheses to suggest 6:2 FTOH activity, these tabular summaries for those hypotheses are not presented.

Table 4. Ranked endpoints to evaluate 6:2 FTOH as an estrogen agonist

Rank 1	Rank 2	Rank 3
FSTRA—VTG increase in males[1] In FSTRA-like studies, VTG increased 8-fold in plasma of rainbow trout (14-day) at 250 ppm (Benninghoff et al., 2011); in male Japanese medaka (3-day) at concentrations >1 µM (Ishibashi et al., 2008) and in livers of female zebrafish (7-day) at 0.03, 0.3, and 3.0 mg/L, and in livers of males at 0.03 and 0.3 mg/L. There was a decrease in liver VTG in males at 3 mg/L, which is not unexpected since the exposure concentration is near the 96hr LC50 value in fish (Liu et al., 2009).	**Ovarian weight decrease[1]** Decreased P1 absolute weight in mice at 100 mg/kg (Mukerji et al., 2015). No change in rats dosed for 28 or 90 days (Kirkpatrick, 2005; Serex et al., 2014) or in P1 rats administered prior to and during mating, gestation, and lactation (O'Connor et al., 2014).	**Estrous cyclicity** No change (Kirkpatrick, 2005; O'Connor et al., 2014; Mukerji et al., 2015).
	Ovarian histopathology No abnormalities (Daikin study, 2007; Serex et al., 2014; O'Connor et al., 2014; Mukerji et al., 2015).	**Uterus weight increase[1]** No change (Serex et al., 2014) or decreased weights recorded at 250- or 100- mg/kg dose levels reported by O'Connor et al., 2014 and Mukerji et al., 2015, respectively.
	Testis weight decrease[1] Testes weights were increased or not changed (Kirkpatrick, 2005; Daikin study, 2007; Serex et al., 2014; O'Connor et al., 2014; Mukerji et al., 2015).	**Uterus histopathology** No abnormalities (Kirkpatrick, 2005; Daikin study, 2007; Serex et al., 2014; O'Connor et al., 2014; Mukerji et al., 2015).
	Testis histopathology No abnormalities (Kirkpatrick, 2005; Daikin study, 2007; Serex et al., 2014; O'Connor et al., 2014; Mukerji et al., 2015).	**Vaginal histopathology** No abnormalities (Kirkpatrick, 2005; Daikin study, 2007; Serex et al., 2014; O'Connor et al., 2014; Mukerji et al., 2015).
	ERTA Positive activity in the E-Screen assay; activation of cell proliferation in MCF-7 human cells (Maras et al., 2006; Vanparys et al., 2008), positive ER agonism in Yeast cells transfected with Medaka ERα (Ishibashi et al., 2007) and positive ER agonism in yeast cells transfected with hERα and hERβ (Ishibashi et al., 2008).	**Epididymis histopathology** No abnormalities (Kirkpatrick, 2005; Daikin study, 2007; Serex et al., 2014; O'Connor et al., 2014; Mukerji et al., 2015).
	***In vitro* estrogen activity** Positive increase in VTG with exposure suggest estrogen activity with inhibition of this change int the presence of tamoxifen or estradiol, suggesting anti-estrogenic activity in Tilopia hepatocytes (Lui et al., 2007).	
	ERTA and ERBA Positive activity in 2/15 assays integrated ER binding and transactivation assays. The AUC was integrated with a score of 0.007[1] (ToxCast/ Tox21/EDSP21).	**Mammary histopathology** No abnormalities (Kirkpatrick, 2005; O'Connor et al., 2014), or changes characterized by lactational hypertrophy and hyperplasia of glandular epithelium in 8/11 dams in mice dosed 100 mg/kg (Mukerji et al., 2015).
		FSTRA—GSI decrease in males and increase in females[1] FSTRA-like study GSI decreased in male and increased in female Zebrafish (Liu et al., 2009).
		ERBA Negative competitive binding to rainbow trout hepatic ER, weak *in silico* binding to human, mouse, and trout estrogen receptor ligand binding domain (Benninghoff et al., 2011).
		Steroidogenesis Assay Estradiol increased in H295R human cell-line at 6.3 µM (Rosenmai et al., 2016).

[1] Expected direction of endpoint. [2] As reported by Judson et al. (2015), a score of less than 0.01 indicates that the agonist pathway would be inactive.

Table 5. Ranked endpoints to evaluate 6:2 FTOH as an inhibitor of steroidogenesis

Rank 1	Rank 2	Rank 3
No ranked 1 endpoints identified across mammalian and non-mammalian studies.	**Steroidogenesis Assay** Increased estradiol levels in H295R human cell-line (Rosenmai et al., 2016).	**Testis weight** Testes weights were increased or not changed (Kirkpatrick, 2005; Daikin study, 2007; Serex et al., 2014; O'Connor et al., 2014; Mukerji et al., 2015).
	Steroidogenesis Assay No change in testosterone levels in H295R human cell-line (Rosenmai et al., 2016).	**Prostate weight** No change (O'Connor et al., 2014).
	FSTRA—VTG decrease in females[1] In FSTRA-like studies, VTG increased in female zebrafish livers (7-day) at 0.03, 0.3, and 3.0 mg/L, and male livers at 0.03 and 0.3 mg/L. There was a decrease in liver VTG in males at 3 mg/L (Liu et al., 2009).	**FSTRA—GSI** In a FSTRA-like study, GSI decreased in male and increased in female zebrafish (Liu et al., 2009).

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

Study Category Summaries

In vitro assays

- HTS assay data indicated there was overall negative bioactivity across the ER pathway, but a few assays in the peer-reviewed literature did show positive, although weak activity (**Tables 1, 2 and Figure 3**).
- 6:2 FTOH was not bioactive in the HTS assays across the ER, AR, or TR pathways and did not inhibit aromatase activity. In the peer-reviewed literature 6:2 FTOH was negative for AR transactivation activity, with no evidence of binding to the thyroid hormone transport protein or activity in the T-Screen cell proliferation assay (**Tables 1, 2 and Figure 3**).
- In a steroidogenesis assay, there was an increase in estradiol, but not testosterone levels measured in 6:2 FTOH–exposed H295R cells (**Table 2**).

In vivo mammalian studies summary

- All the *in vivo* mammalian studies evaluated (**Table 3**) were of the highest quality (score of 1) and were relevant for evaluating endpoints across the E, A, T, and S pathways. Four of the five mammalian studies (Level 4) were guideline studies, and all the studies were conducted under Good Laboratory Practices (GLP) with Rank 2 and 3 endpoints identified as negative across the eight hypotheses evaluated.
- In mammalian species, 6:2 FTOH is metabolized to several metabolites including PFHxA (Russell et al., 2015). As noted in Poster number RP021, PFHxA does not have activity across the E pathway.

In vivo non-mammalian study summary

- The *in vivo* non-mammalian (aquatic) studies with 6:2 FTOH are of low quality and reliability, which hinders their overall utility in the screening the potential endocrine activity of 6:2 FTOH (**Table 3**).
- Reported in these *in vivo* studies is a mild change in VTG concentration with exposure to high concentrations of 6:2 FTOH (compared to the positive control estradiol), as well as other endocrine changes (e.g., plasma hormone levels, mRNA expression). The shortcomings of the available aquatic toxicity studies in which 6:2 FTOH was evaluated include:
  - The use of dimethyl sulfoxide [DMSO] at high levels (~0.01%) within aquatic toxicity experiments can potentially increase the uptake of chemicals into aquatic biota (Hutchinson, 2006).
  - The absence of a control group without solvent since DMSO itself has been sometimes shown to affect fish reproductive capacities at low concentrations (Pawlowski et al., 2004) similar to those used in both Ishibashi et al. [2008] and Liu et al. [2009]. The presence of a solvent-free control group helps to establish a true baseline response, which is a basic "principle of sound ecotoxicology" established by Harris et al. [2014].
  - 6:2 FTOH exposure concentrations were not measured in any of the aquatic toxicity studies, so the concentration of 6:2 FTOH to which the fish were exposed cannot be confirmed.
  - It is critical to note that the exposure concentrations that altered VTG levels and other estrogenic signals (i.e., plasma hormone levels, mRNA expression) in these studies were well above environmentally relevant concentrations.
  - A positive control was not used in two of the aquatic toxicity studies.
- In the three non-mammalian studies, there were changes in VTG and the gonado-somatic index (GSI) (**Table 4**), with Liu et al. [2009] reporting changes in gene expression. mRNA expression changes were increased or decreased depending on dose and tissue examined, which suggested changes in ERα, GnRH, FSH and aromatase. While, these targets are pivotal for a normal functioning HPG axis in fish, the expression of these genes is not normally used within the ranking exercise but it suggests that further investigation for the potential of 6:2 FTOH to modulate the E pathway is warranted.

Conclusion

- Based on data available from both *in vitro* and *in vivo* mammalian and non-mammalian studies, 6:2 FTOH did not show any evidence of endocrine activity across the A, T, or S pathways.
- There was no evidence that 6:2 FTOH had E activity in mammalian studies (Level 4) across Rank 2 and 3 endpoints. However, there was weak evidence for E activity in three separate Level 1 non-guideline, non-mammalian studies. This weak activity was supported *in vitro*, with indications of positive ER activity in selected assays. Given the indication for positive, though weak, E activity, further conduct of a guideline assay is warranted, in which multiple validated estrogen endpoint data would be collected with characterized 6:2 FTOH exposure conditions.
- A screening-level ecotoxicology assessment such as the FSTRA (OECD TG 229, Level 3) is recommended to fully examine whether 6:2 FTOH interferes with the E pathway.

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