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HORMETIC RESPONSES OF FOOD-SUPPLIED PCB 31 TO ZEBRAFISH *(DANIO RERIO)* GROWTH

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□ Hormesis is commonly defined as a beneficial or stimulatory effect caused by exposure to low doses of a chemical known to be toxic at high doses. Hormetic responses of food-supplied PCB 31 (2, 4', 5-Trichlorobiphenyl) was studied by using zebrafish (*Danio rerio*) growth as an end point. The results in general followed the hormesis hypothesis, PCB 31 at lower concentrations (0.042 μg/g and 0.084 μg/g) exhibited beneficial effects on the growth of zebrafish by weight and length while higher concentrations (10μg/g and 20μg/g) revealed inhibitory effects. The magnitude of stimulatory responses of zebrafish growth by weight and length at lower concentrations (0.01-0.084 μg/g) on days 14 and 21 were in the range 9.09-18.18%; 10-38.09% and 4-14.4%; 6.25-10.93%, respectively as compared to control. Growth and conditions indices also suggested that the zebrafish was healthier at lower concentrations as compared to those at higher concentrations. The results of the present study will elaborate fish toxicological evaluation regarding the hormetic model.

Keywords: Hormesis, Inverted U-shaped, Xenobiotics, Zebrafish growth.

INTRODUCTION

Polychlorinated biphenyls (PCBs) are widespread in the environment and applied in a range of industrial applications such as used as insulating materials and coolant fluids in transformer, capacitors and motors, and also produced as a byproduct during electric waste recycling (Jursa *et al.* 2006, Bordajandi *et al.* 2008). The global occurrence of PCBs is the result of ocean currents and atmospheric deposition (Aono *et al.* 1997, Breivik *et al.* 2004). PCBs consist of up to 209 different congeners, and the toxicity of individual PCB is structure dependent. Congeners with no or one chlorine substituent in the *ortho* positions may assume a coplanar configuration, bind to the aryl hydrocarbon receptor (AhR), and have pattern of toxicity similar to 2,3,7,8-tetrachlorodibenzo-p-dixoin (TCDD) (Chen *et al.* 2010, Shen *et al.* 2011). PCB 31 have coplanar configuration with toxicity similar to TCDD or dioxin like PCBs and the hormetic toxicity of

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dioxin like compounds has been reported previously for cell lines (Shen *et al.* 2011). However, to our best of knowledge there is no information of hormetic toxicity to fish. Upon release into the environment, PCBs can enter the food chain via ingestion by benthic animals owing to their high affinity for adsorption to particles, which settle out and accumulate as a result of sediment deposition. PCBs are hydrophobic in nature and accumulate in the lipids as a result of ingestion and can cause dermal toxicity, hepatotoxicity, immunotoxicity and carcinogenesis in addition to endocrine and reproduction effects in both humans (Vater *et al.* 1995, Safe *et al.* 1997, Carpenter 2006, Tiido *et al.* 2006) and animals (De Flora *et al.* 1991, Kozie and Anderson 1991, Tanabe *et al.* 1994, Eqani *et al.* 2013).

During the past few decades biphasic dose/response relationships that are characterized by a stimulatory response in the measured parameter at low doses of a stressor while inhibition at higher doses are well recognized in toxicology and pharmacology (Calabrese and Baldwin 2001, Calabrese and Blain 2005). This dose/response phenomenon is termed hormesis and represents an evolutionarily conserved process of adaptive, potentially beneficial responses to low doses of a stressor agent/condition (Calabrese *et al.* 2007). Recently, several researchers made considerable advances in hormetic mechanisms to prove hormesis a highly plausible phenomenon (Hashmi *et al.* 2014b; Calabrese 2013), and suggested that hormetic responses may be activated by receptor-mediated or cell signaling-mediated hormetic mechanisms, affecting a broad range of cell types and endpoints. It has been observed with diverse range of both inorganic and organic compounds such as As, Cd, Cr, Hg, higher chlorinated PCBs and some pesticides (Kushida *et al.* 2005, Cedergreen *et al.* 2007, Belz *et al.* 2008, Shen *et al.* 2009, Shen *et al.* 2010, Spoljaric *et al.* 2011, Wang *et al.* 2012) with a wide range of endpoints like carcinogenicity, life span, growth in plants and algae and fish behavior (Perez-Benito 2006, Puatanachokchai *et al.* 2006, Kurta and Palestis 2010, Spoljaric *et al.* 2011) and in essentially all organisms studied so far. However, in the context of fish toxicology studies regarding hormetic effects to chemicals in general and particularly to PCBs are still scarce (Hashmi *et al.* 2014a).

Growth rates are fundamental life-history traits of organisms (West *et al.* 2001). Variations in growth help differentiate the evolutionary success of individuals, population growth (Phillips 2009), and play vital role in ecosystem function, e.g., production (Waters 1977). Factors such as nutrients, temperature, food availability, competition, predation, and hydrology are traditionally thought to leverage growth rates (Rypel and Bayne 2009). Increasingly, human activities, including certain types of pollution, are being linked to growth processes (Migliore *et al.* 2010, Rypel and Bayne 2010). Still, there is scarce information regarding hormetic dose responses in fish growth for chemicals. So, this study was designed to investigate: 2,4',5-Trichlorobiphenyl (PCB31) hrometic responses to fish

growth and the influence of different concentrations of PCB 31 on fish growth index and condition factors.

MATERIALS AND METHODS

Chemicals

PCB 31 was purchased from Accustandard, Inc. (New Haven, CT, USA). PCB 31 was dissolved in dimethyl sulfoxide (DMSO). A 50 μg/g stock solution of PCB 31 was prepared with DMSO and further working concentrations were prepared using stock solution.

Experimental Design

Wild-type, adult zebrafish *(Danio rerio)* of same age (2 months old) were purchased from a local pet store or hatchery (Animal Pantry, Zhejiang province, China) and maintained in 15 glass tanks. All the experimental conditions were same as described previously(Laiz-Carrión *et al.* 2005). Briefly, the zebrafish were acclimatized in two clean tanks (70 L) in semi static conditions for two weeks. The tanks were kept heated at approximately 24ºC, continuously aerated and filtered with standard aquarium pumps. The photoperiod was set at a 10 h light and 14 h dark cycle. Due to the hydrophobic and hydrophilic nature of PCBs, we spiked PCB 31 in the fish food. Fish PCBs exposure through feeding has already been reported previously (Bengtsson 1979, 1980; Daouk *et al.* 2011), and confirmed that feeding is suitable method to assess the effects of PCBs to fish. The fish food was spiked with different concentrations of PCB 31 such as 0.001μg/g (t1), 0.01μg/g (t2), 0.014μg/g (t3), 0.028μg/g (t4), 0.042μg/g (t5), 0.084μg/g (t6), 10μg/g (t7) and 20 μg/g (t8) (Bengtsson 1980). Briefly, food was spread out in different thin layer Petri dishes and was then soaked in DMSO. After 5 to l0 min, different concentrations of PCB 31 were spiked in fish food. After a careful stirring for a couple of minutes, the dishes were set for freeze drying to remove water or DMSO contents. The control diet was made by mixing food with an equal volume of only DMSO. A freeze dryer (Dura-Dry™ MP, FTS Systems, Stone Ridge, NY, USA) was used to remove water or DMSO from the diets. Freeze dried diets were crushed into flakes which were used as source of diet (Daouk *et al.* 2011). Food was supplied in surplus amount to ensure its availability to fish for the whole period of experiment and it was commercial flake food. Food was purchased from a local pet store and was free from any chemical contamination (Spinello *et al.* 2013). Our facilities and procedures have been approved by the Institutional Animal care and use committee of the Animal Science department Zhejiang university, China.

Before undertaking the experiment, ten fish of same age were randomly taken out, weighed (0.01 g accuracy), and measured for total length (1 mm accuracy) to establish d_0. The 28-day experimental assay

consisted of a static glass tanks with a capacity of 15 L, in which 13 L of clean deionized water was allocated. The assay was performed for eight different concentrations of PCB 31 with one DMSO control, thus nine tanks were used in total. The treatment conditions remained same as those applied during the acclimation period. Sixty fish per treatment were randomly distributed in each tank. In order to maintain an acceptable quality of overlying water, a daily water change (1/3 of total water volume) was performed as PCBs are hydrophobic. Water parameters (temperature, pH, oxygen, salinity and turbidity) were monitored on daily basis before renewal of water.

Analysis of PCB 31

Analysis of PCB 31 was accomplished by use of previously established methods with modifications(Bengtsson 1979, Bengtsson 1980). Briefly, food samples were collected at the end of experiment. Food samples were then spiked with a mixture of decachlorobiphenyl (PCB-209) as the surrogate standard. Food samples (1 g) were soxhlet-extracted with 120 mL high purity acetone/hexane (1:1, v/v) for 24h at 4-6 cycles/h. Each extract was cleaned up by a Florisil column with a top layer of anhydrous sodium sulfphate. The column was subsequently eluted with 100mL hexane and further concentrated to 1mL. PCB 31 concentrations in the food samples for each tank were quantified by GCMS (an Agilent 7890A apparatus equipped with an Agilent 5975C mass spectrometry detector and autosampler). The recoveries of surrogate standards for PCB determinations were in ranges of 82–105%. All the chemicals used in the study were of analytical grade and purchased from Merck, Germany. The glassware used was baked at 450 °C for 6 h before use. The detectable concentrations of PCB 31 are given in Table 1.

Growth Trial

At time 0 (start of experiment), 7, 14, 21 and 28 days (end of experiment), 10 fish from each tank (60 fish per treatment) were captured by netting, slightly anaesthetized with 2-phenoxyethanol (0.5 ml/L water), weighed and measured. No mortality was observed during this manipulation. Following parameters were calculated to evaluate the effects of different concentrations of PCB 31 on the growth of the zebrafish:(Kerambrun *et al.* 2012).

Growth index (GI)

Zebrafish specific growth rates in weight (% per day) were estimated as:

$$GW = 100(lnW2 - lnW1)\,t2 - t1$$

TABLE 1. PCB 31 supplied and measurable concentrations in zebrafish food

	Concentration	
Treatments	Supplied	Measurable*
Control	0	0
t1	0.001	0.002
t2	0.01	0.04
t3	0.014	0.05
t4	0.028	0.06
t5	0.042	0.09
t6	0.084	0.1
t7	10	11
t8	20	30

*only one gram of food sample was left at the end of experiment

where W1 and W2 are zebrafish total body weight at times t_1 (start of experiment) and t_2 (time of collection). Similarly, the specific growth rate in length was estimated as:

$$GL = 100(lnL2 - lnL1)\,t2 - t1$$

where L_1 and L_2 are total length of zebrafish at times t_1 and t_2 respectively.

Condition indices (CI)

We estimated Fulton's condition index (k) as an indicator of the general well being of the zebrafish. This morphometric index assumes that heavier fish, for a given length, are in better condition. We calculated Fulton's condition index (K) using the formula:

$$K = 100(W/L3)$$

where W is the body mass (mg) and L is the total length (mm).

Statistical Analysis

One way analysis of variance (ANOVA) followed by the Duncan's Multiple Range Test was used to sort out the differences in growth increase in zebrafish during different days and PCB 31 exposures. The results were presented in the form of box and whisker plots. The probability level value ($p < 0.05$) was accepted as significant. Statistical software (SPSS 16) was used for statistical analysis.

RESULTS

Hormetic responses of PCB 31

A typical low-dose (hormetic) stimulation by a low concentration of a chemical normally inhibitory to growth at higher concentrations is shown in Fig. 1 for zebrafish. Zebrafish growth increased in some experimental days in response to low PCB 31 doses when compared with control zebrafish values. However, the growth of the control zebrafish was also varied between experimental times. During the four experimental times with media exposure, the control zebrafish were not significantly different from each other ($F_{4, 36} = 0.27$, $P = 0.89$).

However, after two weeks, on day 14 and 21 concentrations such as 0.042 µg/g and 0.084 µg/g showed significant ($F_{4, 36} = 2.91, 4.50$, $P = 0.04$ & 0.02 respectively) stimulatory responses in the weight and length of zebrafish (Fig. 1). While, the higher concentrations such as 10 µg/g and 20 µg/g exhibited significant inhibitory responses in weight and length of zebrafish with the decreasing trends in growth. The results in general depicted the picture of inverted U-shaped dose response phenomenon. The increase/stimulatory response in weight of zebrafish were 10-38.09% higher at concentration of 0.084 µg/g on 21 days as compared to the control one.

Environmental Parameters

No mortality was observed in any of the exposure tanks except in the last week at 10 µg/g and 20 µg/g of PCB 31. Temperature (24.5ºC), pH (7.44), turbidity (46.1 NTU) and oxygen (20.87 mg/L) levels were constant in the different exposure tanks throughout the experimental assay. All the environmental parameters were monitored on daily basis and any change in pH, turbidity and oxygen was adjusted by supplying the fresh water.

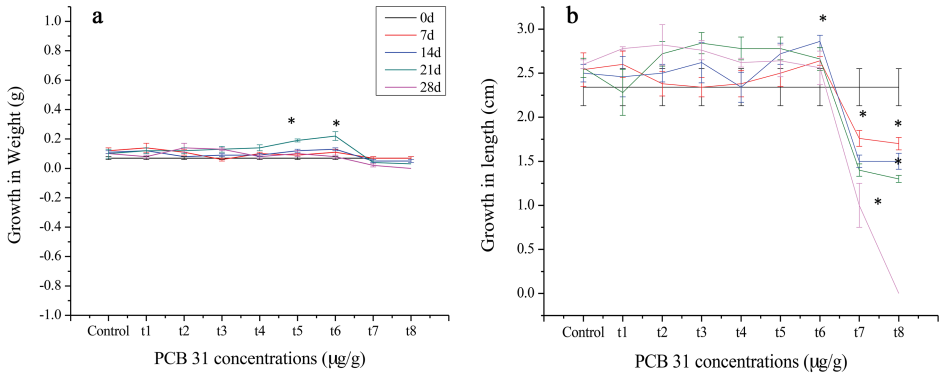

FIGURE 1. Hormetic responses of PCB 31 upon zebrafish growth in (a) weight (g) and (b) length (cm).

Physiological Biomarkers

The zebrafish exposed to different concentrations of PCB 31 for 7 and 28 days revealed a positive specific growth rate in weight (GW) (Table 2). The zebrafish GW was significantly higher on 28 days for 0.01 μg/g (23.15±15.03) and 0.014 μg/g (25.41±9.20) of PCB 31 as compared to the other days and treatments. Zebrafish indicated a loss in weight after 20 μg/g of PCB 31 for 21 days (Fig. 2). The specific growth rate in length (GL) of zebrafish was significantly higher (1.92±0.07) for 28 days exposed to 0.001μg/g of PCB 31 as compared to the other treatments and exposure times. The zebrafish exposed to the 10 μg/g and 20 μg/g of PCB 31 revealed significantly lower GL as compared to the other concentrations.

For both, different PCB 31 concentrations and exposure times, the control fish showed a similar Foulton's k index to that of d_0, d_7, d_{14}, d_{21} and d_{28} (Fig. 2). The Foulton's k index value after 21 days was significantly higher 8.97±3.01 at 0.084 μg/g of PCB 31 as compared to other concentrations and time periods. However, after 28 days of exposure to 10 μg/g and 20 μg/g of PCB 31 zebrafish indicated lower k index values 1.55±1.13 and 0.00±0.00, respectively compared to the d_0 and control.

DISCUSSION

Hormetic responses of PCB31

In the present study, zebrafish growth in weight and length was used to examine the effects of PCB 31 on growth. The results supported the hormesis hypothesis, as stimulatory effects of PCB 31 on zebrafish growth were observed at low concentrations and inhibitory effect at the high concentration, resulting in a biphasic dose-response curve. Regarding growth in weight (Fig. 1a) and growth in length (Fig. 1b), inverted U-shaped dose response curve was observed after exposure to orally supplied PCB 31. After 14 and 21 days the concentrations of PCB 31 such as 0.042 μg/g and 0.084 μg/g showed significant (ANOVA, P= 0.02 & 0.04 respectively, Fig. 3) increased in stimulatory responses by weight and length of the zebrafish (Fig. 1). While, the higher concentrations (10 μg/g & 20 μg/g) of PCB 31 exhibited significant inhibitory responses by weight and length of the zebrafish. Low dose stimulatory effects were also reported under laboratory studies to minnow fish for certain PCBs mixture (Clophen A 50) (Bengtsson 1979, Bengtsson 1980). However, for the first time Rypel and Bayne (2010) in field conditions found that mixture of PCBs burden at low concentrations were related to increase in fish growth. The remarkable increase in growth of zebrafish in our study after 14 and 21 days at 0.084 μg/g PCB 31 concentration may be related to a stimulation of different osmoregulatory hormones such as growth hormone, cortisol, etc, which are also related to growth in teleosts (McCormick 2001) including gilthead sea bream (*Sparus aurata*) (Mancera *et al.* 1994, Miguel Mancera

TABLE 2. Growth and condition indices of zebrafish growth at different time periods.

Index	PCBs concentrations (µg/g)	d0	d7	d14	d21	d28	P
Specific growth rate in weight	Control	-	2.60±1.55	0.76±0.36	-0.52±0.43	0.94±0.21	NS
	0.001	-	5.43±2.94	2.14±1.11	1.50±1.40	-2.38±0.85	NS
	0.01*	-	5.48±2.98	1.50±1.01	2.64±2.00	23.15±15.03	0.00
	0.014*	-	-2.49±0.67	2.65±0.10	2.63±0.85	25.41±9.20	0.00
	0.028*	-	6.66±5.90	2.92±1.60	2.41±1.72	8.99±2.24	0.00
	0.042	-	3.37±0.08	3.66±1.78	2.03±1.45	8.38±1.97	NS
	0.084	-	6.51±4.23	4.18±2.34	2.82±0.34	4.85±1.54	NS
	10	-	-6.39±0.66	-3.64±0.23	-3.62±0.11	-1.86±0.81	NS
	20*	-	5.21±3.38	-2.81±3.45	-17.26±12.87	9.61±0.49	0.00
Specific growth rate in length	Control	-	0.34±0.09	0.57±0.16	0.98±0.83	0.58±0.65	NS
	0.001	-	1.00±0.81	0.03±0.89	-0.38±0.38	1.92±0.07	NS
	0.01	-	0.57±0.56	0.57±0.18	0.82±0.74	0.73±0.55	NS
	0.014	-	0.00±0.00	0.73±0.55	0.91±0.38	0.59±0.40	NS
	0.028	-	0.66±0.11	0.16±0.02	0.96±0.58	0.53±0.05	NS
	0.042	-	0.74±0.91	0.99±0.25	0.76±0.45	0.37±0.07	NS
	0.084	-	0.66±0.35	0.90±0.88	0.33±0.07	0.02±0.09	NS
	10	-	-0.07±0.29	-1.17±0.85	-1.11±0.60	-1.39±0.52	0.00
	20	-	-0.49±0.28	-1.17±1.27	-1.44±0.44	-2.01±0.35	0.00
Foulton's condition factor	Control	4.04±1.15	4.68±1.52	4.05±1.29	3.62±2.44	3.34±0.31	NS
	0.001	3.75±0.77	5.40±2.71	5.12±1.67	5.56±1.61	3.02±0.56	NS
	0.01*	3.05±0.60	4.41±1.40	3.40±0.60	4.59±1.45	4.73±1.54	0.00
	0.014*	2.74±0.58	2.51±1.12	3.60±0.87	4.36±1.48	4.70±0.66	0.00
	0.028	2.85±0.70	4.21±0.30	4.10±0.78	3.99±1.43	3.21±0.48	NS
	0.042	3.14±0.95	3.77±0.94	4.51±0.79	4.15±1.48	3.96±2.16	NS
	0.084	2.84±0.91	4.34±1.33	4.46±0.97	8.97±3.01	3.14±0.78	NS
	10*	4.55±0.44	4.16±0.53	3.27±0.72	2.78±0.82	1.55±1.13	0.00
	20*	3.97±0.91	4.08±0.55	3.23±0.99	2.27±0.60	0.00±0.00	0.00

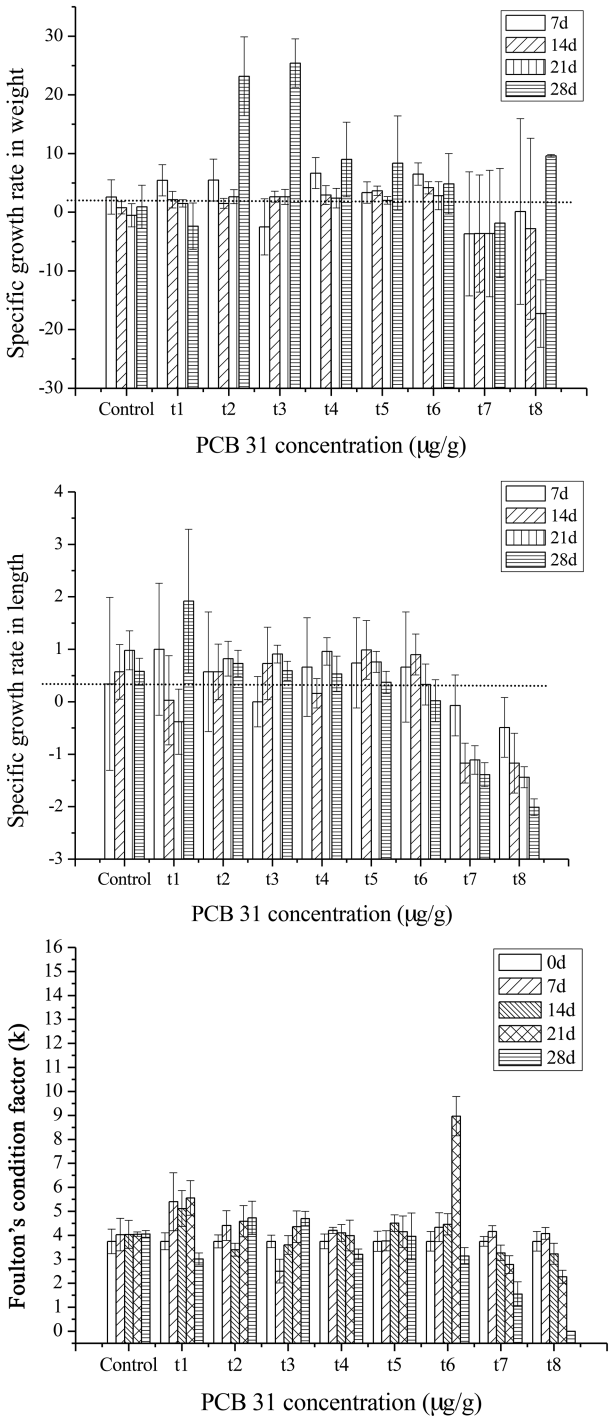

FIGURE 2. Specific growth rate in weight (GW), length (GL) and Foulton's condition factor (k) of zebrafish exposed to different concentrations of PCB31.

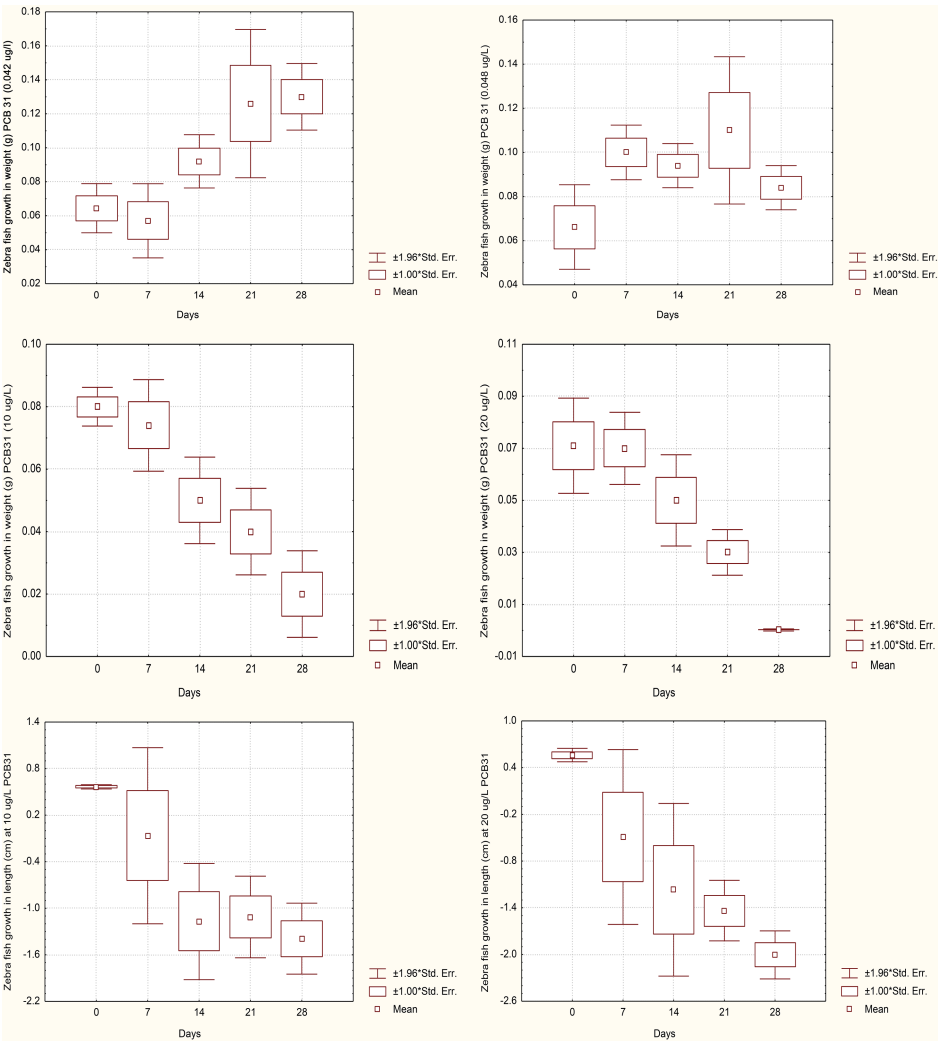

FIGURE 3. Box and whisker plots of zebrafish growth in weight (g) and length (cm) significantly different among days and different PCB 31 concentrations.

et al. 2002). Other possibilities related to increase in zebrafish growth may be metabolic reorganization (Sangiao-Alvarellos *et al.* 2003) at lower concentrations of PCB 31.

The results revealed that at higher concentrations of PCB 31 zebrafish growth showed inhibitory responses, suggesting that may be due to the inactivation of growth hormones at higher concentrations. However, future studies are necessary to investigate the molecular mechanisms involved in the decrease of zebrafish growth. It has been reported that a well-known effect of dioxin-like PCBs in many species is the 'wasting syndrome' which results in enhanced weight loss in spite of a normal appetite. This 'wasting syndrome' has also been reported in fish (Kleeman

et al. 1998; Ginneken *et al.* 2009). We speculate therefore that the PCB-treatment affected intermediary metabolism and metabolic processes and made the zebrafish somehow economize on energy expenditure. Several studies have also shown that the inhibitory responses in growth of fish in a number of species may be due to higher concentration of chemicals, especially at early life stages, such as larvae and juveniles (Bengtsson 1979, Bengtsson 1980, Al-Yakoob *et al.* 1996, Rypel and Bayne 2010). Similarly, a decrease in growth and condition of different fish species exposed to higher PCBs mixtures and heavy metals levels has been observed previously (Rowe *et al.* 2001, Alquezar *et al.* 2006). Further, the results of our study suggested that differences in fish growth could represent a sum-up of the sub-lethal responses to chemical contaminants as growth integrates many processes (Morales-Nin *et al.* 2007). In particular, exposure to chemicals could lead to a change in energy allocation which would be used preferentially for resistance to chemical stress to the detriment of growth in zebrafish (Rowe *et al.* 2001).

Growth and condition indices

Growth and condition indices are used to estimate the weight gain and general well being of fish. In the present study, the effects of PCB 31 on the growth and condition of zebrafish were also studied. The results for growth index showed that the positive GW and GL were observed at lower concentration (0.001μg/g) of PCB 31 for 28 days. The results trends in growth index recorded in the present study were different from the previous study (Kerambrun *et al.* 2012), where the author observed the positive GW and GL for 7days only for control fish group. Therefore, results of present study suggested that lower concentrations of PCB 31 also positively influence the growth index by weight and length of zebrafish.

The Foulton's k index value in our study showed that after 21 days it was higher at 0.084 μg/g of PCB 31 while k index exhibited decreasing trends after 28 days of exposure to 10 μg/g and 20 μg/g of PCB 31 (Fig. 2). However, the Foulton's k condition factor of juvenile turbot (*Scophthalmus maximus*) investigated by Kerambrun *et al.* (2012) indicated that in control group it was higher as compared to the different exposure of chemicals. The author observed a decrease in k factor after each successive time period. The results in general suggested that condition and health of zebrafish was good at relatively lower concentrations. On the contrary, the exposure of zebrafish to the higher concentrations of PCB 31, led to a decrease of their biological performance. Indeed, zebrafish growth and condition indices decreased with the level of PCB 31 contamination. A decrease of growth and condition indices were observed in relation with the chemical contamination in sediments, a dose-dependent effect of chemical contamination on fish health appeared evident (Kerambrun *et al.* 2012). Hence, the results suggested that growth and condition indices

were positively influenced at low concentrations of PCB 31 and negatively at high concentrations.

CONCLUSIONS

The phenomenon of hormesis, which is characterized by stimulatory effects of chemicals at low doses and inhibitory effects at high doses, was studied by using zebrafish (*Danio rerio*) growth as an end point. It was concluded from the study that PCB 31 at lower concentrations (0.042 μg/g and 0.084 μg/g) stimulated zebrafish growth by weight and length while higher concentrations (10μg/g and 20μg/g) revealed inhibitory effects. The proposed growth and condition index also suggested that at lower doses fish growth was good as compared to the higher doses for the 28 days experimental period. However, long term studies are needed to check the chemical low doses stimulation in the context of fish toxicology. Further it is suggested that the underlying mechanisms that enhanced or inhibited the growth of zebrafish after exposure to PCB 31 should be investigated further.

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