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Perfluorobutanesulfonate Exposure Causes Durable and Transgenerational Dysbiosis of Gut Microbiota in Marine Medaka

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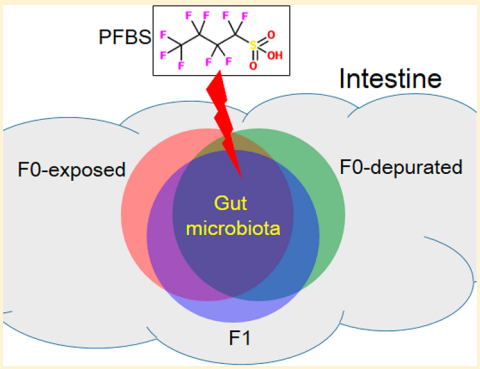
Supporting Information

ABSTRACT: Environmental pollutants are known as disruptors of gut microbiota. However, it remains unexplored whether the dysbiosis of gut microbiota by pollutants is durable and transgenerational in teleost. Therefore, this study exposed eggs of marine medaka to environmentally realistic concentrations (0, 1.0, 2.9, or 9.5 μg/L) of perfluorobutanesulfonate (PFBS), a persistent organic pollutant of emerging concern, until sexual maturity. A proportion of F0 adults was dissected after exposure (F0-exposed). Remaining fish were depurated in clean seawater (F0-depurated). F1 offspring were also cultured in clean seawater for a complete life-cycle. Substantial amounts of PFBS were accumulated in F0-exposed intestines, while F1 intestines contained no PFBS. Significant alterations were observed in physiological activities of F0-exposed and F1 medaka. The gut microbial community in F0-exposed, F0-depurated, and F1 medaka were restructured in a concentration-dependent manner by PFBS exposure. Dysbiosis of gut microbiota caused by PFBS exposure was durable in parents and persisted in the offspring. Significant positive correlations were constructed for the genus *Cetobacterium* with host intestinal epithelial permeability and production of endotoxin lipopolysaccharides. Overall, this study provided the first insight into durable and transgenerational dysbiosis of gut microbiota and intestinal health by PFBS, highlighting the particular susceptibility of gut to xenobiotic stresses.



INTRODUCTION

Environmental pollutants are increasingly documented to disrupt the dynamics of gut microbiota in animals.[1−3] Albeit with varying physicochemical properties, diverse pollutants, including persistent organic pollutants, antibiotics, and pesticides, can potentially alter compositions and metabolic activities of intestinal microbiota. Gut microbes are closely involved with physiological regulation of hosts, including metabolism of nutrients, immune function, and neurobehavior.[4−8] Changes in composition and metabolism of gut microbiota by environmental pollutants can eventually compromise the health of host organisms, by inducing the onset of various diseases, including obesity and diabetes. However, although long-lasting perturbation of gut microbiota by chemicals is observed in mouse and human,[9−12] it is still unknown whether dysbiosis of gut microbiota by environmental pollutants is recoverable in teleost after the exposure has ceased. Furthermore, whether parental exposure to pollutants will transgenerationally dysregulate gut microbiota of offspring also needs to be elucidated.

Perfluorobutanesulfonate (PFBS) is a persistent organic pollutant of emerging concern in aquatic environments. Following worldwide phasing-out of perfluorooctanesulfonate (PFOS),[13,14] PFBS has been increasingly used as a replacement in diverse industrial and commercial products.[15] Consequently, it has been ubiquitously detected in environmental abiotic and biotic matrices. Concentrations of PFBS as great as 1.9 μg/L have recently been reported in leachate of a landfill site at Singapore.[16] In Tangxun Lake at Hubei Province of China, discharges of municipal and industrial wastewater result in point-source pollution of PFBS, where concentrations up to 8.0 μg/L have been observed.[17] Increasing accumulation

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of PFBS has also been observed in cetacean samples from 2002 to 2014, causing a shift in bioaccumulation pattern from PFOS to PFBS.[18] Although PFBS has been considered less accumulative and less toxic than PFOS due to shorter chain length,[19−21] potent and multigenerational disruption of the thyroid endocrine system by PFBS in marine medaka has been recently reported after parental life-cycle exposure to environmentally realistic concentrations.[22] PFBS is also found to accumulate in the eyes and impair the visual function of medaka fish.[23] Therefore, hazards of PFBS to aquatic wildlife cannot be neglected, which necessitates more toxicological studies for a comprehensive risk assessment.

In order to clarify whether environmental pollutants will cause persistent or even transgenerational dysbiosis of gut microbiomes, the present study exposed eggs of marine medaka (*Oryzias melastigma*) to various waterborne concentrations of PFBS (0, 1.0, 2.9, or 9.5 μg/L) for an entire life-cycle. A portion of the F0 fish was dissected immediately after exposure, while another portion of F0 medaka was transferred to clean seawater to depurate for another two months. F1 offspring were also cultured in PFBS-free seawater until sexual maturity. Intestines from F0-exposed, F0-depurated, and F1 adults were all dissected. Concentrations of PFBS in F0 and F1 intestines were quantified. Potential changes in physiological activities of medaka were also monitored by an array of sensitive biomarkers. Compositions of intestinal microbial communities were profiled and intercompared among F0-exposed, F0-depurated, and F1 adults to distinguish the durable and transgenerational effects of PFBS.

MATERIALS AND METHODS

Chemicals. PFBS was purchased from Tokyo Chemical Industry (Tokyo, Japan; purity >98.0%). Stock solutions of PFBS were prepared using dimethyl sulfoxide of high-performance liquid chromatography-grade (DMSO; Sigma-Aldrich Corp., St. Louis, MO, USA). Other chemicals used in the present study were of analytical grade.

Fish Maintenance and Life-Cycle Exposure. Culture and exposure of marine medaka *O. melastigma* were conducted following a previously described protocol,[22] in a semistatic system containing charcoal-filtered, fully aerated artificial seawater (salinity: 25‰) at 24 ± 0.5 °C with a photoperiod of 14-h light:10-h dark. Marine medaka eggs were nominally exposed to environmentally realistic concentrations of PFBS (0, 1.0, 3.0, or 10.0 μg/L). Actual waterborne concentrations of PFBS were previously measured to be 0, 1.0, 2.9, and 9.5 μg/L, respectively.[22] Each tank equivalently contained very low concentration of DMSO (<0.001% v/v). Approximately, 150 eggs were included in 100 mL of exposure medium per glass beaker. Each exposure group had three replicates (n = 3). Juvenile medaka were transferred to 4-L media at one-month old and later to 20-L media at two-month old. Exposure media were renewed daily to maintain constant concentrations of PFBS. F0 fish were exposed until sexual maturity when males and females can be easily discerned by secondary sexual characters after males develop larger and parallelogram-shaped anal fins. During the last 1 week of exposure, F0 adults were paired to spawn F1 eggs, which were collected and cultured for a life-cycle in clean seawater without further exposure to PFBS (approximately 50 eggs per dish and three replicates per group). A portion of the F0 adults was dissected right after exposure to obtain the intestine, liver, and blood, which were defined as F0-exposed samples. The remaining F0 medaka were depurated in PFBS-free seawater for another two months. After depuration, all F0 medaka were dissected and intestines were collected, which were defined as F0-depurated samples. Sexually mature F1 adults were also dissected to collect the intestine, liver, and blood. All the tissues from F0-exposed, F0-depurated, and F1 medaka were snap-frozen in liquid nitrogen and then stored at −80 °C for further analytic and molecular analyses.

Quantification of PFBS in Intestines. Intestines from five individual fish of the same sex were pooled as a replicate (n = 3). PFBS in intestine was quantified according to previous methods.[24] In brief, PFBS was extracted by sonication and cleaned up using ENVI-Carb graphitized carbon cartridges (250 mg; Supelco, Bellefonte, PA). After elution and concentration in 0.5 mL of methanol, PFBS extracts were analyzed and quantified on an Agilent 1290 Infinity ultra-performance liquid chromatograph (Agilent, Palo Alto, CA, USA) coupled with a 5500 QTRAP mass spectrometer (AB Sciex, Foster City, CA, USA). The spike recovery of PFBS in nonexposed fish was at 93 ± 7%.

Monitoring of Host Health. A battery of sensitive biomarkers was measured to indicate the physiological disturbances in intestine, liver, and blood of hosts, including interleukin 1β (IL1β) for inflammatory response; serotonin for neural signaling; tight junction protein 2 (TJP2) for intestine epithelial permeability; reactive oxygen species (ROS), superoxide dismutase (SOD) and catalase (CAT) for oxidative damage; triglyceride (TG) and free fatty acid (FFA) for lipid metabolism; and lipopolysaccharides (LPS) for endotoxin production. Detailed procedures have been previously described.[25]

16S rRNA Amplicon Sequencing. Intestines of five individuals of the same sex were pooled as a replicate. There were three replicates for each exposure group (n = 3). Genomic DNA of whole intestines was extracted using a DNeasy Blood & Tissue Kit based on the manufacturer's instructions (Qiagen, Hilden, Germany). The 16S rRNA gene was amplified using the primer pair 515 F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 909R (5′- CCCCGYCAATTCMTTTRAGT-3′), which target V4 V5 hypervariable regions. The amplicons were sequenced on an Illumina MiSeq PE250 platform (Illumina, San Diego, CA, USA). Raw reads were filtered, clustered to Operational Taxonomic Units (OTUs) of 97% similarity following the UPARSE pipeline, and phylogenetically annotated using the RDP classifier against the Greengenes database to obtain the microbial abundances at each taxonomic level (e.g., phylum and genus).[26]

Statistical Analyses. Values are presented as mean ± SEM of three replicates. Data were checked for normal distribution and homogeneous variance using Shapiro-Wilk and Levene's tests, respectively. One-way analysis of variance (ANOVA) was employed to differentiate significant difference between exposure groups and the control group, followed by the post hoc LSD test. If assumptions of normality and homogeneity could not be met after log-transformation, the nonparametric Kruskal−Wallis ANOVA test with Dunn-Bonferroni posthoc comparison was used to determine significances of differences among groups. Statistical analyses were performed using SPSS version 22.0 software (IBM SPSS Statistics, IBM Corporation, Armonk, New York). A P value <0.05 was selected as the criterion for significant difference.

Unweighted pair group method with arithmetic mean (UPGMA) clustering analysis was conducted based on

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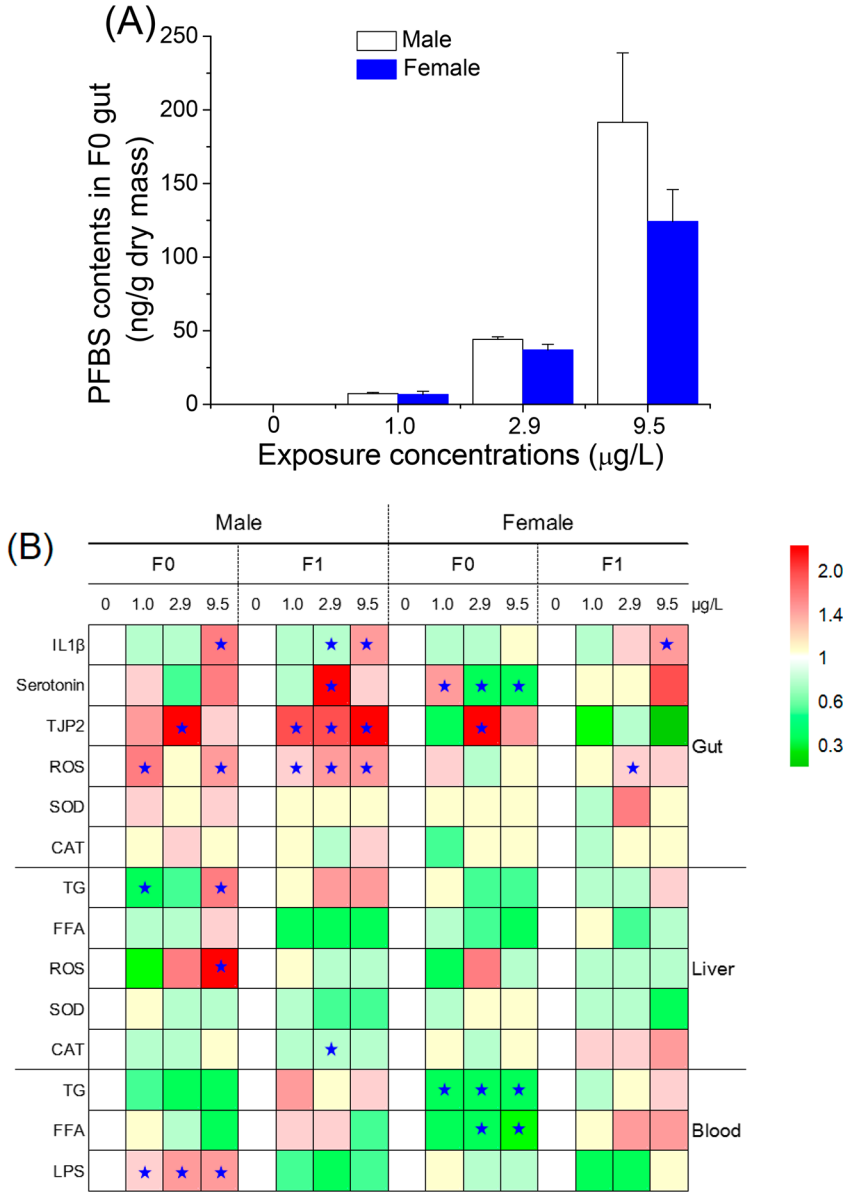

Figure 1. (A) Significant concentrations of PFBS observed in intestines of F0-exposed medaka and (B) a heatmap demonstrating substantial disturbances in the physiological conditions within gut, liver, and blood of F0-exposed and F1 medaka after a life-cycle exposure to various environmentally realistic concentrations of PFBS (0, 1.0, 2.9, or 9.5 μg/L). Values are presented as mean ± SEM of three replicates. In the heatmap, red represents up-regulation, while green represents down-regulation. Color intensity is proportional to extent of change. Significant differences are indicated by $^*P < 0.05$ between exposure groups and the control group. Abbreviations: interleukin 1β, IL1β; tight junction protein 2, TJP2; reactive oxygen species, ROS; superoxide dismutase, SOD; catalase, CAT; triglyceride, TG; free fatty acid, FFA; lipopolysaccharides, LPS.

unweighted UniFrac distances using QIIME 1.9.0 software. Principal component analysis (PCA) was conducted based on the variance−covariance matrix of genera abundances (>1%) using PAST software. Pearson correlation analysis and linear regression analysis among toxicological indices were also evaluated using SPSS version 22.0 software (IBM SPSS Statistics).

RESULTS AND DISCUSSION

Concentrations of PFBS in F0 and F1 Intestines. In intestines of F0-exposed male medaka, 7.3, 44.2, and 191.5 ng PFBS/g dry mass were observed after exposure to 1.0, 2.9, or 9.5 μg/L, respectively (Figure 1A). Intestines of F0-exposed females contained 6.8, 37.0, and 124.3 ng/g dry mass of PFBS after a life-cycle exposure to 1.0, 2.9, or 9.5 μg/L, respectively (Figure 1A). No PFBS was observed in intestines of control F0 medaka that were not exposed to PFBS. In intestines of F1 offspring, there was also no PFBS after culturing in PFBS-free seawater until sexual maturity, which indicated that PFBS transferred from F0 to F1 offspring was eliminated during the period of depuration.

Health of F0 and F1 Generations. Mortality of F0 during entire life-cycle was not significantly affected by PFBS waterborne exposure. However, parental exposure to PFBS (2.9 and 9.5 μg/L) increased the mortality rate of F1 generation, which were reared in clean seawater until sexual maturity (Figure S1A). Within the last week of exposure, egg production of F0 adult was significantly decreased at exposure groups relative to control fish (Figure S1B). In F0-exposed male or female medaka, both the values of condition factor (K

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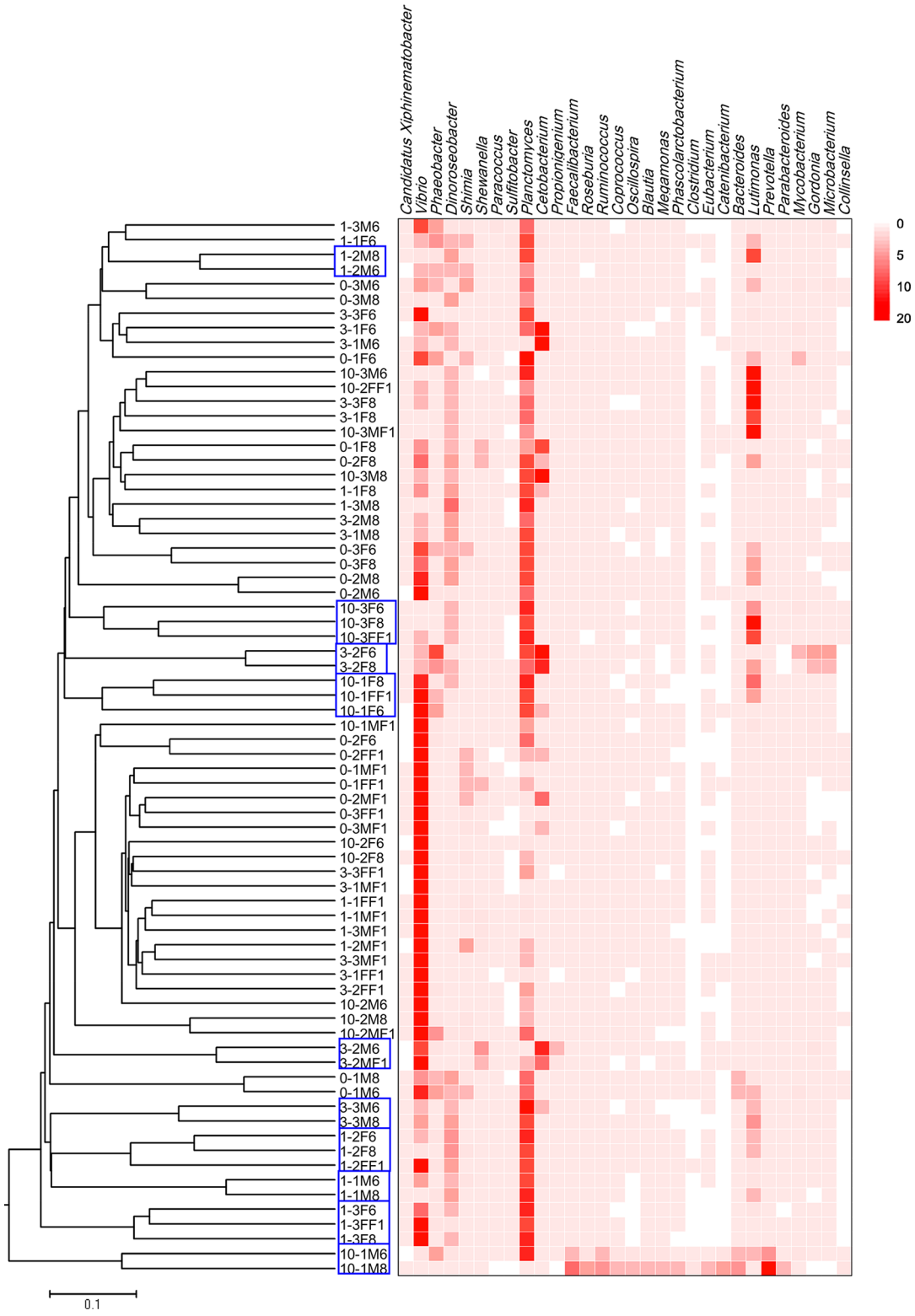

Figure 2. Unweighted UniFrac tree and heatmap profile of genera compositions (relative abundances >1%) in F0-exposed (M6/F6), F0-depurated (M8/F8), and F1 intestines (MF1/FF1) after a life-cycle exposure to various environmentally realistic concentrations of PFBS (nominal 0, 1, 3, or 10 μg/L). Representative clusters are highlighted in blue circles. Each group includes three replicates (each replicate containing five intestines). In the heatmap, red represents up-regulation, and intensity is proportional to the extent of changes. Abbreviations: M, male; F, female.

factor) and hepatosomatic index (HSI) were significantly decreased after direct exposure to PFBS (Figure S1), possibly indicating excessive energetic expenditure in a suboptimal environment.[27] In contrast, recovery of F1 offspring exhibited increases in both K factor and HSI values after parental exposure to PFBS (Figure S1). Physiological activities in F0-exposed and F1 adults were also reflected by the use of sensitive biomarkers in three tissues, including intestine, liver,

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and blood. Although F1 offspring were cultured in PFBS-free seawater, significant alterations in the health of intestines were observed in both the F0 and F1 generations (Figure 1B), including inflammation (IL1β), neural signaling (serotonin), intestinal epithelial integrity (TJP2), and oxidative stress (ROS). Based on the measurements of physiological markers, effects of PFBS on livers of both F0 and F1 fish appear to be less severe than those on the intestines (Figure 1B), which is consistent with previous observations.[28] In blood of F0-exposed male medaka, greater toxicity was caused by exposure to PFBS, as indicated by increased levels of LPS (Figure 1B). LPS is produced by microbes in the gut[29] and can act as an endotoxin to potently stimulate the secretion of pro-inflammatory cytokines in host organisms.[30] Therefore, increased production of toxic LPS by PFBS exposure will result in the induction of inflammatory responses, which is here supported by concurrent greater amounts of LPS and IL1β at 9.5 μg/L in F0-exposed males (Figure 1B). Decreased concentrations of TG and FFA were also observed in blood of F0-exposed females (Figure 1B), which indicates that metabolism of lipids has been impaired. Furthermore, Pearson correlation analysis showed that TJP2 expression was significantly positively associated with SOD activity in intestines (R = 0.7; P < 0.001). This tight relationship between intestinal TJP2 and SOD is also supported by previous toxicological studies about gut microbiota.[25,26,28] Additionally, SOD activity showed significantly positive correlation with ROS levels in both intestine (R = 0.8; P < 0.001) and liver (R = 0.7; P < 0.001), to remove excessive free radicals.

Some physiological changes did not have a dose-dependent response, which may be explained by the differential responsiveness or sensitivities of certain tissues and toxic indices toward the challenges of pollutants at various concentrations.[31,32] Additionally, a sex-specific response to PFBS exposure was observed in male and female medaka of F0 or F1 generation (Figure 1B). The inherent distinction of males and females in levels of sex hormones would impart different detoxifying capacity through the crosstalk between ER and AhR signals, which presumably accounts for the sex-specificity of toxic responses.[22,32]

Durable and Transgenerational Dysbiosis of the Intestinal Microbiome. Intercomparisons among commensal microbial communities in guts of F0-exposed, F0-depurated, and F1 medaka allowed the differentiation of durable and transgenerational effects of PFBS on gut microbiota. Rarefaction curves verified that current sequencing had a good representation of microbial diversity in the guts (Figure S2). PFBS exposure decreased the observed species and diversity of gut microbes in F0-depurated male medaka after life-cycle exposure to 2.9 and 9.5 μg/L, while F1 male or female intestines contained increased microbial species and diversity (Table S1). At the phylum level, exposure to PFBS varied the composition of microbial communities in intestines of both F0 (exposed or depurated) and F1 individuals (Figure S3). Persistent and transgenerational dysbiosis of gut microbiomes by PFBS was also manifested at the level of genera (Figure 2). Profiles of relative proportions of individual genera varied among F0-exposed, F0-depurated, and F1 intestines. F0-exposed intestines were clustered with F0-depurated replicates (e.g., 10-1M6 and 10-1M8) and F1-unexposed replicates (e.g., 1-3F6, 1-3F8, and 1-3FF1) based on the unweighted UniFrac method (Figure 2), indicating the incomplete recovery and transgenerational dysbiosis of gut microbiota by PFBS exposure. PCA plots of microbial communities in intestines of F0-exposed and F0-depurated medaka exhibited similar patterns (Figure 3A and 3B, respectively). In a concentration-dependent manner, PFBS life-cycle exposure caused deviation of microbiota in exposed intestines relative to that in

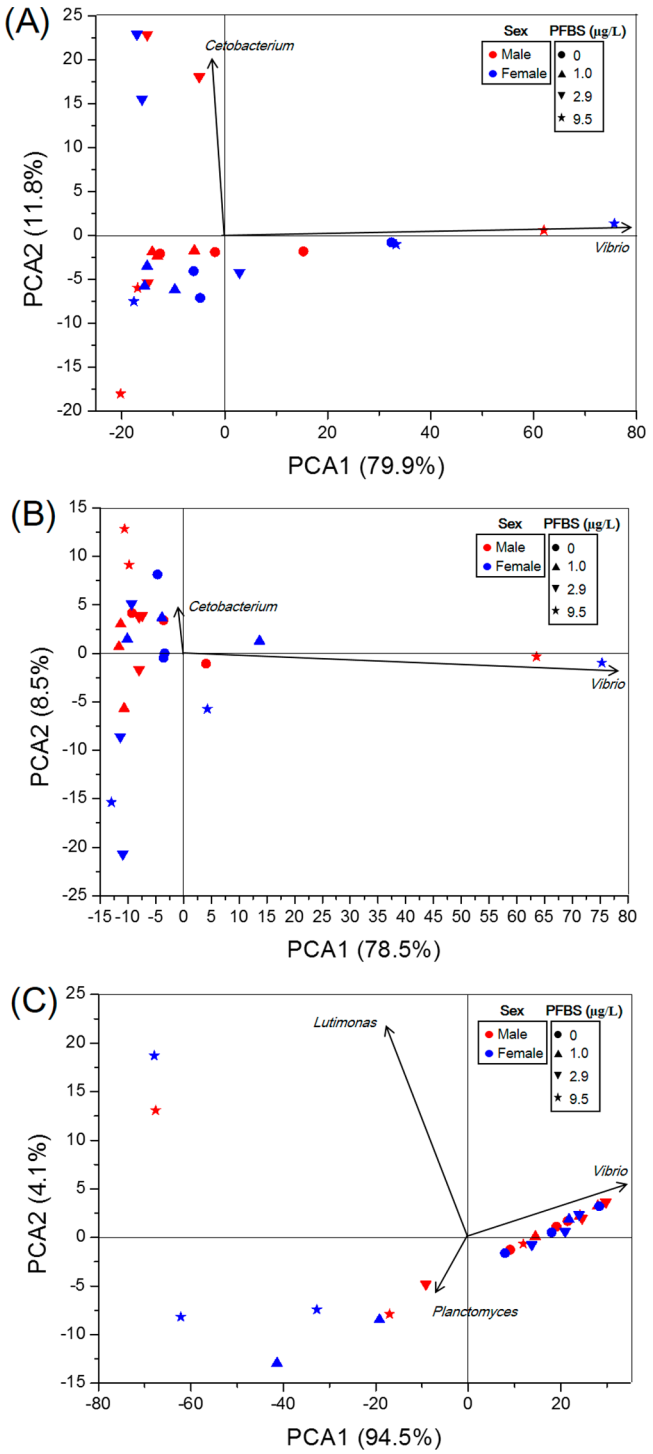

Figure 3. Principal component analysis (PCA) based on relative abundances of genera (>1%) in F0-exposed (A), F0-depurated (B), and F1 intestines (C) after a life-cycle exposure to various environmentally realistic concentrations of PFBS (0, 1.0, 2.9, or 9.5 μg/L). There are three replicates per group (each replicate containing five intestines).

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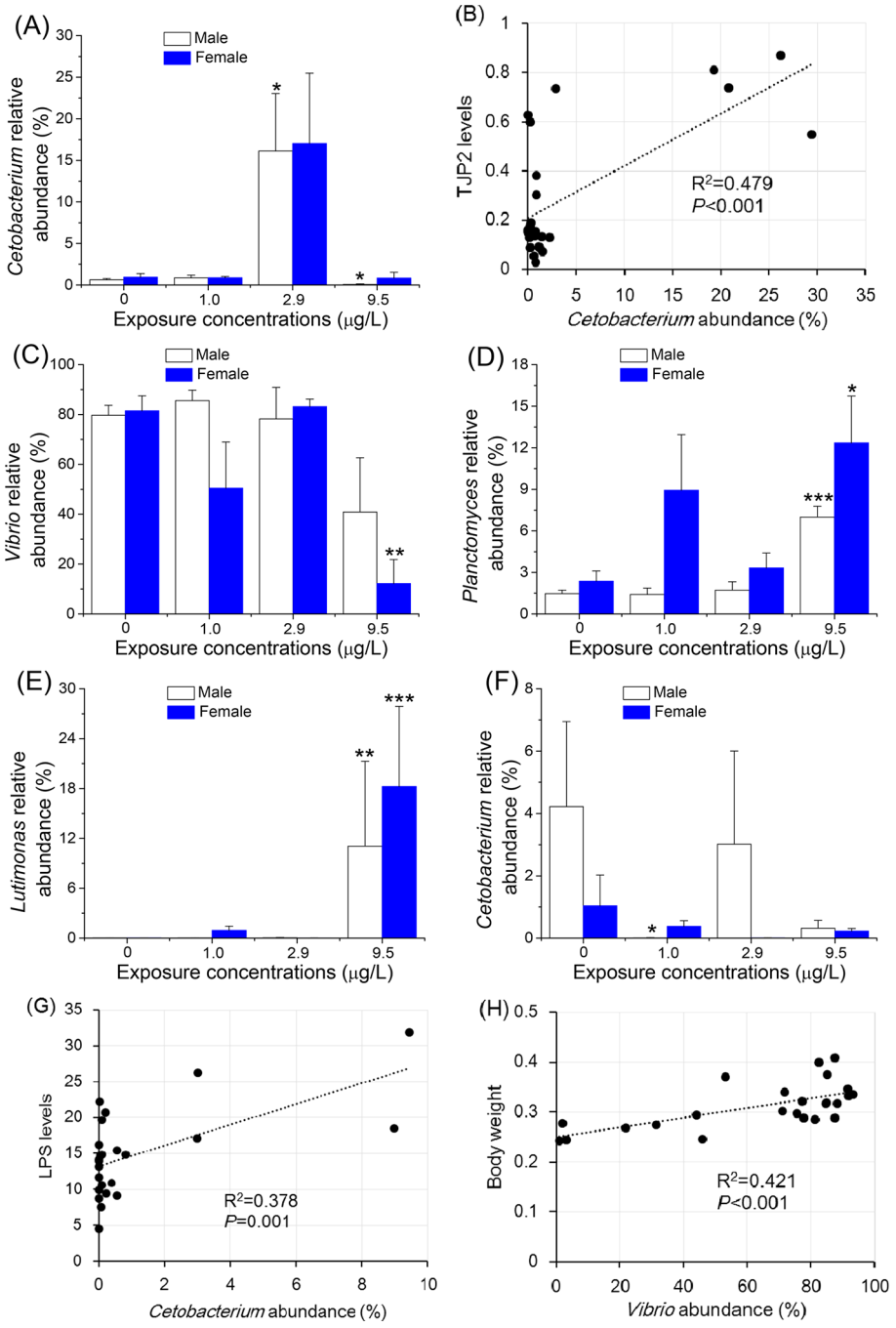

Figure 4. Differential changes in relative abundances of representative genera and their correlations with biomarkers of physiological status in intestines from F0-exposed and F1 medaka after a life-cycle exposure to various environmentally realistic concentrations of PFBS (0, 1.0, 2.9, or 9.5 μg/L). (A) Abundances of *Cetobacterium* in F0-exposed intestines; (B) correlation between *Cetobacterium* abundances and TJP2 expressions in F0-exposed intestines; (C) abundances of *Vibrio* in intestines of F1; (D) abundances of *Planctomyces* in intestines of F1; (E) abundances of *Lutimonas* in intestines of F1; (F) abundances of *Cetobacterium* in intestines of F1; (G) correlations between abundances of *Cetobacterium* and concentrations of LPS in intestines of F1; (H) correlation between abundances of *Vibrio* and body masses of F1. Values are presented as mean ± SEM of three replicates. Significant differences are indicated by $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ between exposure groups and the control group. Abbreviations: tight junction protein 2, TJP2; lipopolysaccharides, LPS.

unexposed, control intestines. After depuration in clean seawater for two months after PFBS exposure, profiles of microbial communities in previously exposed guts still did not completely recover to the conditions of unexposed controls. This result demonstrated that dysbiosis of microbiota in the gut by exposure to PFBS was durable and long-lasting. Compared to F0 parents, the PCA plot of microbiome in F1 guts after parental exposure to PFBS was significantly different from that of the control group (Figure 3C). Although F1 offspring were cultured in PFBS-free seawater for an entire life-cycle, transgenerational dysregulation of gut microbiota and intestinal health was still notable. Because intestines of F1 adults did not contain PFBS, dysbiosis of microbiota in their guts could result from the indirect influences from host anomalies. Furthermore, variation in physiological status between male and female medaka may influence gut microbial

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composition differentially. In F1 male adults, correlation analysis showed that intestinal expressions of IL1β (R = −0.5; P = 0.04) and TJP2 (R = −0.5; P = 0.03) were negatively associated with the abundance of *Vibrio* genus. Serotonin levels in F1 female intestines were significantly and positively correlated with *Lutimonas* abundance (R = 0.9; P < 0.001).

Correlation between Intestinal Microbes and Host Health. Changes in the abundances of major contributing genera to PCA variation were listed herein. Correlation of bacterial genera with host health was also constructed. In intestines of F0-exposed males, abundances of *Cetobacterium* were increased after exposure to 2.9 μg/L PFBS relative to controls but were significantly decreased after exposure to 9.5 μg/L PFBS (Figure 4A). *Cetobacterium* genus was significantly and positively correlated with host TJP2 expressions, a biomarker of epithelial barrier integrity (Figure 4B). Intestines of F1 females exhibited significantly lesser abundances of *Vibrio* after exposure of F0 parents to 9.5 μg/L PFBS (Figure 4C). However, the genera *Planctomyces* and *Lutimonas* had increased abundances in F1 intestines after parental exposure to 9.5 μg/L PFBS (Figure 4D and 4E, respectively). Significantly negative correlations between abundances of *Vibrio* and *Planctomyces* (R = −0.8; P < 0.001) or *Lutimonas* (R = −0.8; P < 0.001) were observed. These results indicated that PFBS parental exposure induced niche transition of microbes in the gut ecosystem. Decreased relative abundances of *Cetobacterium* were observed in intestines of F1 males derived from parents exposed to 1.0 μg/L PFBS (Figure 4F). Furthermore, the abundances of the genus *Cetobacterium* were significantly and positively correlated with production of toxic LPS in F1 blood (Figure 4G). In intestines of teleosts, *Cetobacterium* bacteria can efficiently produce vitamin B_{12} upon the demand of host.[33] Therefore, fluctuations in abundances of *Cetobacterium* might affect the supply of vitamin B_{12} in the intestine, which could result in deficiencies and mental disorders.[34] The positive correlations of *Cetobacterium* with both TJP2 and LPS levels probably indicate the pros and cons of certain bacterium in the intestines. A significant positive correlation was also observed between abundances of *Vibrio* and body weight of F1 individuals, which is indicative of an apical adverse effect (Figure 4H). *Vibrio* is the dominant genus in the gut of the marine medaka. While some species of *Vibrio* are pathogenic to human and fish, this genus may also participate in the normal metabolism and growth of marine organisms.[35]

■ ASSOCIATED CONTENT

Supporting Information

This material is available free of charge online at The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.estlett.8b00597.

Changes in survival, fecundity, K factor, and HSI values in F0 and F1 generations (Figure S1), rarefaction curves of representation of microbial diversity in guts (Figure S2), alterations in microbial community of F0 and F1 intestines at phylum level (Figure S3), alpha diversities of intestinal microbiota of F0 and F1 medaka after exposure to PFBS (Table S1) (PDF)

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Notes

The authors declare no competing financial interest.

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Supporting Information

PFBS Exposure Causes Durable and Transgenerational Dysbiosis of Gut Microbiota in Marine Medaka

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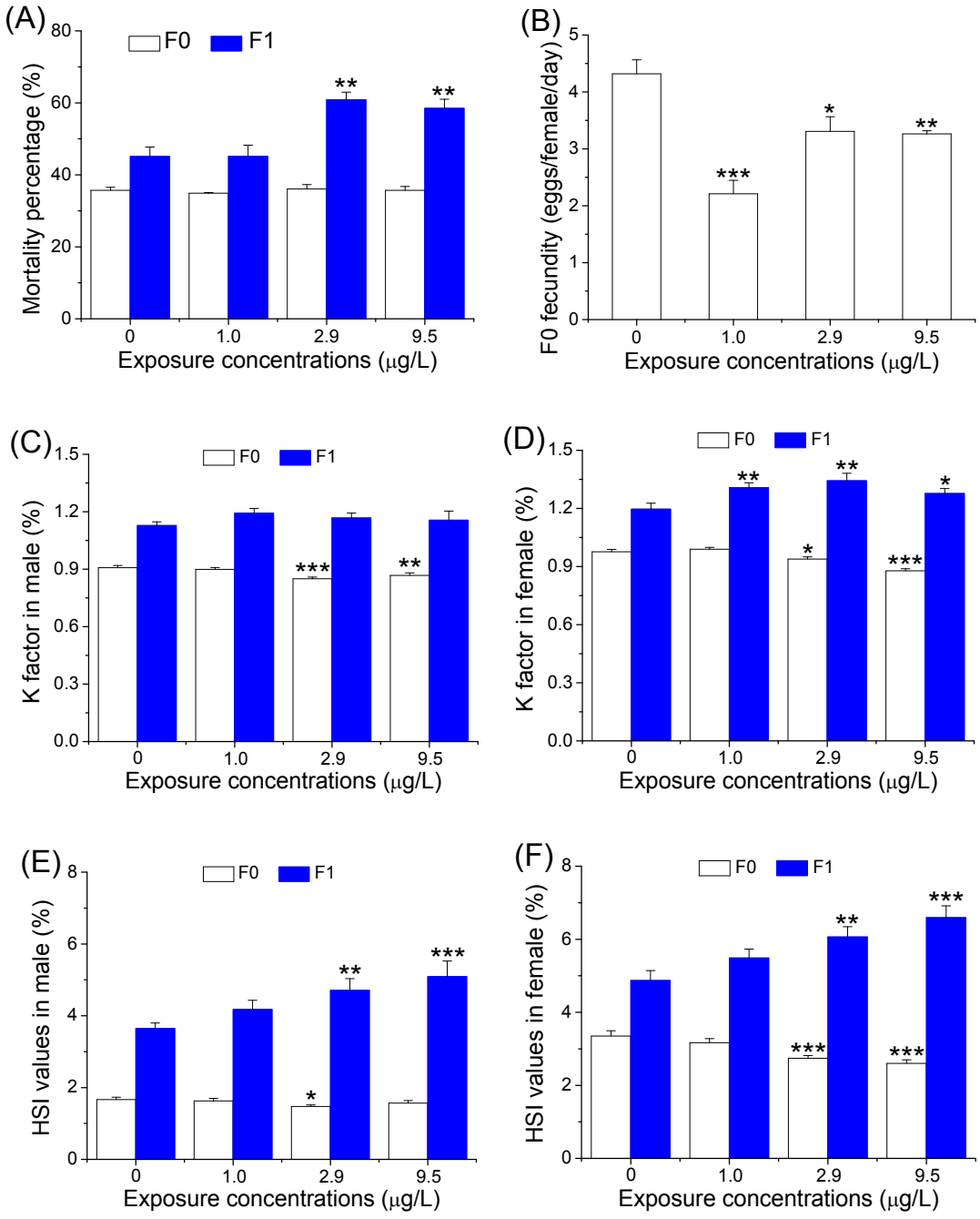

Figure S1. Changes in mortality through entire duration (A), egg production within the last week of exposure (B), K factor (C and D) and hepatosomatic index (HSI; E and F) of male or female, F0-exposed or F1 marine medaka after a life-cycle exposure to various concentrations of PFBS (0, 1.0, 2.9 or 9.5 μg/L). K factor = mass (g)/length $(cm)^3 \times 100$; HSI = liver mass/body mass × 100. Values represent the mean ± SEM of three replicates. **P* <0.05, ***P* <0.01 and ****P* <0.001 indicate significant difference

between exposure groups and the corresponding control group.

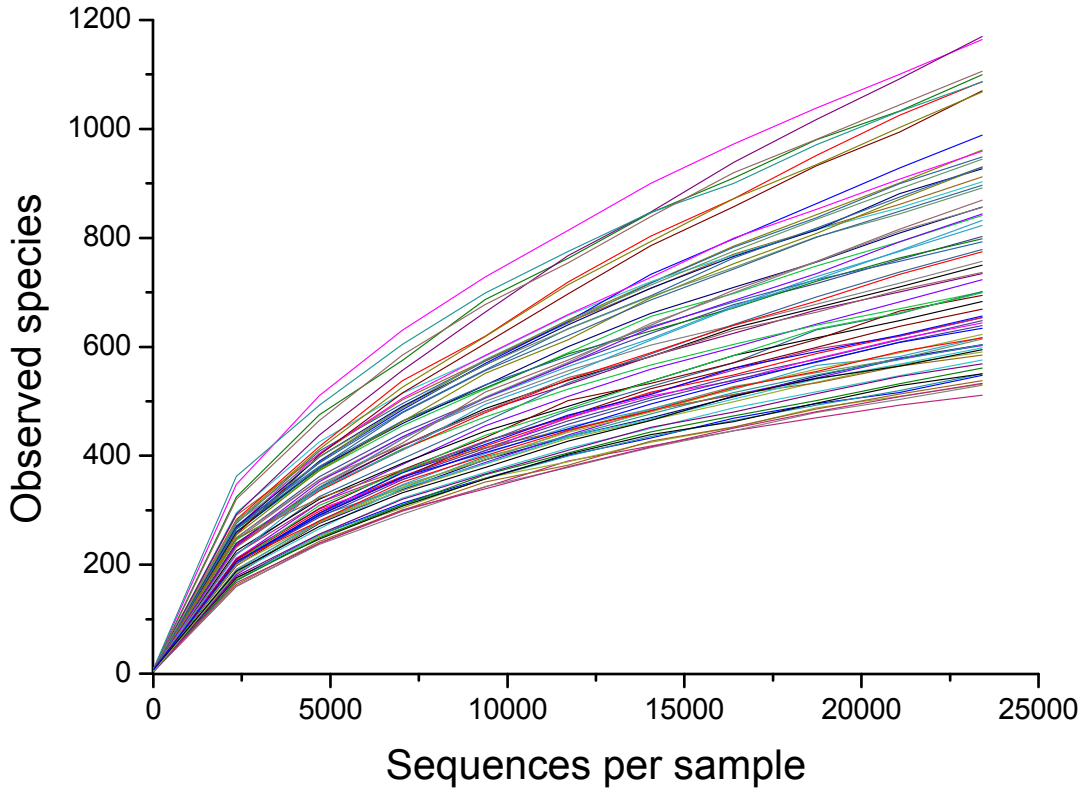

Figure S2. Rarefaction curves based on numbers of sequences using 16S rRNA amplicon sequencing and observed species to demonstrate diversity of the microbial community of the intestine.

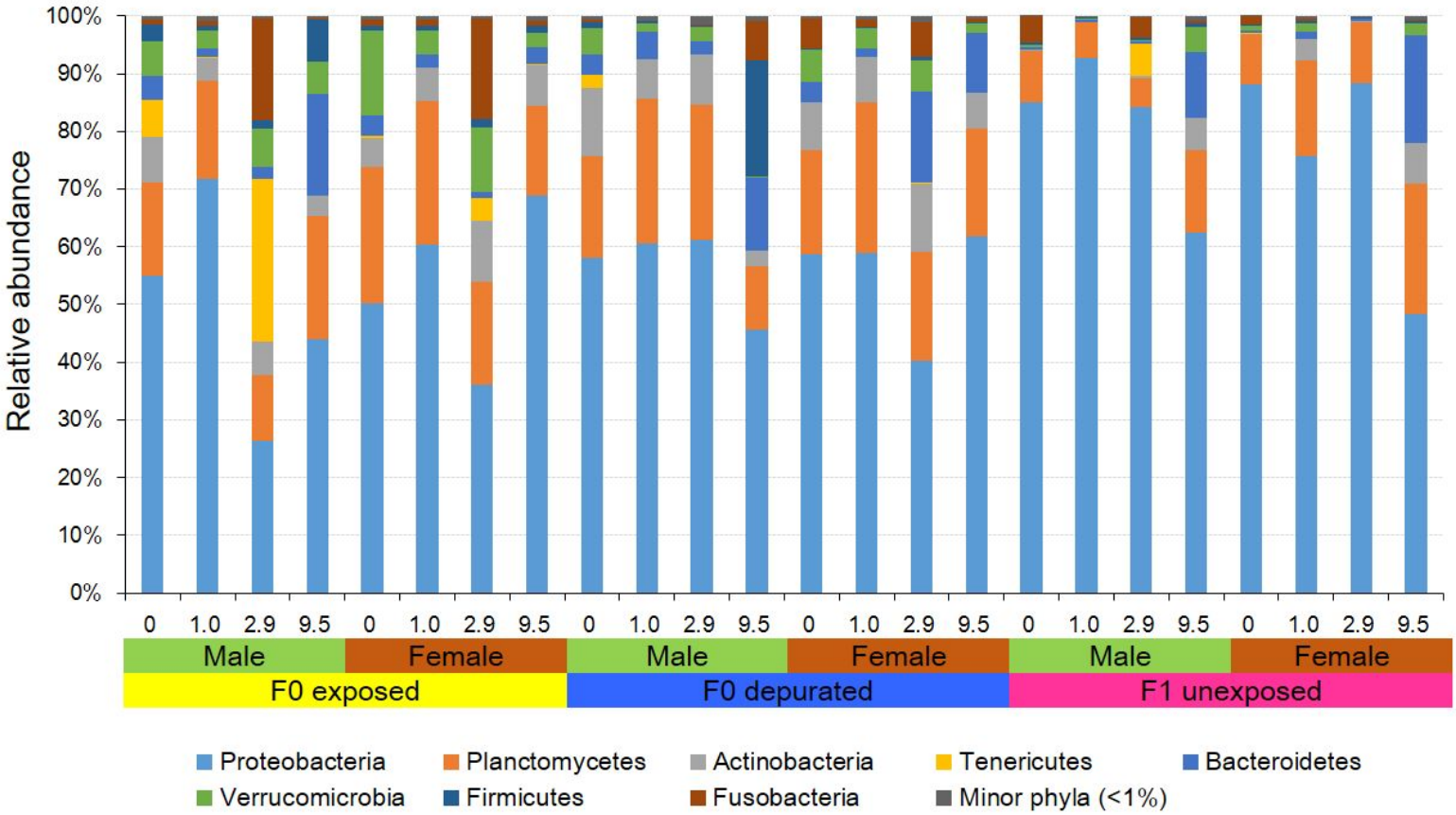

Figure S3. Alterations in relative abundances (%) of bacterial phyla in F0-exposed, F0-depurated and F1 intestines after a life-cycle exposure to various environmentally realistic concentrations of PFBS (0, 1.0, 2.9 and 9.5 µg/L). Values are represented as mean of three replicates (each replicate containing five intestines).

Table S1. Alpha diversity in intestines from F0-exposed, F0-depurated and F1 medaka after parental exposure to environmentally realistic concentrations of PFBS (0, 1.0, 2.9 or 9.5 μg/L).[a]

Male		Observed species	Shannon [b]	Simpson [b]	PD whole tree [b]	Chao1 [c]	Good's Coverage% [d]
F0-exposed	**0**	1018.3±80.5	6.2±0.2	1.0±0.0	62.8±3.5	2167.7±292.6	97.8±0.0
	1.0	806.3±9.7	5.6±0.3	0.9±0.0	50.5±1.1	1506.6±159.9	98.4±0.0
	2.9	764.0±89.8	5.4±0.4	0.9±0.0	47.3±3.9	1484.3±274.3	98.3±0.0
	9.5	771.0±158.8	5.3±0.9	0.9±0.1	49.4±11.0	1499.6±393.6	98.4±0.0
F0-depurated	**0**	949.0±83.2	6.1±0.2	1.0±0.0	56.6±3.9	2064.3±274.1	97.9±0.0
	1.0	745.7±68.6	5.5±0.1	0.9±0.0	44.9±5.2	1450.1±192.9	98.4±0.0
	2.9	700.0±68.4*	5.7±0.2	1.0±0.0	39.5±2.8*	1406.5±265.9	98.5±0.0
	9.5	685.7±87.9*	5.6±0.6	0.9±0.1	42.3±4.5*	1377.7±372.1	98.6±0.0
F1	**0**	563.0±22.0	3.7±0.2	0.8±0.0	33.9±1.7	937.4±20.6	98.9±0.0
	1.0	561.3±24.9	3.7±0.2	0.8±0.0	34.9±1.6	980.7±27.0	98.9±0.0
	2.9	620.7±71.4	3.8±0.6	0.8±0.1	37.2±3.7	1142.2±168.0	98.7±0.0
	9.5	817.0±148.7*	5.1±0.6*	0.9±0.1	47.8±8.5	1685.9±509.6	98.2±0.0
Female							
F0-exposed	**0**	785.3±60.3	5.7±0.4	1.0±0.0	48.1±2.8	1465.8±109.7	98.4±0.0
	1.0	1007.3±130.6	6.0±0.2	1.0±0.0	60.0±6.5	2667.7±711.9	97.5±0.0
	2.9	739.0±68.6	5.6±0.3	0.9±0.0	47.2±3.8	1502.0±390.1	98.4±0.0
	9.5	712.0±99.0	4.8±0.8	0.9±0.1	46.2±8.0	1264.9±193.3	98.5±0.0
F0-depurated	**0**	745.3±52.6	5.9±0.3	1.0±0.0	41.1±2.6	1434.3±188.6	98.5±0.0
	1.0	919.3±103.2	5.9±0.0	1.0±0.0	51.3±5.0	2326.7±526.0	97.8±0.0
	2.9	747.3±103.4	5.8±0.3	1.0±0.0	43.8±7.0	1582.8±441.5	98.4±0.0
	9.5	783.3±95.5	5.0±0.7	0.9±0.1	47.7±7.2	1829.4±405.9	98.1±0.0
F1	**0**	581.3±47.2	3.6±0.5	0.8±0.0	35.2±3.1	993.4±142.0	98.9±0.0
	1.0	889.0±141.6*	5.2±0.8*	0.9±0.1*	52.1±7.4*	2088.8±579.5	97.9±0.0
	2.9	585.0±25.2	3.6±0.2	0.8±0.0	34.6±1.1	997.1±31.3	98.8±0.0
	9.5	818.7±95.5	5.7±0.3*	0.9±0.0*	50.6±6.9	1880.3±385.1	98.1±0.0

[a] Values represent the mean ± SEM of three replicates;
[b] indicative of bacterial community diversity;
[c] indicative of bacterial community richness;
[d] indicative of bacterial sequencing coverage;
*P <0.05 indicates significant difference between exposure groups and the control group.