1   **Bioconcentration and metabolic effects of emerging PFOS alternatives in**

2   **developing zebrafish**

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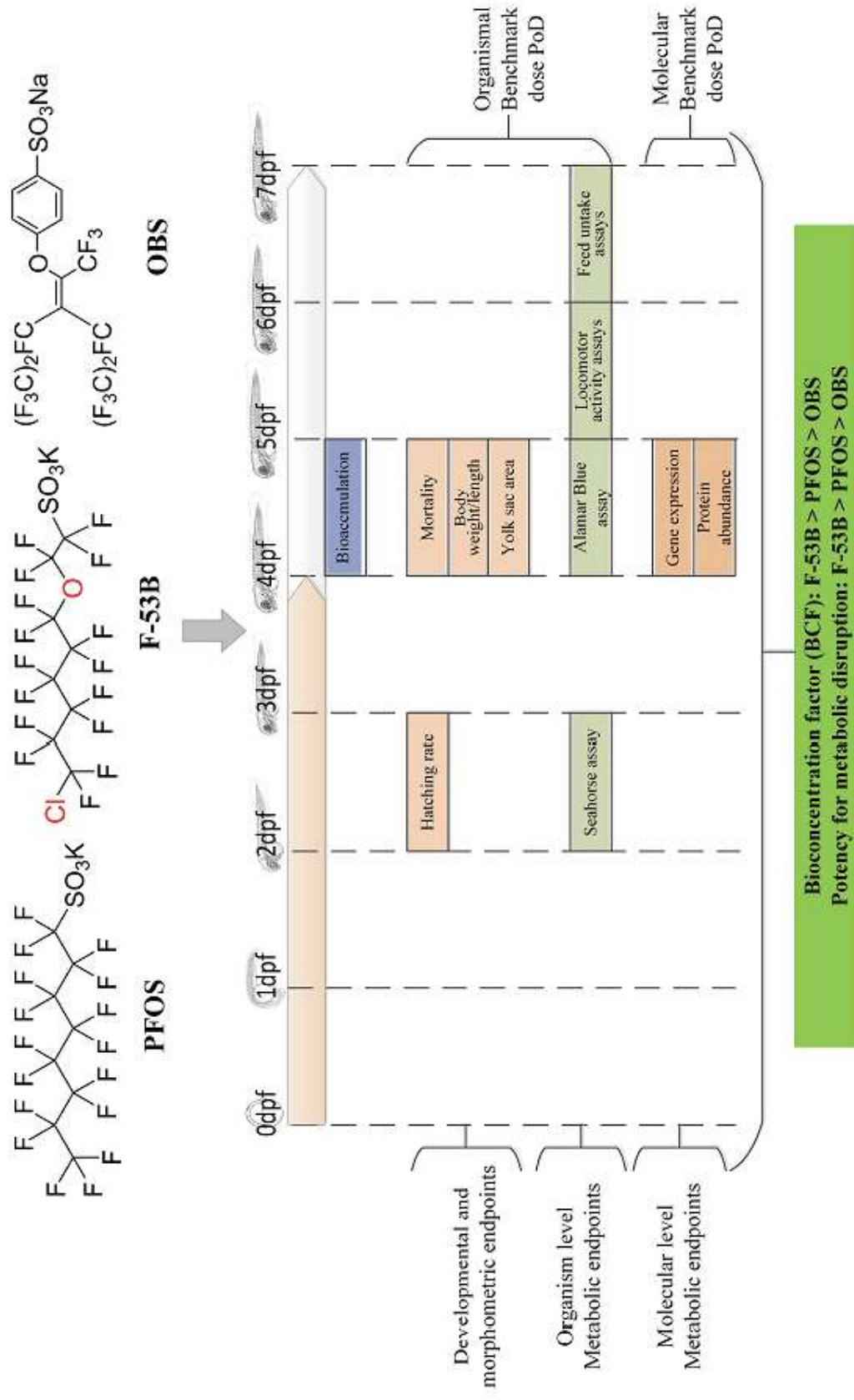18   Dr. Jan A. Mennigen

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20

Abstract

22 The novel PFOS alternatives 6:2 chlorinated polyfluorinated ether sulfonate (F-53B) and sodium

23 *p*-perfluorous nonenoxybenzene sulfonate (OBS) are emerging in the Chinese market, but little is

24 known about their ecological risks. In this study, zebrafish embryos were exposed to PFOS, F-

25 53B and OBS to evaluate their bioconcentration and acute metabolic consequences. Per- and

26 polyfluoroalkyl substances (PFASs) accumulated in larvae in the order of F-53B > PFOS > OBS,

27 with the bioconcentration factors ranging from 20 to 357. Exposure to F-53B and PFOS, but not

28 OBS, increased energy expenditure, and reduced feed intake in a concentration-dependent

29 manner and the expression of genes involved in metabolic pathways at the transcriptional and

30 translational levels. Molecular docking revealed that the binding affinities of PFASs to

31 glucokinase were decreased in the following order: F-53B > PFOS > OBS. Finally, the results of

32 Point of Departure (PoD) indicate that metabolic endpoints at the molecular and organismal level

33 are most sensitive to F-53B followed by PFOS and OBS. Collectively, F-53B has the highest

34 bioconcentration potential and the strongest metabolism-disrupting effects, followed by PFOS

35 and OBS. Our findings have important implications for the assessment of early developmental

36 metabolic effects of PFOS alternatives F-53B and OBS in wildlife and humans.

37 **Keywords:** PFOS alternatives; bioconcentration; metabolic disruption; zebrafish; energy balance

Environmental Science & Technology

38   **TOC art**

PFOS

F-53B

OBS

Developmental and morphometric endpoints

Organism level Metabolic endpoints

Molecular level Metabolic endpoints

0dpf   1dpf   2dpf   3dpf   4dpf   5dpf   6dpf   7dpf

Hatching rate

Seahorse assay

Bioaccumulation

Mortality

Body weight/length

Yolk sac area

Alamar Blue assay

Locomotor activity assays

Feed untake assays

Gene expression

Protein abundance

Organismal Benchmark dose PoD

Molecular Benchmark dose PoD

Bioconcentration factor (BCF): F-53B > PFOS > OBS
Potency for metabolic disruption: F-53B > PFOS > OBS

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1. Introduction

41

42   In recent years, 6:2 chlorinated polyfluorinated ether sulfonate (F-53B) and sodium *p*-

43   perfluorous nonenoxybenzene sulfonate (OBS) have emerged as novel substitutes for

44   perfluorooctanesulfonic acid (PFOS) in the Chinese market (**Figure S1**). F-53B, which is

45   structurally similar to PFOS, is used as a chrome mist suppressant in Chinese electroplating

46   industry, with an annual production of 20-30 tons,[1] while OBS is used in fire-fighting foams and

47   photographic materials, with an annual production capacity of 3500 tons.[2] Without the same

48   regulations and restrictions as PFOS on their use, F-53B has been identified in industrial

49   wastewater, river water and municipal sewage sludge at concentrations ranging from 43 µg/L to

50   112 µg/L, 2.0 ng/L to 7.6 µg/L and 0.02 ng/g to 209 ng/g, respectively.[3-6] Furthermore, the

51   accumulation of F-53B was reported in the blood of crucian carp (20.9-41.9 ng/g), marine

52   mammals (0.023-0.27 ng/g) and human serum (0.8-2.3 ng/mL).[1, 7, 8] In 2017, Xu et al.[9] first

53   reported the detection of OBS in lake water with a concentration up to 3.2 µg/L. With the

54   continued use of F-53B and OBS, it is critical to investigate the toxic effects of these

55   alternatives.

56   In comparison to PFOS, few studies have been conducted to evaluate the bioconcentration

57   and negative effects of F-53B and OBS. Previous studies have shown that F-53B could

58   accumulate in aquatic organisms and induce developmental and reproductive toxicity as well as

59   thyroid endocrine disruption.[10-13] Recently, gut barrier dysfunction and hepatic metabolism

60   disorder have been found in mice after exposure to OBS.[14] Increasing evidences suggest that

61   exposure to PFOS has been linked to metabolic disruption. For example, PFOS has been

62   associated with reduced feed intake and increased spontaneous activity in rodents,[15-18] and a

63   negative correlation between PFOS exposure and birth weight,[19, 20] and a positive correlation

64   between PFOS exposure and circulating cholesterol and type II diabetes in humans have been

65   reported. [21, 22] In addition, adult zebrafish exposure to PFOS has been also reported to reduce

66   body weight,[23] induce spontaneous hyperactivity[24-26] and disturb lipid and lipoprotein

67   metabolism.[27-29] In contrast to these comparatively well-characterized metabolism-disrupting

68   effects of PFOS, the potential metabolic disrupting potential of the emerging PFOS alternatives

69   F-53B and OBS is currently unknown.

70   Zebrafish has become an increasingly popular model to study human metabolic diseases

71   since it possesses conserved metabolic pathways at the genetic level and high homology of

72   pathways involved in metabolic regulation including energy intake, storage and usage.[30] In this

73   study, the bioconcentration, developmental toxicity, morphometric endpoints, energy balance at

74   the levels of feed intake, oxidative energy expenditure and locomotion of F-53B, OBS and PFOS

75   were studied in early developing zebrafish. To probe metabolic effects at the molecular level, the

76   key transcripts and proteins involved in the regulation of feed intake and energy expenditure,

77   glucose and lipid metabolism and somatic growth were quantified. Additionally, molecular

78   docking was used to further explore the interactions between glucokinase and per- and

79   polyfluoroalkyl substances (PFASs). Finally, in order to compare metabolic disruption thresholds

80   at the organismal and transcript level in developing zebrafish, we used endpoints in a benchmark

81   dose approach to determine the point of Departure (PoD).[31]

82   ## 2. Materials and methods

83   ### 2.1 Chemicals and reagents

84   PFOS (purity ≥ 98%), F-53B (purity ≥ 98%) and OBS (purity ≥ 98%) were purchased from

85   Sigma-Aldrich (Oakville, ON, Canada), Shanghai Maikun Chemical Co., Ltd. (Shanghai, China)

86   and Ningbo Yongshen Trading Co., Ltd. (Zhejiang, China), respectively. $^{13}C_4$-labeled L-PFOS

87   was obtained from Wellington Laboratories Inc. (Guelph, ON, Canada). All other chemicals and

88   solvents were of HPLC grade or analytical grade.

89   *2.2 Animal exposure*

90   Wild-type adult zebrafish (AB strain), housed at the University of Ottawa Aquatic Care

91   Facility, were bred in 1L breeding tanks separated with dividers. Fertilized zebrafish embryos (3

92   h post-fertilization, hpf) were randomly exposed to various concentrations of PFOS (0.025, 0.25

93   and 2.5 mg/L), F-53B (0.015, 0.15 and 1.5 mg/L) and OBS (0.04, 0.4 and 4 mg/L). The three

94   exposure concentrations of each compound were chosen based on 0.1%, 1% and 10% of the

95   previously established 96 h-LC_{50} of each respective compound (unpublished results of W. Tu).

96   Each treatment and the control (only contained system water; pH 7.5 ± 0.5, conductivity 500 ±

97   50 μS) were performed in triplicate. Zebrafish embryos/larvae were maintained at 28.5 °C with a

98   12-h light/12-h dark cycle. All exposure solutions were renewed daily until 4 dpf, after which the

99   larvae were raised in clean system water. The exposure solutions were sampled at the beginning

100   of exposure (T_0) and before the first renewal (T_{24}) to measure the actual concentrations of PFASs

101   in exposure solutions. A rapid decrease in the concentration of PFASs was observed at T_0, which

102   was mainly attributed to their adsorption on the surface of glass beakers,[10] while a small

103   fluctuation was measured at T_{24} (**Table S1**). All experimental protocols were approved by the

104   University of Ottawa's Animal Care and Veterinary Service (ACVS) protocol for the use of

105   animals in research and teaching (BL-3006).

106   *2.3 Quantification of PFASs in exposure solutions and zebrafish larvae*

107   The extraction of PFASs from the exposure solutions and zebrafish larvae were carried out

108   in accordance with our previously established protocol for F-53B.[13] Chromatographic separation

109   was performed on an Eclipase Plus C18 column (1.8 μm × 2.1 mm ×50 mm, Agilent, CA, USA).

110   The mobile phase was 10 mM aqueous ammonium acetate (A) and methanol (B). The solvent

111   gradient started at 40% B to 100% B over 3 min and was held for 1 min, followed by equilibrium

112   at 40% B for 1 min. The identification and quantification were carried out on an Agilent 1290

113   Infinity HPLC System consisting of an Agilent 6420 Triple Quadrupole (Santa Clara, CA, USA)

114   in the negative ESI mode with multiple reaction monitoring (MRM). Additional details are given

115   in the Supporting Information (**Text S1**).

116   **2.4 Developmental toxicity and morphometric endpoints**

117   The number of dead embryos/larvae was determined daily. The hatching rate at 48 hpf was

118   calculated by dividing the number of hatched larvae by the total number of exposed larvae. After

119   4 days of exposure, 18 larvae from each treatment were randomly selected for body length and

120   yolk sac area measurement. The yolk sac region contains the entire visible contour surrounding

121   the yolk sac and the yolk sac extension. Three pools of 60 larvae from each treatment were used

122   to measure body weight.

123   **2.5 Energy expenditure assays**

124   *2.5.1 Seahorse assay*

125   At 2 dpf, zebrafish larvae (n = 15 larvae/treatment) were placed in 500 μL system water in

126   24-well Seahorse islet capture plates as previously described.[32] Following 1 h of acclimation,

127   plates were run in a Seahorse XF24 machine (Agilent, USA) using the standard energy

128   phenotype protocol to obtain baseline and stressor-induced oxygen consumption rate (OCR) and

129   extracellular acidification rate (ECAR). The injected stressor mix (FCCP + oligomycin,

130   Agilent, CA, USA) resulted in a final well concentration of 3 μM FCCP and 8 μM oligomycin,

131   respectively. Data were obtained by repeated measurements at baseline and following the

132   injection of the stressor mix using the standard Seahorse Agilent energy phenotype kit protocol.

133   Metabolic potential of OCR and ECAR were expressed as ratios between stressor induced and

134   baseline values for each parameter obtained from the standard Seahorse Agilent energy

135   phenotype kit protocol.

136   *2.5.2 Alamar Blue assay*

137   Alamar Blue assay, which is dependent on a NADH-, NADPH-, $FADH_2$- and $FMNH_2$-

138   based reduction of Alamar Blue (resazurin), has been described as proxy to quantify zebrafish

139   oxidative metabolism.[33-35] At 4 dpf, zebrafish larvae (n = 72 larvae/treatment) were transferred

140   into black 96-well plates. This assay was adapted according to the protocol developed by

141   Renquist et al.[33] The fluorescence at 0 h and 24 h was measured in a fluorescence reader

142   (Molecular Devices, USA) with excitation and emission wavelengths of 530 nm and 590 nm,

143   respectively.

144   *2.5.3 Locomotor behavior assay*

145   At 5 dpf, zebrafish larvae (n = 36 larvae/treatment) were transferred into 48-well plates.

146   Each well contained one larva and 500 μL of system water. The swimming behavior and average

147   locomotor activity of zebrafish larvae were quantified using the ZebraBox (Viewpoint Behavior

148   Technology, France). The movement was recorded for a 20-minute period under light condition.

149   The ViewPoint software (ViewPoint Behavior Technology, France) was used to record the

150   distance of the movement.

151   **2.6 Feeding assay**

152   At 6 dpf, zebrafish larvae (n = 36 larvae/treatment) were over-fed with commercial

153   zebrafish food labeled with fluorescent 4-(4-(didecylamino)styryl)-N-methylpyridinium iodide

154   (4-Di-10-ASP, Sigma-Aldrich, Canada) in Petri dishes. After one hour of feeding, the larvae

155   were collected, washed and homogenized with 300 μL of double-distilled water. The 250 μL

156   homogenate was then transferred to a black 96-well plate. The fluorescence was determined by a

157   fluorescence reader (Molecular Devices, USA) with excitation and emission wavelengths of 485

158   nm and 535 nm, respectively.

159   ***2.7 Targeted metabolic gene expression analysis***

160   At 4 dpf, zebrafish larvae (n = 45 larvae/treatment) were harvested and the total RNA

161   extraction, first-strand cDNA synthesis and real-time RT-PCR were performed according to the

162   protocol previously described.[10] The primer sequences for mRNA real-time RT-PCR are shown

163   in **Table S2**. Prior to the analysis, we carried out a trial and confirmed the stability of β-actin

164   under our experimental conditions. Thus, the housekeeping gene β-actin was used as reference

165   gene for normalization.

166   ***2.8 Targeted tissue-specific miRNA analysis***

167   Total RNA was used to synthesize cDNA with HiFlex buffer using the miScript II RT kit

168   (Qiagen, Toronto, ON, Canada) and Specific miRNAs were subsequently quantified using the

169   miScript SYBR Green PCR kit (Qiagen, Toronto, ON, Canada) with miRNA-specific forward

170   primers and a universal reverse primer (**Table S3**). Relative transcript abundance was

171   normalized to *U6* transcript abundance. More details are provided in the Supporting Information

172   (**Text S2**)

173   ***2.9 Protein extraction and Western blot analysis***

174   The total protein was extracted from zebrafish larvae (4 dpf, n = 45 larvae/treatment)

175   according to our previously described procedure.[10] Approximately 50 μg protein was separated

176   by SDS-PAGE, then transferred to a polyvinylidene difluoride (PVDF) membrane and probed

177   with rabbit antibodies raised against neuropeptide Y (NPY; Cell Signaling Technologies,

178   Beverly, MA) or glucokinase (GK; Abcam, Cambridge, UK). Protein levels were quantified by

179   densitometry analysis, with the results normalized to GAPDH abundance.

180   *2.10 Molecular docking analysis*

181   The difference in toxicity of a compound may be attributable to the difference in binding

182   affinity to the target protein.[36] In this study, since we identified a consistent and strong decrease

183   in gk gene expression in response to all PFASs tested and the important role of GK in the

184   metabolism of glucose and lipid energy metabolism, GK was selected for molecular docking to

185   investigate the interactions of PFASs with GK at the atomic level. The 3D structure of zebrafish

186   glucokinase (zfGK) was built by homology modeling strategy (PDB ID 3FR0) using Modeller

187   9.14.[37] The quality of the zfGK was evaluated by PROCHECK, ERRAT and Verify 3D in

188   SAVES (http://servicesn.mbi.ucla.edu/SAVES/) and the results indicated that modeled zfGK was

189   a good quality model (Figure S2). The docked active site of zfGK was defined as the location of

190   the ligand of the template protein. PFASs were docked into the active site of zfGK using

191   AutoDock Vina.[38] AutoDock Tools 1.5.6 package (http://mgltools.scripps.edu) was employed for

192   the generation of docking input files and identify the grid center. The superior pose with the

193   lowest docked energy was selected and visually analyzed by Discovery Studio Visualizer 4.5

194   (San Diego, CA, USA). More details are provided in the Supporting Information (Text S3).

195   *2.11 Statistical analysis*

196   All individual endpoint data are reported as the mean ± standard error of the mean (SEM).

197   A One-way analysis of variance (ANOVA) followed by Dunnett's post hoc test was conducted to

198   assess statistical differences between the treatment groups and the control. For Seahorse data, a

199   two-way ANOVA was used for analysis of treatment groups and stress mix, and their interaction.

200   Statistical significance was determined with a cut-off of $p < 0.05$. Data analysis and graphs were

201   performed using GraphPad Prism version 7.0 (GraphPad, Inc., San Diego, CA, USA).

202   *2.12 Bench mark dose modelling and PoD determination*

203   The benchmark dose (BMD) and Point of Departure (PoD) approaches were utilized to

204   integrate organismal and molecular endpoints into a comparable endpoint for environmental risk

205   assessment of metabolic disruption of the PFASs in zebrafish. Briefly, BMDExpress version 2.2

206   software (https://www.sciome.com/bmdexpress/) was used for the benchmark dose

207   calculations.[39-41] The best BMD value for each compound and parameter was chosen by the

208   BMDExpress software between those established by a third order polynomial, a third order

209   exponential and a Hill equation, depending on the best-fitted model for each endpoint (**Table**

210   **S4**).

3. Results

212   *3.1 Bioconcentration of PFASs in developing zebrafish*

213   The concentrations of PFASs in zebrafish larvae showed clear dose-dependent manners

214   (**Figure 1A**), with 1.4, 24.3 and 160.7 mg/kg wet weight (ww) for PFOS, 1.0, 27.1 and 137.3

215   mg/kg for F-53B, and 0.6, 14.4 and 114.7 mg/kg for OBS. The calculated bioconcentration

216   factors (BCFs) were 113-193 for PFOS, 125-358 for F-53B and 20-48 for OBS (**Figure 1B**).

217   *3.2 Zebrafish developmental toxicity and morphometric endpoints are minimally affected by*

218   *PFASs*

219   Exposure to PFASs did not significantly affect hatching rate and mortality (**Figure S3**).

220   However, the yolk sac area was significantly reduced in high F-53B exposure group compared to

221   the control (**Figure S4A**). PFASs exposure did not significantly affect larval body weight and

222   body length (**Figure S4B-C**).

223   ### 3.3 PFASs induce energy balance parameter changes in developing zebrafish

224   Several aspects of the organismal level metabolic phenotype were significantly affected by

225   PFASs exposure in early developing zebrafish. Embryo oxygen consumption rate (OCR) was

226   significantly reduced in high PFOS exposure group (**Figure 2A**) and all OBS exposure groups

227   irrespective of concentration (**Figure 2C**) compared to the control. The application of the

228   stressor mix significantly increased OCR in PFASs ($p < 0.001$), while no significant interaction

229   between treatment and stressor was found (**Figure 2A-C**). This lack of interaction is also evident

230   in the observed lack of significant effects of OCR potential (**Figure 2D-F**), calculated as the Δ

231   between stressor-induced and baseline OCR. Only PFOS significantly increased the extracellular

232   acidification rate (ECAR) at the low concentration compared to the control (**Figure 2G**). While

233   exposure to the stressor mix significantly increased ECAR in PFASs ($p < 0.01$), no interaction

234   effects between PFASs exposure and stressor (**Figure 2G-I**), and no effects on ECAR potential

235   (**Figure 2J-L**), calculated as the Δ between stressor-induced and baseline ECAR, were

236   identified.

237   Indirect measurement of oxidative energy expenditure using the 24 h Alamar Blue assay

238   increased significantly in zebrafish exposed to medium and high PFOS concentrations and all F-

239   53B concentrations (**Figure 3A**). Locomotory behavior, assessed as total distance travelled, was

240   not significantly affected by exposure to PFASs at any concentration (**Figure 3B**). Feed intake

241   was significantly reduced in medium and high PFOS exposure groups and all F-53B exposure

242   groups (**Figure 3C**).

243   ### 3.4 PFASs differentially affect metabolic gene expression profiles in developing zebrafish

244   The expression of several transcripts involved in central regulation of energy expenditure

245   was profiled (**Figure 4A-F**). Compared to the control, *npy* transcript abundance was significantly

246   reduced by high PFOS, low and high F-53B, and medium and high OBS exposure. The *pomca*

247   transcripts were significantly reduced in larvae exposed to high PFOS and all OBS

248   concentrations compared to the control. Transcript abundance of *cart* was significantly reduced

249   in medium OBS exposed larvae compared to the control. No changes in transcript abundance

250   were observed for *agrp*, *cart2* and *mc4r*. The expression of transcripts involved in glucose

251   metabolism (**Figure 4G-J**) revealed a significant reduction on *gk* expression in all exposed

252   zebrafish compared to the control. Transcript abundance of *pck1*, *insa*, and *insb* did not change

253   significantly in larvae exposed to PFASs. The expression of transcripts involved in lipid

254   metabolism (**Figure 4K-M**) revealed that PFASs significantly reduced *cpt1a* transcript

255   abundance in low and high PFOS and F-53B exposure groups and in all OBS exposure groups

256   compared to the control. No significant differences in transcript abundance of *fasn* and *ppara*

257   were identified. Both profiled transcripts involved in the hypothalamus-pituitary-somatotropic

258   (HPS) axis, *gh* and *igf2*, were significantly affected by exposure to PFASs (**Figure 4N-O**).

259   Transcript abundance of *gh* was significantly reduced by high F-53B and medium OBS

260   exposure. Transcript abundance of *igf2* was significantly reduced by medium OBS exposure.

261   Finally, PFASs exposure did not affect the transcriptomic expression of *ucp2* (**Figure 4P**).

262   *3.5 PFASs differentially affect tissue-specific miRNA expression profiles in developing*

263   *zebrafish*

264   The expression of several tissue-specific and enriched miRNA transcripts was profiled

265   (**Figure S5**). Significant changes in response to PFASs exposures were identified for muscle-

266   specific *miRNA-1* (**Figure S5A**), but not muscle-specific *miRNA-133* (**Figure S5B**), liver-

267   specific *miRNA-122* (**Figure S5C**) or pancreas-enriched *miRNA-375* (**Figure S5D**). Compared to

268 the control, *miRNA-1* transcript abundance (**Figure S5A**) was significantly reduced by high OBS

269 exposure.

3.6 PFASs exposure reduce GK and NPY protein levels in developing zebrafish

271 Exposure to PFASs resulted in a dose-dependent decrease in GK protein abundance, but a

272 significant reduction was observed only in medium and high OBS exposure groups compared to

273 the control (**Figure 5A-C**). NPY protein abundance (**Figure 5D-F**) was significantly reduced by

274 high PFOS exposure compared to the control (**Figure 5D**)

3.7 Binding potential of PFASs toward zebrafish GK (zfGK)

276 As illustrated in **Figure 6**, PFASs were docked into the active site of zfGK. The binding

277 energy and hydrogen bond interactions obtained from docking analysis are listed in **Table S5**. In

278 the docked complexes, hydrogen bonds were formed between PFOS and residues of Asn70,

279 Ser138, Thr215 and Gly431; F-53B and residues of Thr155, Asn191, Gly216 and Cys217; and

280 OBS and residues of Gly68, Thr69, Gly216 and Ser432. The binding affinity of PFASs toward

281 zfGK in the order of F-53B > PFOS > OBS, and the corresponding binding energies were -7.8, -

282 6.8 and -6.2 kcal/mol, respectively.

3.8 Transcriptomic and organismal PoD of metabolic disruption

284 The transcriptomic point of departure (PoD) was calculated to be 0.0038, 0.0080 and

285 0.00141 mg/L for F-53B, PFOS and OBS, respectively (**Figure 7A**). Regarding the PoD for the

286 organismal level endpoints, the order of PoD thresholds from most sensitive to least sensitive

287 was maintained albeit at higher thresholds of 0.0063, 0.0122 and 0.9412 mg/L for F-53B, PFOS

288 and OBS, respectively (**Figure 7B**). A summary of the median BMD and BMDL values for

289 PFASs can be found in **Table S6**.

4. Discussion

291   *4.1 Differential accumulation of PFASs in developing zebrafish*

292   The high body burden of PFOS, F-53B and OBS in zebrafish larvae indicates their

293   bioconcentration potential as other PFASs. The accumulation of PFOS and F-53B has been

294   previously reported in fish and mammals,[1,7,13] and OBS has been recently found to accumulate in

295   the liver and gut of mice (unpublished results of W. Tu). To compare the bioconcentration potential

296   of the three PFASs in developing zebrafish, their respective BCFs were determined. The results

297   showed that the BCFs of PFOS and F-53B are of the same order of magnitude, which are one

298   order of magnitude higher than OBS. This suggests chemical structure-specific differences in

299   uptake, bioconcentration and metabolism. In the terms of medium exposure concentration, F-53B

300   exhibited twice the bioconcentration potential than PFOS. This is not surprising since F-53B has

301   been reported to be the most bio-persistent PFASs to date,[42] and the BCF of F-53B was also

302   found to be significantly higher than that of PFOS in wild crucian carp.[1]

303   *4.2 PFASs differentially affect organism level metabolic endpoints*

304   Exposure to PFASs did not significantly affect developmental and morphometric

305   parameters, except for a significant reduction in yolk sac area observed in high F-53B exposure

306   group. Control group mortality did not exceed 5%, respecting OECD Test 212 guideline

307   stipulations for fish embryo testing.[43] In contrast, several metabolic endpoints were differentially

308   affected by PFASs exposures at the organismal level. Reductions in oxygen consumption were

309   observed in pre-hatching zebrafish embryos at 2 dpf exposed to high PFOS, and all OBS

310   exposure groups. These results suggest a compound and dose-specific reduction in standard

311   metabolic rate, with the strongest and most consistent effects in OBS. Extracellular acidification,

312   a measure of glycolytic flux, was only significantly increased by low PFOS exposure. Together

313   this data suggests a reduction in energy expenditure, especially in OBS-exposed larvae. Both

314   PFOS and F-53B, but not OBS, concentration-dependently increased 24 h oxidative energy

315   expenditure and decreased feed intake in post-hatching larvae at 4 dpf, suggesting similar modes

316   of action of these structurally similar compounds on larval energy balance. While the 24 h

317   Alamar Blue assay has been described as proxy to quantify zebrafish oxidative metabolism,[33-35] it

318   is, however, important to acknowledge that locomotion may contribute to increased oxidative

319   energy expenditure quantified by this assay. While not significant, we observed a tendency for

320   increased locomotor activity in all PFOS and F-53B, but not OBS exposed larvae compared to

321   the control, suggesting that the tendency for increased locomotor activity may have contributed

322   to increased energy expenditure at this developmental stage. Indeed, developmental and adult

323   PFOS exposure has been linked to increase spontaneous activity and a hyperactive phenotype in

324   rodents[17,18] and zebrafish.[24-26] Furthermore, in addition to mitochondrial reductases indicative of

325   oxidative metabolism, other enzymes, including enzymes involved in cellular detoxification and

326   oxidative stress responses, may affect the reduction of Alamar Blue.[44] Induction of oxidative

327   stress in zebrafish larvae has recently been reported for both PFOS and F-53B exposures, [13,45]

328   raising the possibility that active enzymes in detoxification oxidative stress response may have

329   contributed to the observed significant increase in energy expenditure in PFOS and F-53B

330   exposed larvae. The concentration-dependent decrease in larval feed-intake exposed to PFOS

331   and F53B, but not OBS, is in line with previously reported effects in rodent models, where PFOS

332   was shown to suppress feed intake by stimulating hypothalamic transcript urocortin 2 *(ucn2)* and

333   brown adipose tissue *ucp2*.[15,16] While brown adipose tissue does not exist in fish, our study

334   suggests that F-53B, similarly to PFOS, exhibits early onset anorexic and energy expenditure

335   promoting effects after hatching, potentially via central mechanisms. Together, our findings

336   show that structurally similar PFOS and F-53B promote a negative organismal energy balance in

337   post-hatch larvae, an early onset effect that, at least in the highest F-53B exposure group, is

338   linked to increased yolk sac utilization, and may play a role in previously reported PFOS- and F-

339   53B-dependent decreases in body weight reported in zebrafish.[10, 23]

340   **_4.3 PFASs affect transcript and protein abundance involved in energy metabolism_**

341   To investigate potential molecular mechanisms that may underlie the organismal metabolic

342   phenotype in PFASs-exposed larvae, we profiled transcript abundance of genes involved in the

343   central regulation of energy expenditure, metabolic pathways implicated in glucose and lipid

344   metabolism, the HPS axis, and mitochondrial uncoupling. A differential reduction in larval

345   transcript abundance of the orexigenic factor _npy_ and the anorexigenic factors _pomc_ and _cart_

346   were observed in groups exposed to PFASs, suggesting potential molecular level effects on

347   central mediators of energy balance. A significant decrease of _npy_ was observed in response to

348   all PFASs, albeit at different exposure concentrations. Subsequent Western blot analysis

349   confirmed that at least in high PFOS exposure group, these transcript changes were translated

350   into a decrease in NPY protein abundance, suggesting a possible role for NPY in the

351   significantly reduced feed intake in this group. However, it is important to keep in mind that

352   while zebrafish _npy_ is expressed in brain regions involved in the regulation of feed intake and

353   energy expenditure, it is widely expressed in other regions.[46] This warrants a cautionary

354   interpretation, and future _in-situ_ hybridization studies will be needed to link these changes

355   specifically to brain areas involved in feed intake and energy expenditure. Significant reductions

356   of _pomc_ and _cart_ transcript abundance were largely found in OBS-exposed zebrafish, raising the

357   possibility that a reduced anorexigenic transcript abundance may have contributed to the lack of

358   effect on food intake in OBS exposed larvae. Developmental _pomc_ expression is centrally

359   restricted to the anterior pituitary region,[47-49] allowing for a more precise interpretation of *pomc*

360   gene expression changes in whole larvae compared to *npy*.

361   With regard to key metabolic pathways involved in the metabolism of glucose and lipid

362   energy metabolism, we identified a consistent and strong decrease in *gk* gene expression in

363   response to all PFASs tested, irrespective of dose. Consistent with this pattern, a dose-dependent

364   trend for a decrease in GK protein was observed across all exposure groups. Furthermore,

365   molecular docking studies showed that PFASs fitted well into the active site of zebrafish GK and

366   form stable interactions through hydrogen bonding, indicating that PFASs may directly affect

367   GK protein abundance through protein-ligand interactions. GK is a low-affinity enzyme that

368   distributes glucose to the cytoplasm by catalyzing the initial step of glycolysis by conversion of

369   glucose to glucose-6-phosphate under high glucose conditions.[50] It is primarily expressed in

370   liver, pancreas and glucosensing parts of the brain, and contributes to developmental[51] and adult

371   systemic glucoregulation by acting in all of these tissues in mammals.[52-54] In zebrafish,

372   ontogenetic *gk* expression is detected at 4 dpf.[55] However, its functional roles other than

373   inducibility in response to glucose in adult zebrafish liver[56,57] have not been characterized in this

374   model. While a trend for a decrease in pancreas-specifically expressed *insa* was observed in

375   zebrafish larvae exposed to PFASs, this decrease was not significant, suggesting *gk* reduction is

376   not completely due to pancreatic development effects, which have been reported for PFOS.[58] In

377   spite of relatively well characterized, temporospatial expression profiles obtained from *in situ*

378   hybridization studies, differential allometric growth may be a factor in affecting tissue-specific

379   expression profiles, exemplified by *gk*, which is predominantly expressed in hepatopancreatic

380   tissue and glucosensing areas of the brain.[52-54] Therefore, we quantified several highly tissue-

381   specific miRNA markers, including liver-specific *miRNA-122*, pancreas-enriched *miRNA-375*,

382　　and muscle-specific *miRNA-1/133* to delineate possible effects on tissue differentiation from the

383　　described ubiquitous downregulation of *gk* gene expression transcripts in zebrafish exposed to

384　　PFASs. Because neither *miRNA-122* nor *miRNA-375* significantly altered the expression levels in

385　　groups exposed to PFASs, it is likely that observed *gk* effects are specific and not mediated by

386　　effects of PFASs on the liver or pancreatic tissue differentiation. Conversely, the OBS-specific

387　　decrease on muscle-specific *miRNA-1*, but not *miRNA-133*, suggests muscle-specific effects of

388　　OBS exposure. Indeed, *miRNA-1* and *miRNA-133* have been shown to play central roles in the

389　　balance of striated muscle proliferation and differentiation.[59, 60] Future work should therefore

390　　explore possible functional consequences of OBS induced downregulation of *miRNA-1*. The

391　　transcript abundance of *pck1*, the rate-limiting enzyme in *de novo* gluconeogenesis, which is

392　　expressed and functions in zebrafish glucoregulation in early development,[61] and in liver and

393　　pronephros of developing zebrafish,[62] did not change significantly across groups exposed to

394　　PFASs. Transcripts related to lipid-metabolism revealed that PFASs affected both rate-limiting

395　　lipogenic (*fasn*) and β-oxidation (*cpt1a*) transcripts, however, only *cpt1a* transcript abundance

396　　revealed significant changes compared to control groups. Together, these transcriptional changes

397　　point to early onset effects of PFASs exposure on rate-limiting glucose and lipid metabolic

398　　pathways. PFASs exposure differentially affected transcript levels of the HPS axis, with a

399　　consistent decrease in both *gh* and *igf2* in zebrafish exposed to medium OBS. This response is

400　　indicative of a concentration- and the compound-specific response of the HPS axis to OBS, at

401　　least at the transcript level. PFASs exposure did not affect transcript levels of *ucp2*, suggesting

402　　that mitochondrial uncoupling, at least at the transcriptional level, was not affected by PFASs

403　　exposure in post-hatch larvae.

404   *4.4 PoD analysis reveals higher sensitivity for metabolic disruption in developing zebrafish*

405   *exposed to F-53B and PFOS compared to OBS*

406   In order to integrate quantified transcript and organismal level endpoints to assess sublethal

407   metabolic effects of PFASs exposure, we determined separate PoDs using a Benchmark dose

408   approach for transcriptomic and organism level responses. While transcript PoDs were more

409   sensitive compared to organismal PoDs, the order of sensitivity from lowest to highest threshold

410   is maintained at both levels of biological organization (F-53B < PFOS < OBS; **Figure 7A-B**). In

411   the case of the PFOS (0.0080 and 0.0122 mg/L, respectively) and F-53B (0.0038 and 0.0063

412   mg/L, respectively), the transcript and organismal level PoDs are in the same order of

413   magnitude, and relatively similar between both compounds compared to OBS. This observation

414   points to the fact that metabolic transcript responses to PFOS and F-53B exposure are a good

415   approximation of organismal metabolic effects, and that F-53B and PFOS have similar PoDs for

416   sublethal metabolic effects at both levels of organization, likely caused by their similar chemical

417   structure, which allows them to interact similarly with cellular targets. Interestingly, a recent

418   transcriptomic level analysis of PFOS at different concentrations resulted in a similar PoD in the

419   same order of magnitude (0.011 mg/L), whereas the PoD for morphometric parameters was

420   identified to be 2.53 mg/L.[63] This suggests that while the targeted metabolic subset of transcripts

421   is reflective of whole transcriptome level PoD for PFOS in developing zebrafish, the organism-

422   level metabolic phenotype is more susceptible to alteration in response to low concentration

423   PFOS exposure compared to strictly morphometric endpoints. In case of gene expression and

424   whole organism parameters, F-53B elicited sublethal metabolic responses at a two-fold lower

425   threshold compared to PFOS. Taking into account that F-53B is found in the environment at

426   concentrations comparable to PFOS[3] and the F-53B PoD threshold concentration of metabolic

427   disruption at both the organismal and molecular level is in the range of reported aquatic

428   environmental F-53B concentrations,[3-5] our study raises concerns over F-53B-induced sublethal

429   metabolic disruption in early development in wild-life and humans. Clearly, long-term studies in

430   the zebrafish and other models are warranted to address potential long-term metabolic

431   consequences, especially given the reported environmental persistence and bioconcentration of

432   the compound across development and even generations,[10, 12, 13] as well as reported long-term

433   metabolic consequences of developmental PFASs exposure in manipulative rodent and human

434   epidemiological studies.[64, 65]

435   On the other hand, OBS presented the lowest PoDs for sublethal metabolic effects of the

436   three PFASs tested (0.0141 and 0.9412 mg/L for the transcriptomic and organismal level

437   endpoint, respectively), suggesting that the different chemical structure of this compound affects

438   transcript and organism-level metabolic responses differently and with lower potency. It is,

439   however, possible that OBS affects other pathways not related to energy metabolism with higher

440   potency, and future transcriptome level studies should investigate this possibility.

441   Interestingly, a consistently lower threshold for transcript compared to organismal

442   metabolic disruption PoD exists in developing zebrafish, suggesting that molecular markers can

443   be used as a sensitive early indicator that precedes the onset of organismal level metabolic

444   disruption effects. While the order of sensitivities (F-53B < PFOS < OBS) is maintained at both

445   levels, the margin between molecular and organism level metabolic disruption PoD is much

446   higher for OBS (approximately two orders of magnitude) than the differences between the

447   respective PoDs for F-53B and PFOS (in the same order of magnitude), suggesting a smaller

448   safety margin between identification of molecular level metabolic effects and organismal level

449   effects for F-53B and PFOS.

450   In conclusion, our study reveals previously unknown sublethal effects of the Chinese PFOS

451   replacement compound F-53B on energy metabolism across early development in the zebrafish

452   model. F-53B exposure affects both metabolic transcript level and organismal metabolic

453   phenotype at a two-fold lower PoD both at both levels of the biological organization compared to

454   PFOS, a known metabolic disruptor. Given the widespread use of F-53B as PFOS replacement in

455   China, this raises concerns about metabolic disruption during early development, and future

456   studies are warranted to assess potential long-term consequences of the metabolic phenotype

457   within and across generations. Compared to F-53B, exposure to OBS elicits metabolic disruption

458   in larvae only at higher concentrations, especially at the organismal level, suggesting a

459   comparatively lower potency for metabolic disruption in early development. Nevertheless, our

460   study identified glucokinase transcript abundance as an endpoint potently disrupted by all

461   PFASs, and future studies are warranted to validate this transcript as a potential sensitive marker

462   for developmental PFASs exposure in general.

463   **Acknowledgements**

469   **Supporting Information Available**

470   Additional information including supplemental texts (**Text S1-3**), supplemental tables

471   (**Table S1-6**) and supplemental figures (**Figure S1-6**). This information is available free of

472   charge via the internet at http://pubs.acs.org.

473   **Disclosures**

474   The authors declare no competing financial interest.

475

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685   **Figures captions**

686   **Figure 1.** The concentrations of PFASs (A) and their corresponding bioconcentration factors (B)

687   in developing zebrafish after 4 days of exposure.

688   **Figure 2.** Effects on oxygen consumption rates (**A-C**), oxygen consumption rate metabolic

689   potential (**D-F**), extracellular acidification rates (**G-I**) and extracellular acidification-rate

690   potential (**J-L**) in 2 dpf zebrafish exposed to various concentrations of PFASs. Asterisks are

691   indicative of significant differences compared to the control at the $P < 0.05$ (*) and $P < 0.01$ (**)

692   level, respectively.

693   **Figure 3.** Effects on oxidative metabolism related energy expenditure measured by Alamar Blue

694   assay in 4 dpf zebrafish (**A**), locomotor activity assessed as total distance travelled in 20 min in 5

695   dpf zebrafish (**B**) and feed intake measured as ingestion of fluorescently labelled food (**C**) in 6

696   dpf zebrafish exposed to various concentrations of PFASs. Asterisks are indicative of significant

697   differences compared to the control at the $P < 0.05$ (*) and $P < 0.01$ (**) level, respectively.

698   **Figure 4.** Effects on abundance of transcripts involved in central regulation of energy

699   expenditure (**A-F**), glucose metabolism (**G-J**), lipid metabolism (**K-M**), the hypothalamus-

700   pituitary growth axis (**N-O**) and mitochondrial uncoupling (**P**) in 4 dpf zebrafish exposed to

701   various concentrations of PFASs. Asterisks are indicative of significant differences compared to

702   the control at the $P < 0.05$ (*) and $P < 0.01$ (**) level, respectively.

703   **Figure 5.** Effects on protein abundance of GK (**A-C**) and NPY (**D-F**) in 4 dpf zebrafish exposed

704   to various concentrations of PFASs. Asterisks are indicative of significant differences compared

705   to the control at the $P < 0.05$ (*) and $P < 0.01$ (**) level, respectively.

706   **Figure 6.** The active binding site of PFASs toward zebrafish GK (within red ball) (A) and

707   molecular docking result of PFOS (B), F-53B (C) and OBS (D) with zebrafish GK.

708 **Figure 7.** Accumulation plot of the best calculated BMDs for metabolic transcript- (**A**) and

709 organism-level endpoints (**B**) The best BMD value for each endpoint was determined by the

710 BMDExpress software by identifying the best fitted model for each endpoint between third order

711 polynomial, third order exponential and a Hill equation models. The median BMDL were used as

712 PoD (Point of Departure) for each compound and are interpolated in the graph as dotted line

713 arrows.

714

715   **Figure 1**
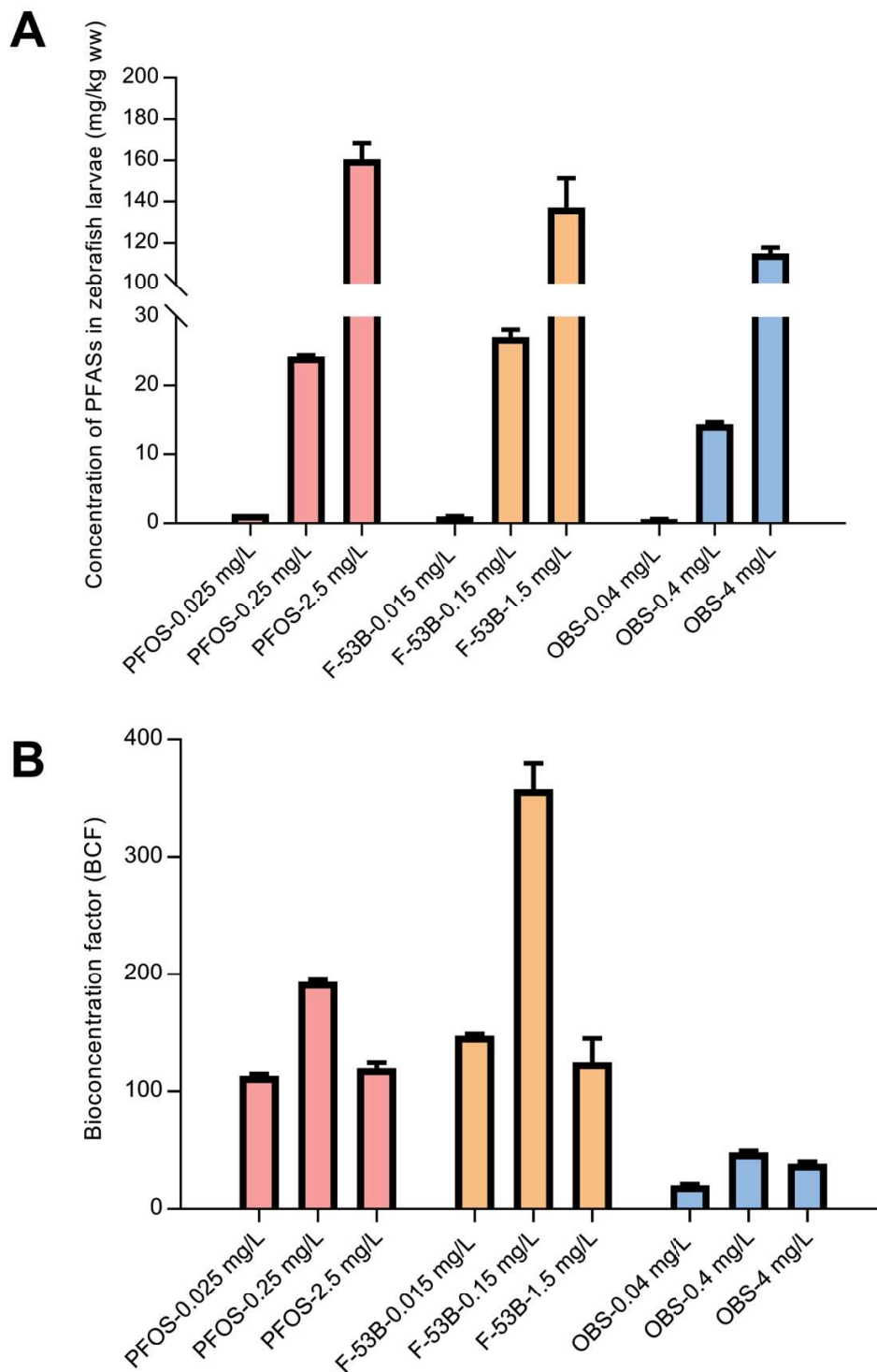
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718   **Figure 2**
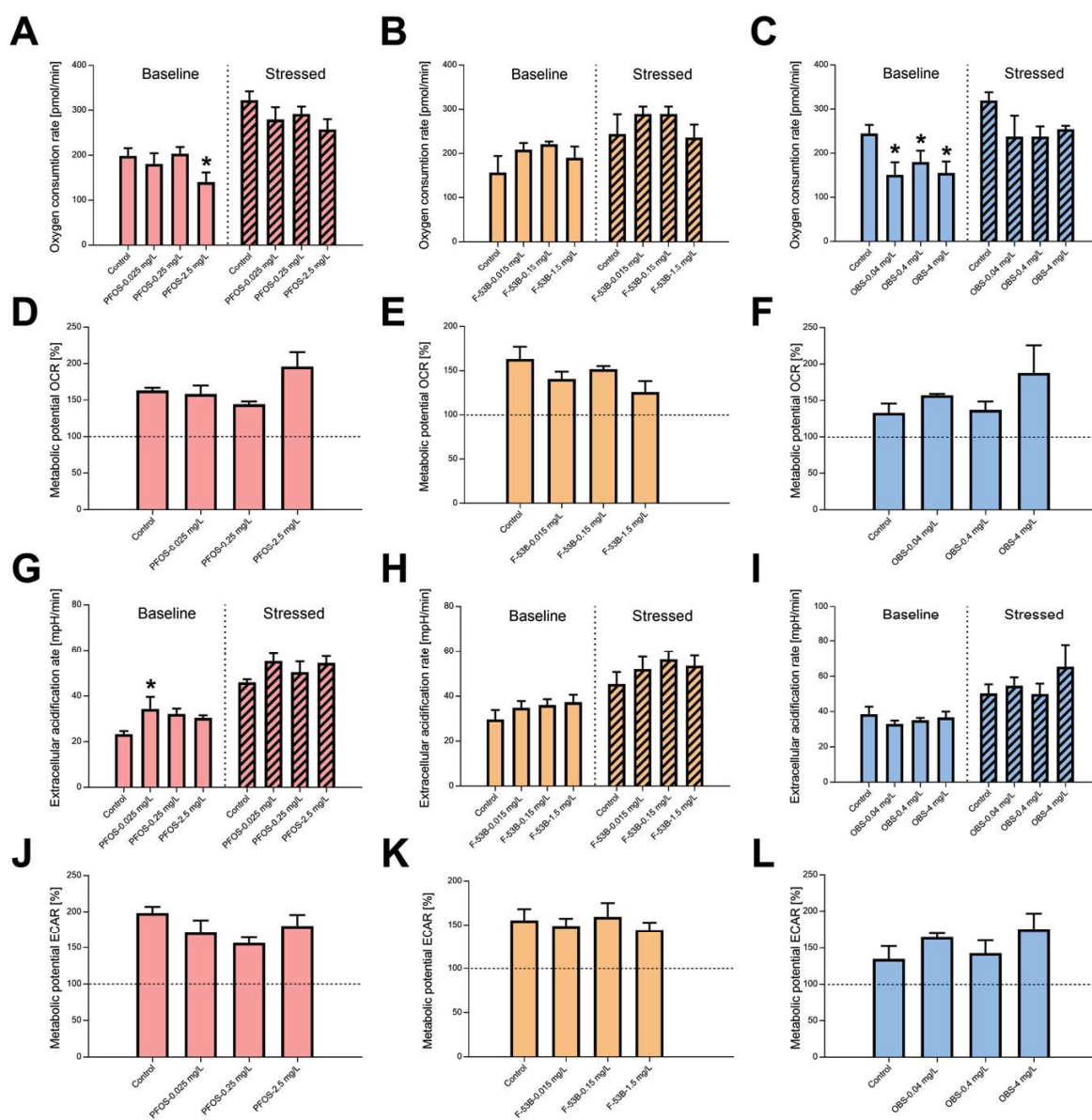
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721　　**Figure 3**
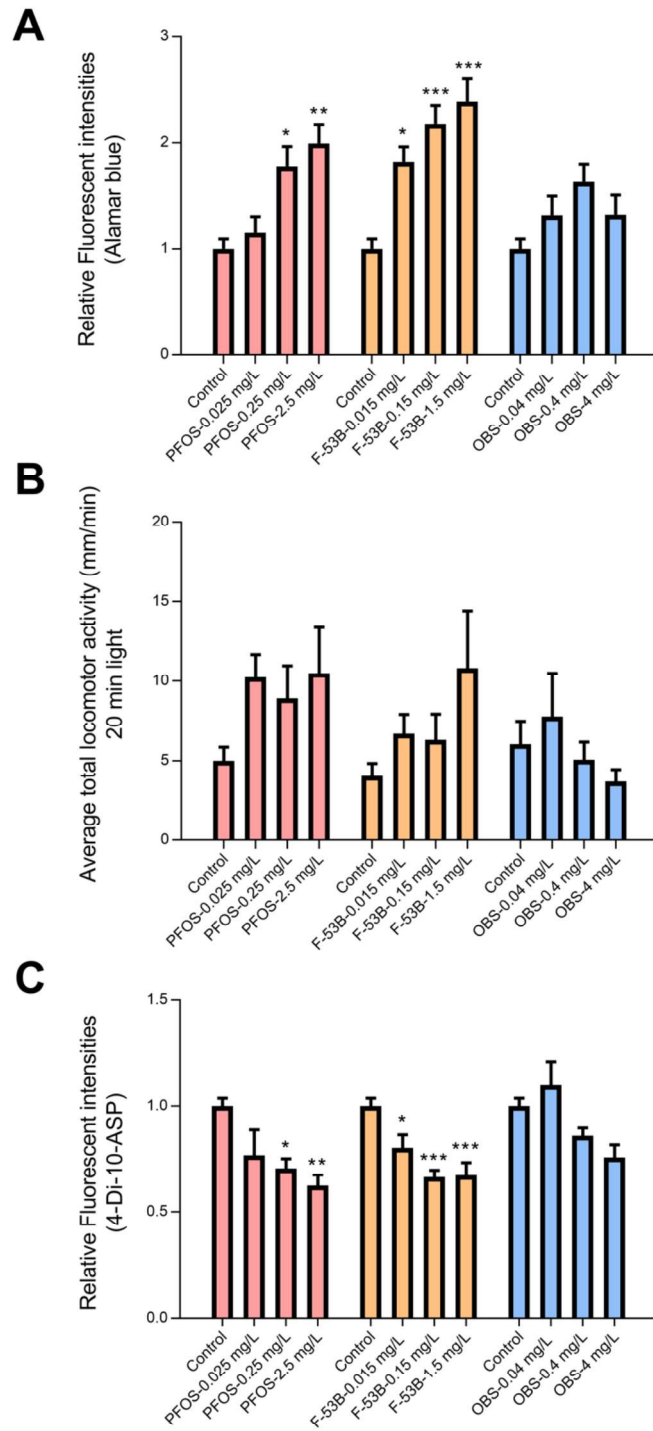
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724   **Figure 4**
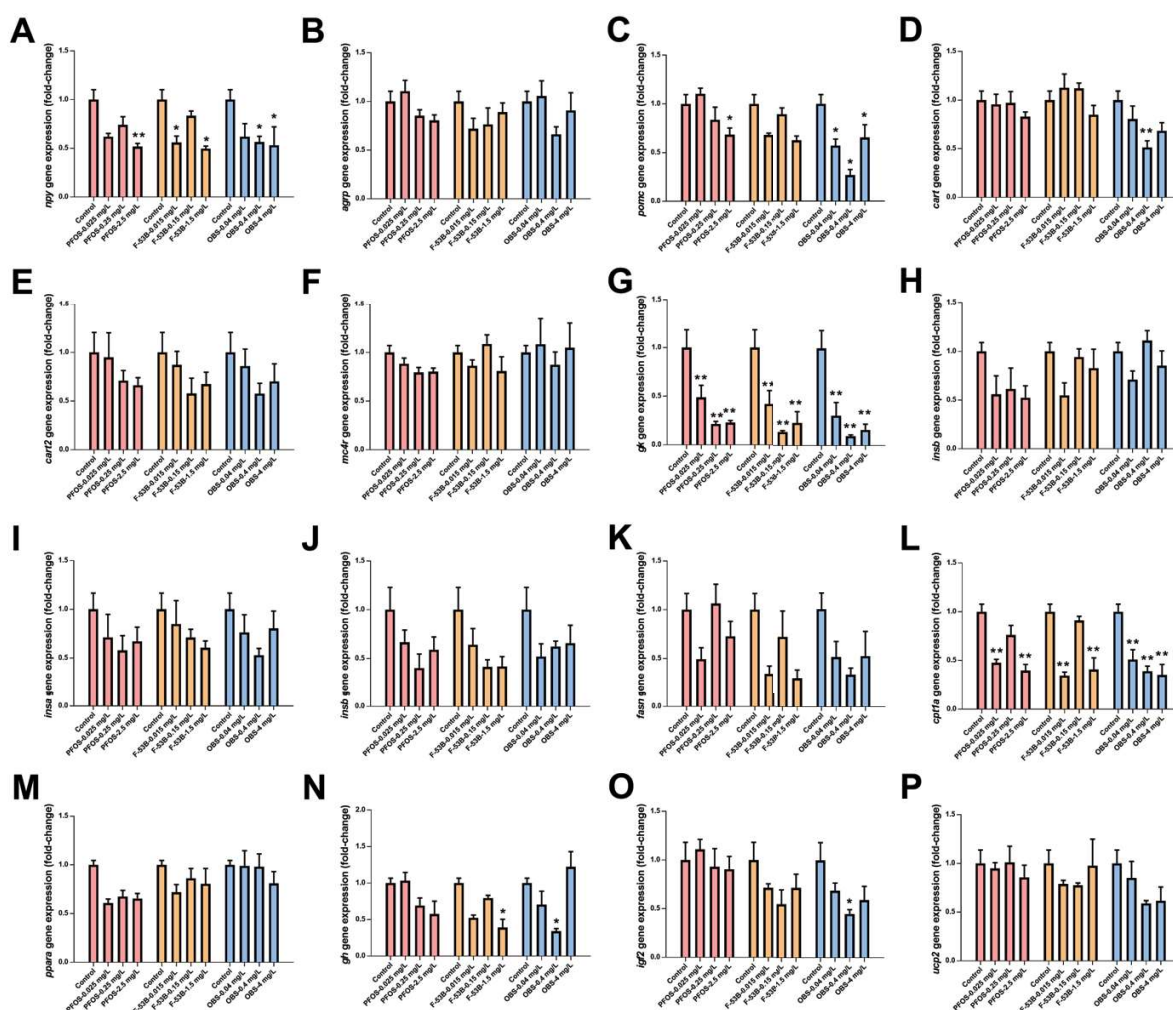
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727　　**Figure 5**

728

729

730   **Figure 6**

731

732

733   **Figure 7**

A

B

734