Preliminary assessment of thermal treatments of Chlorpyrifos and 3,5,6-trichloro -2-pyridinol (a potential precursor of the pyridine analogue of 2,3,7,8-T4CDD) using the early developmental stage embryos of medaka (*Oryzias latipes*)

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ABSTRACT
Chlorpyrifos is one of the major pesticides used today which has a chlorinated aromatic moiety of a pyridine analogue, 2,4,5-trichlorophenol, and can be a potential direct precursor of another pyridine analogue of 2,3,7,8-T4CDD. In the recent study by Holt et al, PCDFs compounds have been detected in chlorpyrifos samples, however, the presence of this analogue of 2,3,7,8-T4CDD has not been assessed yet to our knowledge. Here, we analyzed in a preliminary study the ecotoxicities of this dioxin-like compound in the early developmental stage embryos of medaka (*Oryzias latipes*). Chlorpyrifos and its analogue, 3,5,6-trichloro-2-pyridinol, were thermally treated in closed glass ampoules at 300, 340, and 380 ºC for 10 minutes and exposed to medaka embryos in early developmental stage for 48 hours, to addressed the response of the 20 biomarker-genes expressions. The thermally treated 3,5,6-trichloro-2-pyridinol enhanced expression of CYP1A1 and AhRR as 2,3,7,8-T4CDD and PCBs, suggesting that the potentially ecotoxic compounds like 2,3,7,8-T4CDD is newly produced during thermal treatment of 3,5,6-trichloro-2-pyridinol. The expression profiles of the 20 bio-marker genes in the embryos exposed to 2,3,7,8-T4CDD and 3,5,6-trichloro-2-pyridinol were almost the same, strongly suggesting the thermally treated 3,5,6-trichloro-2-pyridinol sample contained 2,3,7,8-T4CDD-like ecotoxic compounds.

Keywords: chlorpyrifos, thermal treatment, medaka embryos

INTRODUCTION

Pesticide production, use and disposal have contributed significantly to polychlorinated dibenzo-p-dioxins and dibenzofuran (PCDD/F) emissions in the past (Weber, et al., 2008, Masunaga, et al., 2001). In a recent monitoring of current used pesticides in Australia in all assessed formulations, PCDD/PCDF were detected at high concentrations in some pesticide formulation (Hagenmaier, et al., 1986). From historic perspective, the two pesticides with the highest dioxin production and release were 2,4,5-trichlorophenoxy acetic acid (2,4,5-T) and pentachlorophenol (PCP). The production and use of 2,4,5-T and 2,4,5-Trichlorophenol, which can be a precursor of 2,3,7,8-T4CDD, resulted in large contamination during the Vietnam War (366 kg TEQ release from Agent Orange spray) and had a long series of dioxin contamination in the factories with the last accident in Seveso where about 30 kg TEQ have been released (Mocarelli, 2001). Today, 2,4,5-T is not available on the market and the use of 2,4,5-trichlorphenol is restricted to the synthesis of the still available hexachlorophene, one of the major pesticides used today. Another major pesticide used today,

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Received, 2012, Accepted, 2012.

chlorpyriphos, has a chlorinated aromatic moiety of a pyridine-analogue, 2,4,5-trichlorophenol (3,5,6-trichloro-2-pyridinol; pyridinol), making this pesticide to be a potential direct precursor of the other pyridine analogue, 2,3,7,8-T4CDD. In the recent study by Holt et al., (2010), PCDFs have been detected in chlorpyrifos samples and the presence of chlorpyrifos has been assessed with GC/MS analysis by Sakiyama et al., (2011). In this preliminary study, we analyzed the ecotoxicities of this dioxin-like compound in the early developmental stage embryos of medaka (*Oryzias latipes)*. Chlorpyrifos and pyridinol were thermally treated to form their pyrolysates at moderate temperatures and thier precursor potentials were addressed. Medaka embryos in early developmental stage were exposed to these pyrolysates for 48 hours and the ecotoxicogenomics response were assessed by the changes in expressions of the 20 bio-marker specifically chosen for the evaluation of the ecotoxocities of environmental water.

MATERIALS AND METHODS

Thermal treatment

Chlorpyrifos and 3,5,6-trichloro-2-pyridinol (pyridinol) were purchased from Wako Pure Chemicals (chemical standard grade, Osaka, Japan). All pyrolysis experiments were carried out in sealed brown glass ampoules; 0.2mg of chlorpyrifos or 0.8mg of pyridinol were thermally treated in closed brown glass ampoules at 300, 340, and 380 ^{o}C for 10 minutes in a GC-oven (Agilent). After cooled down to the room temperature, the ampoules were opened carefully. Then, the pyrolysates products were extracted with toluene (pesticide grade; Wako Pure Chemicals) and the toluene was evaporated to the volume of 1.5 ml under gentle nitrogen stream. One hundred and fifty μL of the extracts dissolved in the toluene was placed in a vial to be dried up and the pyrolysates was re-dissolved in DMSO 60 μL.

Medaka embryos and exposure to waterborne Chlorpyrifos

Fertilized eggs and embryos were obtained from natural mating of adult orange-red strain medaka fish originally derived from the colony kept in The University of Tokyo and maintained at 26±0.5 ^{o}C under a 14 h-light and 10 h-dark cycle. Newly fertilized eggs (200-300 embryos) were placed in a 100 ml beaker with distilled water and incubated for 24 hours at 26±0.5 ^{o}C to develop. On the next day, live embryos (stage17) were settled into wells of microtiter plates filled with 200μl of waterborne vehicle (DMSO) with 0.32 mg/L of Chlorpyrifos or 1.3 mg/L of pyridinol. The final concentration of the vehicle (DMSO) was to be 0.1% (v/v).

Total RNA isolation and real-time PCR experiments

Total RNA was isolated from the exposed embryos by ISOGEN (Nippon Gene Co., Tokyo, Japan) and then purified with RNeasy Mini Kit (QIAGEN). The quality and quantity of isolated total RNA were verified by agarose gel electrophoresis and a spectrophotometer (ND-1000, NanoDrop Tech.). Total RNA (400–1,000 ng/L) with an OD260/OD280 ratio over 2.0 were obtained from 30–50 embryos and used. cDNAs were synthesized from 1 μg of total RNA using Rever TraAce-α^{TM} (Toyobo, Osaka, Japan) and oligo(dT)20 primers. The relative amounts of the transcripts were quantified using real-time PCR which was performed using SYBR® Premix Ex Taq^{TM} in a Smart Cycler®II System (Takara, Shiga, Japan) and specific primers (Hanno et al., in preparation), according to the manufacturer's instructions. For each sample, gene expression was analyzed in triplicate with the following protocol: 95 ^{o}C for 10 s, followed by 45 cycles of 95 ^{o}C for 5 s and 60 ^{o}C for 20 s. At the completion of each

PCR run, the obtained PCR products were subjected to melting curve analysis to ensure that only a single product was amplified. At least three technical replicates of each RNA sample were conducted. Expression data were quantified based on threshold cycle (Ct) values. For each gene, the Ct values for each sample were averaged and normalized to the mean Ct of EF-1α, which is expressed ubiquitously in tissues and widely used as gene expression control. Relative mRNA expression for each gene was calculated as the fold change compared with the solvent control, exposed to DMSO (0.1% (v/v)) for 48 h, according to the following equation (Livak et al., 2001, Hanno et al., 2010) : Relative mRNA expression = $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = [Ct_{(\text{exposed to chemicals})} - Ct_{(EF\text{-}1\alpha)}]_{\text{exposed for 48 h}} - [Ct_{(\text{solvent control})} - Ct_{(EF\text{-}1\alpha)}]_{\text{exposed for 48 h}}$.

Statistical analysis

Statistical significance was determined by the Student's t-test. The results are presented as fold difference between the chemicals treated groups and the control groups.

RESULTS AND DISCUSSION

We classified the biomarker genes into four groups, using a medaka customized microarray : Group1 (*CYP1A1*, *UDPGT*, *AhR1b-1*, *AhR2a*, *AhRR*, *ER-β*) ; detoxication metabolism genes, Group2 (*CACHD1*, *RAR-α*, *ER-α*, *VEGF-R*) ; endocrine / reproduction genes, Group3 (*AGXT*, *MTF1*, *Tropomyosin*, *HSP90*) ; cell proliferation genes, Group4 (*TBP*, *TNF-R*, *CDC37*, *HSP70*, *MT*, *Ependymin*) ; immunity / nervous system genes, considering the physiological functions and induction by chemicals (Hanno et al., in preparation). In particular, Group1 genes were highly inducible by dioxins and dioxin-like compounds exposure.

Induced expression of biomarker genes mRNA in medaka embryos exposed to pyrolysates of chlorpyrifos and 3,5,6-trichloro-2-pyridinol

1. *CYP1A1* (Group1)

Cytochrome P450 1A1 (*CYP1A1*) and aryl hydrocarbon receptor repressor (*AhRR*) genes are well known to be highly inducible by dioxin and dioxin-like compounds exposure. Both genes are regulated through the aryl hydrocarbon receptor (AhR)-mediated pathway, which is ubiquitously functional in both mammalian tissues and medaka (*Oryzia latipes*) embryos. Although pyridinol did not enhance the *CYP1A1* expression without thermal treatment, thermally treated pyridinol with higher temperature induced higher expression of *CYP1A1* mRNA in medaka embryos (Fig.1). In contrast, chlorpyrifos didn't enhance *CYP1A1* expression with/without thermal treatment. The relative expression of *CYP1A1* was highly related to the WHO-TEF (the toxic equivalent factor) for mammals (Hanno, et al., 2010) and 380 °C- treated pyridinol enhanced *CYP1A1* expression as dioxine and PCBs did, suggesting that the potentially ecotoxic compounds like 2,3,7,8-T4CDD is newly produced during thermal treatment of pyridinol.

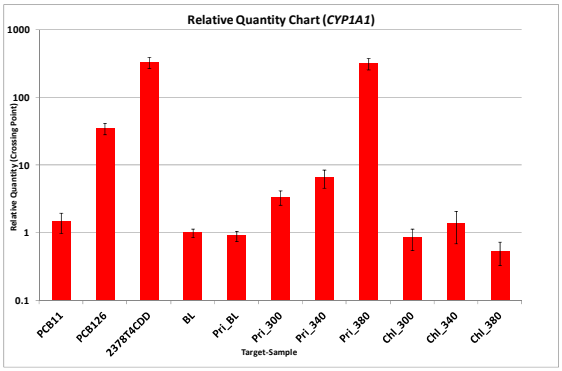

Figure 1. *CYP1A1* mRNA expression in medaka embryos exposed to pyrolysates of chlorpyrifos and 3,5,6-trichloro-2-pyridinol. The amount of cyp1a mRNA was measured by quantitative real-time PCR and normalized to the solvent control (0.1% DMSO) for 48 h exposure, the error bars represents SD (n=3). BL; Blank (DMSO 0.1%), Pri_BL; prydinol-blank (non-thermal treatment), Pri; prydinol, Chl; chlorpyrifos, _300; pyrolysates under 300°C treatment.

2. *AhRR* (Group1)

The induced expression of *AhRR* mRNA was shown in Figure 2. In the adult killfish, *AhRR* mRNA is widely expressed in tissues and is enhanced by both 2,3,7,8-T4CDD and PCBs (Hahn., et al., 2009). *AhRR* might play significant roles in modulating the activities of *AhRs* and mediating the induction of *cyp1a* expression in fish as in mammals. In this study, *AhRR* expression in the exposure of pyridinol thermally treated at 380 °C was enhanced 2.0-2.5 fold over that was the almost the same as PCB-126 (1μg/L) and 2,3,7,8- T4CDD (1μg/L) exposure did, in contrast with chlorpyrifos, supporting the presence of 2,3,7,8-T4CDD like ecotoxic chemicals in the pyridinol pyrolysates.

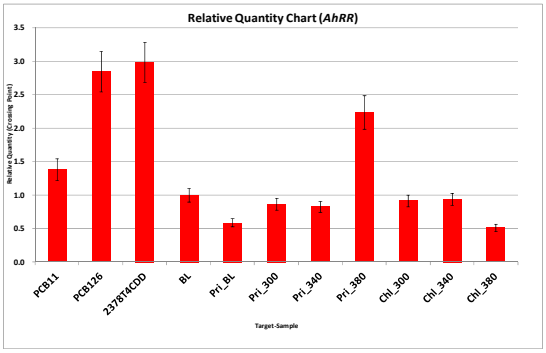

Figure 2. *AhRR* mRNA expression in medaka embryos exposed to pyrolysates of chlorpyrifos and 3,5,6-trichloro-2-pyridinol. The amount of cyp1a mRNA was measured by quantitative real-time PCR and normalized to the solvent control (0.1% DMSO) for 48 h exposure, the error bars represents SD (n=3). BL; Blank (DMSO 0.1%), Pri_BL; prydinol-blank (non-thermal treatment), Pri; prydinol, Chl; chlorpyrifos, _300; pyrolysates under 300°C treatment.

3. *AhR2a* (Group1)

Four AhR genes (*AhR1b-1*, *AhR1b-2*, *AhR2a* and *AhR2b*) were identified in the medaka genome and *AhR2a* expression was investigated in this study because *AhR2a* is most strongly induced by 2,3,7,8-T4CDD and it has been suggested that *AhR2a* plays an important role in meditating the induction of *cyp1a* and the bio-toxicity of 2,3,7,8-T4CDD in medaka embryos (Hanno, et al., 2010). Induction of *AhR2a* mRNA expression was shown in Figure 3. *AHR2a* expression was enhanced by pyridinol thermally treated at 380 °C 1.5 fold induction. In contrast, chlorpyrifos treated at 300 °C induced *AhR2a* with 2.5-3.0 fold mRNA expression of *AhR2a* over vehicle-control (0.1% DMSO; BL) and chlorpyrifos thermally treated at higher temperatures lost the inducibility of *AhR2a* expression.

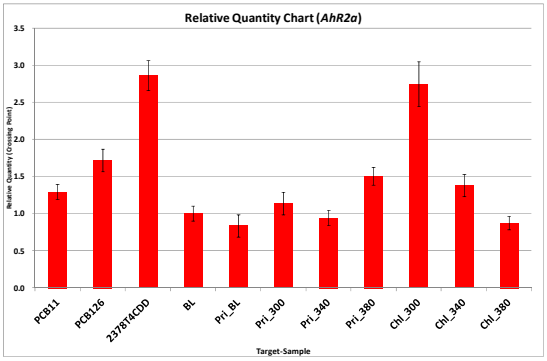

Figure 3. *AhR2a* mRNA expression in medaka embryos exposed to pyrolysates of chlorpyrifos and 3,5,6-trichloro-2-pyridinol. The amount of cyp1a mRNA was measured by quantitative real-time PCR and normalized to the solvent control (0.1% DMSO) for 48 h exposure, the error bars represents SD (n=3). BL; Blank (DMSO 0.1%), Pri_BL; prydinol-blank (non-thermal treatment), Pri; prydinol, Chl; chlorpyrifos, _300; pyrolysates under 300°C treatment.

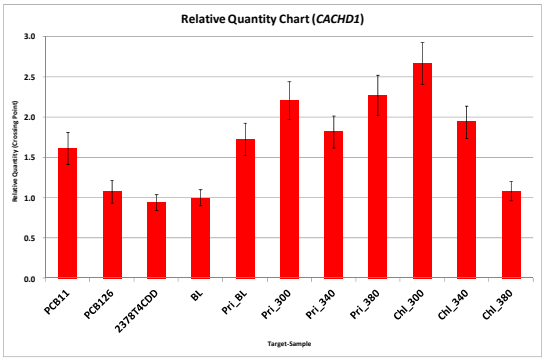

Figure 4. *CACHD1* mRNA expression in medaka embryos exposed to pyrolysates of chlorpyrifos and 3,5,6-trichloro-2-pyridinol. The amount of cyp1a mRNA was measured by quantitative real-time PCR and normalized to the solvent control (0.1% DMSO) for 48 h exposure, the error bars represents SD (n=3). BL; Blank (DMSO 0.1%), Pri_BL; prydinol-blank (non-thermal treatment), Pri; prydinol, Chl; chlorpyrifos, _300; pyrolysates under 300°C treatment.

4. *CACHD1* (Group2)

Induction of *CACHD1* (Cache domain-containing protein 1) mRNA expression was shown in Figure 4. *CACHD1* contains two cache domains and a VWFA (von Willebrand factor A) domain which is known to be involved in hemostasis (Anantharaman, et al., 2000). This gene was induced by the exposure to

heavy-metals or PCBs with low TEF, but was suppressed by PCDDs with high TEF (e.g. 2,3,7,8-T4CDD). *CACHD1* expression was induced by pyridinol exposure and thermal treatment of pyridinol did not change the inductive activity of pyridinol. The induced expression level of *CACHD1* exposed to pyridinol was 1.5 - 2.5 fold induction over vehicle-control, while chlorpyrifos suppressed *CACHD1* expression, as it was thermally treated at higher temperatures.

5. *RAR-α*

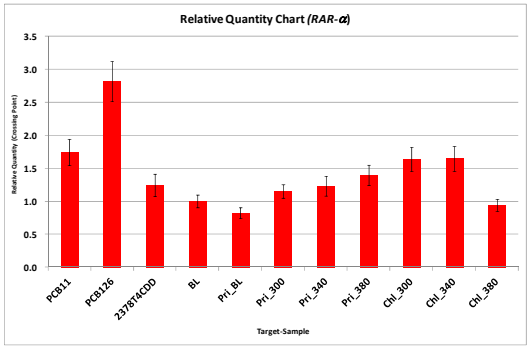

Figure 5. *RAR-α* mRNA expression in medaka embryos exposed to pyrolysates of chlorpyrifos and 3,5,6-trichloro-2-pyridinol. The amount of cyp1a mRNA was measured by quantitative real-time PCR and normalized to the solvent control (0.1% DMSO) for 48 h exposure, the error bars represents SD (n=3). BL; Blank (DMSO 0.1%), Pri_BL; prydinol-blank (non-thermal treatment), Pri; prydinol, Chl; chlorpyrifos, _300; pyrolysates under 300ºC treatment.

Induction of *RAR-α* mRNA expression was shown in Figure 5. Retinoic acid (RA), the active derivative of vitamin A. The lack or excess of RA result in developmental malformation on vascular, blood vessel and bone formation (Sharon et al., 2000) . *RAR-α* expression was enhanced by thermal treated pyridinol exposure and was suppressed by 380 ^{o}C -treated chlorpyrifos exposure.

6. *HSP90* (Group3), *HSP70* (Group4) and *MT* (metallothionein) (Group4)

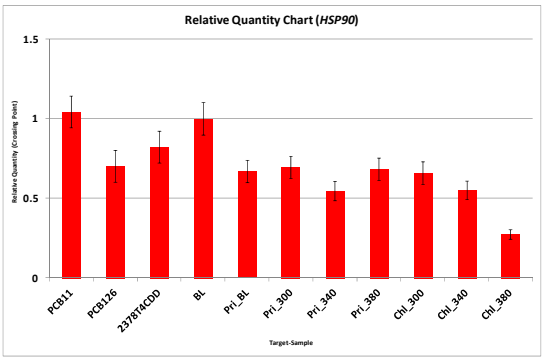

Figure 6. *HSP90* mRNA expression in medaka embryos exposed to pyrolysates of chlorpyrifos and 3,5,6-trichloro-2-pyridinol. The amount of cyp1a mRNA was measured by quantitative real-time PCR and normalized to the solvent control (0.1% DMSO) for 48 h exposure, the error bars represents SD (n=3). BL; Blank (DMSO 0.1%), Pri_BL; prydinol-blank (non-thermal treatment), Pri; prydinol, Chl; chlorpyrifos, _300; pyrolysates under 300ºC treatment.

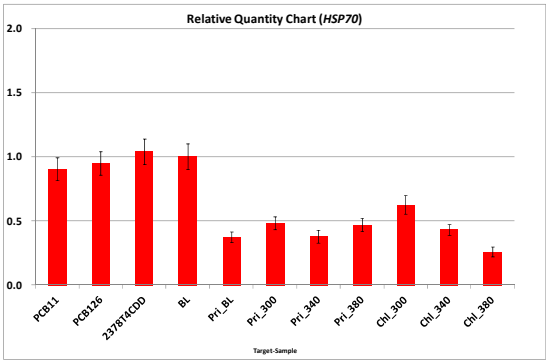

Figure 7. *HSP70* mRNA expression in medaka embryos exposed to pyrolysates of chlorpyrifos and 3,5,6-trichloro-2-pyridinol. The amount of cyp1a mRNA was measured by quantitative real-time PCR and normalized to the solvent control (0.1% DMSO) for 48 h exposure, the error bars represents SD (n=3). BL; Blank (DMSO 0.1%), Pri_BL; prydinol-blank (non-thermal treatment), Pri; prydinol, Chl; chlorpyrifos, _300; pyrolysates under 300ºC treatment.

HSPs (heat shock proteins) are stress-defenssive proteins and are highly induced in response to stresses caused by changes in environmental factors such as high temperature, heavy metal administrations, reactive oxygen production, salinity, etc (Beckmann, et al.,1990) and protect the structure and function of proteins and cells from damages, maintaining the cellular homoeostasis. MT is a protein that has a high cysteine content, a low molecular mass about 7 kDa, and a high affinity for metals. It binds metals and regulates the homeostasis of essential trace metals such as copper and zinc, also taking a part in counteracting the toxic effects of heavy metals such as cadmium, mercury, and silver (Choi, et al., 2007). Induction of *HSP90*, *HSP70*, and *MT* mRNA expressions were shown in Figure 6, 7 and 8, respectively. Expression of *HSP90*, *HSP70* and *MT* genes were suppressed to less than half by both of pyridinol and chlorpyrifos exposure (Fig. 6, 7, 8), as dioxin isomers such as PCB11, PCB126 and 2,3,7,8-T4CDD did. These results

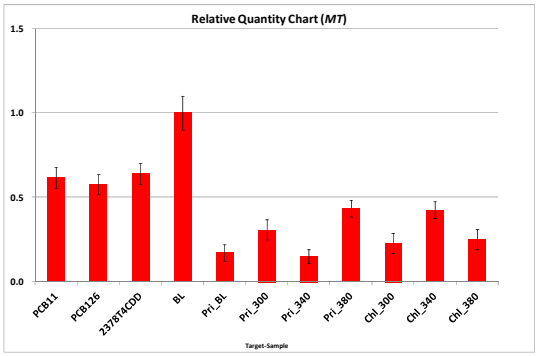

Figure 8. *MT*mRNA expression in medaka embryos exposed to pyrolysates of chlorpyrifos and 3,5,6-trichloro-2-pyridinol. The amount of cyp1a mRNA was measured by quantitative real-time PCR and normalized to the solvent control (0.1% DMSO) for 48 h exposure, the error bars represents SD (n=3). BL; Blank (DMSO 0.1%), Pri_BL; prydinol-blank (non-thermal treatment), Pri; prydinol, Chl; chlorpyrifos, _300; pyrolysates under 300ºC treatment.

indicated that the toxicity inducing mechanisms by pyridinol, chlorpyrifos and dioxins were different from those by heavy metals.

7. The induced expression of biomarker genes mRNA in radar figure of 20 square shapes

Induced expressions of the 20 bio-marker genes mRNA were shown in Figure 9 in radar charts of 20 square shapes. The pyrolysates of 3,5,6-trichloro-2-pyridinol (1.3mg/L) after 380 ^{o}C thermal treatment induced *CYP1A1*, *AhR2a*, *AhRR*: (Group1), *CACHD1*, *RAR-α*: (Group2), gene expression and suppressed *HSP90AA1*: (Group3), *HSP70*, *MT*: (Group4). 2,3,7,8 -T4CDD (1μg/L) induced *CYP1A1*, *AhR2a*, *AhRR*: (Group1), and suppressed *HSP90*: (Group3), *MT*: (Group4). These results strongly suggest that the thermal treatment at 380 ^{o}C of 3,5,6-trichloro-2-pyridinol might form the N-analogue of 2,3,7,8-T4CDD which has the very similar ecotoxicities to those of 2,3,7,8-T4CDD in medaka embryos. In contrast, the pyrolysates formed in thermal treatment of chlorpyrifos (0.32 mg/L) didn't induce *CYP1A1* and *AhRR* gene exression, which were well known to be sensitive target genes of dioxins and dioxin-like compounds exposure. Sakiyama et al., (2011) confirmed that the yield of the N-analogue of 2,3,7,8-T4CDD was considerably higher in the pyrolysate of 3,5,6-trichloro -2-pyridinol than in chlorpyrifos because phosphoric acid ester considerably might reduce the dioxin-precursor potential of the compounds. On the other hand, the pyrolysates of 3,5,6-trichloro-2- pyridinol and chlorpyrifos suppressed gene expression of *HSP90*, *HSP70* and *MT*, in the contrast that dioxins enhance gene expressions of these 3 genes in medaka embryos. These results suggest that the pyrolysates of 3,5,6 -trichloro-2-pyridinol and chlorpyrifos would contain the unknown ecotoxic chemicals

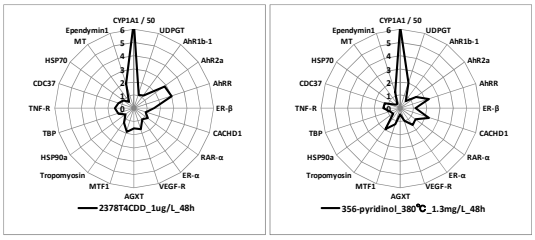

Figure 9. *The biomarker genes mR*NA expressions in medaka embryos exposed to 2,3,7,8-T4CDD and the pyrolysates of 3,5,6-trichloro-2-pyridinol (380℃), presented in radar figure of 20 square shapes. The amount of those mRNA were measured by quantitative real-time PCR and normalized to the solvent control (0.1% DMSO) for 48 h exposure. (n=3)

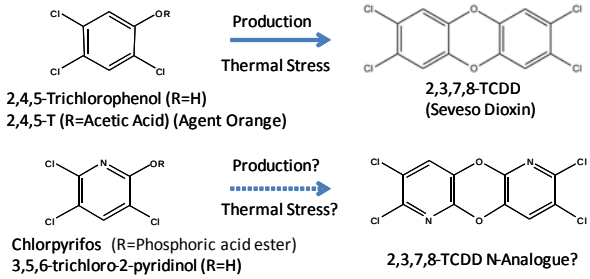

Figure 10. Formation of 2,3,7,8-TCDD from 2,4,5-T/2,4,5-TCP and potential formation of the 2,3,7,8-TCDD pyridine analogue from Chlorpyrifos or 3,5,6-trichloro-2-pyridinol. (quoted figure of Sakiyama et al., 2011)

other than the predicted N-analogue of 2,3,7,8-T4CDD. Further assessment is necessary to make clear the potential ecotoxicities of chlorpyrifos and its analogues and advantages of thermal treatment on these chemicals to reduce their ecotoxicities.

CONCLUSIONS

We have evaluated the ecotoxicities of wastewaters by investigating the biomarker genes expression profiles in the early developmental stage of medaka embryos exposed to wastewaters containing dioxins and dioxin-like compounds. In this study, we evaluated the toxicities of the pyrolysates formed in thermal treatments of 3,5,6-trichloro-2-pyridinol and chlorpyrifos at 300, 340, 380 ^{o}C, obtaining the following conclusions :

1. The N-analogue of 2,3,7,8-T4CDD would be formed in thermal treatment of 3,5,6-trichloro-2-pyridinol at 300, 340, 380 ^{o}C which possess the almost the same ecotoxicities as those of dioxins.
2. The presence of ecotoxic chemicals like 2,3,7,8-T4CDD was not evidenced in the pyrolysate of thermal treated chlorpyrifos at 300, 340, 380 ^{o}C for 10 minutes and the high temperature suppressed the ecotoxicities of chlorpyrifos.

Waste incineration facilities in Japan today, are due to incinerate at 850 ^{o}C or more. Therefore, these pyrolysates of thermal treated pyridinol or chlorpyrifos may have been completely decomposed and detoxified, but inside the waste incinerator is considered that there is much variation in temperature. It is easily to imagine that dioxins and dioxin-like compounds (e.g. the pyridine analogue of 2,3,7,8-T4CDD) may synthesize in the location 'hot-spot' at low temperature (300-500^{o}C) inside the waste incinerator or in illegal incineration (e.g. "open burning", "NOYAKI") . So, temperature dependency of the ecotoxicities formation in chlorypyrifos thermal treatment and identification of the analogues formed and characterization of their ecotoxicities should be cleared in the further assessments.

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