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Characteristics of Vertebral Abnormalities of *Medaka* as a Water Pollution Indicator*)

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ABSTRACT

We exposed red *medaka* at different developmental stages to various levels of cadmium, zinc, oxadiazon emulsion and fenitrothion emulsion to determine whether these fish represent useful biological indicators of water quality. We found that the incidence of vertebral abnormalities was the highest when eggs or newly hatched fry were exposed to the heavy metals or agricultural chemicals, that the chemicals exerted a greater adverse effect on the vertebral structure of *medaka* than did the examined metals, and that the centrum is the most sensitive vertebral indicator of induced damage. Our results suggest that *medaka* represent useful biological indicators of water quality.

INTRODUCTION

Physical and chemical methods have been widely used to assess water purity or pollution. However, there is an increased interest in the efficacy of employing biological indicators to determine water quality[1-9,11-13,15-18]. These indicators can be classified as biological indicators and as physio- or biochemical indicators. The former is based on the saprobien system of Kolkwitz-Marsson[13,14] and the system of Liebmann[15]; both of these have been widely used in Japan. According to these determination methods, the degree of organic pollution is classified into 4 stages, the polysaprobic, α-mesosaprobic, β-mesosaprobic, and the oligosaprobic stages. The normal and pathologic characteristics of the indicator species are examined and the number of species and/or individuals within a species exhibiting abnormal characteristics is used to determine the level of pollution[17,18]. When physio- or biochemical indicators are used, determination of water quality is based on criteria such as growth, proliferation and bioactivity of the indicator species[2].

Nakae[18] collected a bibliographical list on biological indicators of water quality. However, few investigators have employed this method to assess the level of water pollution by heavy metals or agricultural chemicals.

In the present investigation, we exposed red medaka at different developmental stages to various levels of cadmium (Cd) or Zinc (Zn) or to the agricultural chemicals oxadiazon emulsion (Oxa) or fenitrothion emulsion (Fen) and examined the external deformities and vertebral abnormalities induced by these agents.

MATERIALS AND METHODS

Adult *medaka* (the red variety of *Oryzias latipes*) were purchased from Ito fish farm. In control experiments, adult *medaka* obtained from the supplier, eggs spawned in our tank and fry hatched in our laboratory, were maintained in dechlorinated water.

Metal Exposure Experiments

Experiment M-I: Adult fish were allowed to acclimatize to the indoor aquarium for 2 weeks and were then divided into 4 groups. Groups 1 and 2 were exposed for 30 days to 0.01 and 0.05 ppm Cd, respectively; groups 3 and 4 to 1 and 2 ppm Zn, respectively. The water in the tanks was replaced once a week and the Cd and Zn concentration was determined before and after the exchange of rearing water. Dur-

*) 平岡幸夫，奥田久徳：水質汚濁指標としてのメダカの脊椎骨異常の特性

ing the course of the experiment, the aeration and food supplied were irreducible.

Experiment M-II: *Medaka* were raised in the indoor aquarium to the spawning stage, eggs were collected in the morning and the egg clusters were separated into individual eggs. The harvested eggs were divided into 3 groups; group 1 was maintained in a beaker with water containing 0.01 ppm Cd, groups 2 and 3 in water containing 1 and 3 ppm Zn, respectively, until the retinal pigment stage. During the hatching period, the level of aeration was kept irreducible; dead eggs were removed daily. The newly hatched fry were placed in aquariums and raised in dechlorinated water.

Experiment M-III: Separated eggs spawned in indoor aquariums were divided into 4 groups. Groups 1 and 2 were hatched and raised to adulthood in tanks containing 0.001 and 0.01 ppm Cd, respectively, and groups 3 and 4 in water containing 0.3 and 0.5 ppm Zn, respectively. The water was replaced once a week and the Cd and Zn concentration was determined before and after the exchange of rearing water. During the course of the experiment, the aeration and food supplied were irreducible.

Oxa and Fen Exposure Experiments

The Oxa contain 12% 5-t-butyl-3 (2, 4-dichloro-5-isopropoxyphenyl)-3, 4-oxadiazol-2-one and 88% emulsifier and organic solvent. This is the constitution of the herbicide as it is widely used in Japan rice paddies. The Fen contain 50% dimethyl-(3-methyl-4-nitrophehyl) thiophosphate and 50% emulsifier and organic solvent. This is the constitution of the organic phosphorus insecticide as it is widely used in Japan.

Experiment A-I: Adult *medaka* which had been acclimatized to the indoor aquarium were exposed for 48 hr to water containing 16 ppm Oxa. To study the effects of Fen exposure, adult *medaka* which had been spawned in the indoor tank, were divided into 3 groups and exposed for 20 days to water containing 1, 2, or 4 ppm Fen. The water was changed at 3-day intervals to keep the Fen concentration as near the desired level as possible.

Experiment A-II: Eggs spawned in the indoor tank were divided into 8 groups. Groups 1 and 2 were raised to the hatching in water containing 4 and 16 ppm Oxa, respectively. Groups 3-8 were placed until hatching (7-10 days) in water containing 0.1, 0.2, 0.4, 1, 2, or 4 ppm Fen. The water was changed on day 4

Table 1. Incidence of vertebral abnormalities in *medaka* exposed to various concentrations of Cadmium (Cd) or Zinc (Zn)

	No. of fish examined	Body length (mm) (Ave. ± S. D.)	Incidence of deformed fish (%)	Incidence of fish with damaged vertebrae (%)	Incidence of fish with damaged centra (%)
M-I (Adult fish)					
Cd control	48	25.0 2.3	0	42	4
Cd 0.01 ppm	50	24.5 2.8	0	54	5
Cd 0.05 ppm	60	25.4 2.5	0	59	0
Zn control	24	25.9 1.7	0	42	8
Zn 1 ppm	39	24.5 2.0	0	59	0
Zn 2 ppm	37	25.0 1.8	0	46	31
Control	32	20.8 1.5	0	34	9
M-II (Eggs)					
Cd 0.01 ppm	23	18.2 1.2*	9	61	22
Zn 1 ppm	59	16.9 2.9*	0	64*	20
Zn 3 ppm	63	17.1 3.0*	8	52	24
M-III(Egg—Adulthood)					
Cd 0.01 ppm	32	19.1 1.0*	0	66*	38*
Cd 0.01 ppm	46	16.3 3.3*	9	85*	46*
Zn 0.3 ppm	76	17.2 2.2*	5	82*	26
Zn 0.5 ppm	31	14.6 2.8*	13	97*	52*

Statistically significant difference as compared with the control, *: $p<0.01$ by χ^2-test.

to keep the concentration. Aeration was irreducible during the hatching period; dead eggs were removed daily. The newly hatched fry were placed in indoor aquariums and raised in dechlorinated water.

Experiment A-III: Fry hatched in the indoor aquarium were divided into 6 groups. Groups 1-3 were raised in water containing 2, 4, or 8 ppm Oxa, and groups 4-6 in water containing 0.5, 1, or 2 ppm Fen. The water was changed at 3-day intervals to keep the concentration. After being raised for 1 week in the water containing agricultural chemicals, they were placed in aquariums and raised to the adulthood in dechlorinated water.

Preparation and Observation Procedures

At the end of the experimental period, the fish were carefully inspected; bone and cartilage were stained with the Alizarin red S and Alcian blue double-staining method[10] and the vertebral structure was inspected under a stereoscopic mizroscope. Care was taken to avoid damage to the skeletal structure during these procedures. Only those fish which survived the exposure experiments were studied microscopically, however, in experiment A-I, fish which died during Fen exposure were also examined.

RESULTS

Metal Exposure Experiments (Table 1)

M-I: None of the adult fish used in this experiment manifested macroscopically recognizable external deformities. Exposure to Cd or Zn resulted in a slight increase in the number of fish with vertebral damage. Damage to the centra was highest in fish exposed to 2 ppm Zn; the fact that the control population exhibited a higher incidence of centra damage than

Table 2. Incidence of vertebral abnormalties in *medaka* exposed to various concentrations of oxadiazon (Oxa) or fenitrothion (Fen).

	No. of fish examined	Body length (mm) (Ave. ± S. D.)	Incidence of deformed fish (%)	Incidence of fish with damaged vertebrae (%)	Incidence of fish with damaged centra (%)
A-I (Adult fish)					
Oxa control	56	28.3 1.5	0	38	2
Oxa 16 ppm	44	28.8 1.9	0	41	5
Fen control	34	18.9 1.0	0	35	6
Fen 1 ppm	76	19.5 1.5	57	50	12
Fen 2 ppm	74	19.6 1.4	65	50	14
Fen 4 ppm	75	19.4 1.5	3	47	19
Control	34	18.9 1.0	0	35	6
A-II (Eggs)					
Oxa 4 ppm	85	18.0 1.9*	12	80**	38
Oxa 16 ppm	74	19.2 2.5*	41	92**	57
Fen 0.1 ppm	147	17.5 1.7*	0	67**	12
Fen 0.2 ppm	150	18.2 1.9*	0	79**	24
Fen 0.4 ppm	36	14.3 3.0*	78	100**	97
Fen 1 ppm	28	18.8 2.2	25	79**	25
Fen 2 ppm	94	17.2 2.7**	26	87**	59
Fen 4 ppm	181	17.4 2.1**	2	85**	59
A-III (Fry)					
Oxa 2 ppm	32	20.0 1.8**	19	66*	19
Oxa 4 ppm	108	19.0 1.2	2	79**	23
Oxa 8 ppm	24	20.0 1.5**	0	79**	8
Fen 0.5 ppm	132	17.2 3.4**	41	86**	38
Fen 1 ppm	27	21.2 1.8**	85	96**	81
Fen 2 ppm	12	20.3 0.8	0	75**	33

Statistically significant difference as compared with the control; *: $p<0.05$, **: $p<0.01$ by χ^2-test.

some of the test populations is attributed to the random occurrence of vertebral abnormalities in medaka.

M-II: When eggs were exposet to different metal concentration until the retinal pigment stage, the incidence of macroscopic deformities was 9% in the 0.01 ppm Cd group and 8% in the 3 ppm Zn group. Most of the deformities consisted of lateral deflection in the caudal peduncle. Vertebral abnormalities were significantly higher ($p<0.01$) in the 1 ppm Zn group than the control. Damage to the centra was higher in fish exposed to the metals than in the controls.

M-III: When metal exposure lasted from the egg stage until the fish reached adulthood, the incidence of macroscopic deformities was the highest in the 0.05 ppm Zn group. Vertebral damage was significantly more frequent in all the exposed groups as compared to the control ($p<0.01$); upon 0.5 ppm Zn exposure, 97% of the fish manifested damaged vertebrae. Damage to the centra was significantly higher in all the exposed groups except that raised in the presence of 0.3 ppm Zn.

Oxa and Fen Exposure Experiments (Table 2)

A-I: None of the adult fish exposed to Oxa manifested macroscopic deformities and there was no significant difference between the control and the experimental groups with respect to vertebral or centra damage.

In the Fen exposure groups, the incidence of external deformities, especially caudal fin deformities, was high at 1 and 2 ppm Fen. The low incidence of deformation in the 4 ppm Fen group is attributable to the fact that all fish died during the early part of the experiment. Vertebral damage were higher in the Fen- than the Oxa- exposed groups.

A-II: When fish were raised in the presence of different Oxa and Fen concentrations, the incidence of macroscopic deformities was higher than in the controls, excepting exposure to 0.1 and 0.2 ppm Fen. The deformities consisted primarily of deflection of the caudal peduncle. All the exposed groups manifested a significantly higher incidence of vertebral damage than the controls ($p<0.01$) and damage to the centra was from 2-16 times higher than in unexposed fish.

A-III: When fry, hatched in the absence of the agricultural chemicals, were raised in the presence of various Oxa and Fen concentrations, macroscopic deformities ranged from 0-85%. The incidence of vertebral damage was significantly higher in all the exposed groups than in the control and damage to the centra ranged from 8-81%.

DISCUSSION

Medaka is one of those fishes which have been so familiar to us all since long ago. We have great interest in *medaka*, so that we will easily realize something wrong with them. Therefore, the very existence of *medaka* will be regarded as a good indicator. *Medaka*, which is a vertebrate, can indicate bad influences of water pollution on the human body more clearly than invertebrates and floras which have hitherto been largely used as biological indicators. As *medaka* migrates less than other fishes and is so easy to catch that it has almost similar advantage to the existing biological indicators. Verterbal abnormalities caused once in *medaka* will remain as they are as long as it lives.

We have demonstrated two methods by which we can determine vertebral abnormalities in *medaka*; one is detecting the incidence of fish with vertebral abnormalities, and the other is detecting that with the abnormal centra. It was found that the centra were more sensitive than other regions of the vertebrae according to Fen exposure conditions[9]. The centra were more important for fish than other regions of the vertebrae, for they have a great influence on the swimming behaviour of fish. The centra were hardly injured by the processes of preparation and staining and their abnormalities can be detected even by a soft X-ray. It is easy to discern objectively the abnormal centra from the normal ones by using only a small loupe, and moreover, there is little difference between those results obtained by an individual orserver. Therefore, special taxonomic knowledge as is required in using the existing biological indicators is not needed. For the above-mentiooed reasons, we can obtain more reliable results more easily by using the incidence of fish with the abnormal centra than using that with the abnormal vertebrae.

Vertebral abnormalities in red *medaka* were caused most frequently when eggs or newly hatched fry were exposed to concentrations of

TLm (median of Tolerance Limit) value per tenth—near TLm value of the 2 heavy metals and the 2 agricultural chemicals. TLm value in each chemicals toward *medaka* is 0.1 ppm/24h in Cd*, 18 ppm/24h in Zn[19], 7.1 ppm/48h in Oxa[20], and 3.8 ppm/48h in Fen[21]. Many experiments performed on the occurrence of skeletal abnormalities in fishes by using various fishes other than *medaka*, have made us generally accept that skeletal abnormalities occur by various factors, that is the physical and chemical, the hereditary, the nutritional, and so on[11]. Vertebral abnormalities in *medaka* will be caused by those 4 materials used in the experiments as well as other metals and agricultural chemicals and the above-mentioned various factors, so that it is impossible to affirm that vertebral abnormalities in *medaka* can be a biological indicator of a fixed substance within the water pollution. But it may be inferred that their abnormalities are significant as an indicator of water pollution as a whole.

From the results of our experiments, effective concentrations which cause frequently vertebral abnormalities in *medaka* are different according to the 4 causal materials used, but broadly speaking, they are TLm value per tenth—near TLm value. Namely, concentration of TLm value per tenth—near TLm value is roughly equal to the environmental standard for public water areas where Cd is less than 0.01 ppm, and organic phosphorous is undetectable (of which detectable limit is 0.1 ppm), and Zn in drinking water standard less than 1 ppm, or slightly lower than those standards. It may be inferred that there are neither such various pollutants nor factors that can frequently cause vertebral abnormalities in the water area where normal *medaka* inhabit. Therefore, it may be said that the very presence of normal *medaka* indicates a water area being safe also for human beings. As the concentrations of pollutants that can cause vertebral abnormalities in *medaka* are approximate to the environmental standard for public water areas, the vertebrae of *medaka* have a suitable sensitivity in terms of the bad influences of pollutants on the human health.

Automatic apparatuses, which can continuously analyze and determine BOD, pH, heavy metals and so on in the water, have begun to be used to supervise water quality. However, it needs a high technique to analyze agricultural chemicals, and often various kinds of chemicals are mixed and used at the same time. Moreover, some of the components thereof are apt to dissolve and disappear quickly, so that the dgree of water pollution by agricultural chemicals will be analyzed and located by using the physical methods only.

In recent years many kinds of agricultural chemicals are likely to be too much used in rice paddy in Japan. In our experiments the incidence of vertebral abnormalities in fish was higher, and the symptoms were heavier in the case of its exposure to agricultural chemicals than to heavy metals. The cases of skeletal abnormalities caused by agricultural chemicals in fishes other than *medaka* have been reported on by many workers[12]. Agricultural chemicals are one of the important factors that can cause vertebral abnormalities in *medaka*. Our experiments made clear that they had a great influence on the vertebrae of *medaka* at the stages of eggs and newly hatched fry[7,8]. In Japan *medaka* in the field usually spawn in May-August, when a great amount of agricultural chemicals are used in rice paddy. *Medaka* willingly inhabit in irrigation waters for paddy.

From the above-mentioned reasons, it may be inferred that vertebral abnormalities in *medaka* are effective and useful means as a biological indicator, especially of the water pollution by agricultural chemicals.

* unpublished data

REFERENCES

1. **Bengtsson, B. E.** 1975. Vertebral damage in fish induced by pollutants, p. 23-30. *In* Koeman, J. H. and Strik, J. J. T. W. A. (ed.). Sublethal effects of toxic chemicals on aquatic animals. Elsevier Scientific Publishing Company, Amsterdam.
2. **Glass, G. E.** 1973. Bioassay techniques and environmental chemistry. Ann Arbor Science Publishers, Michigan.
3. **Hiraoka, Y.** 1980a. Studies on the vertebral abnormalities of medaka as the biological index of water pollution. I. Time course study at irrigation waters for paddy. Hiroshima M. J. **28** : 611-618.
4. **Hiraoka, Y.** 1980b. Studies on the vertebral ahnormalities of medaka as the biological index of water pollution. II. The comparison among frequency of vertebral abnormalities of medaka in a few water areas. Hiroshima M. J. **28** : 619-624.
5. **Hiraoka, Y.** 1980c. Studies on the vertebral

abnormalities of medaka as the biological index of water pollution. III. Experimental investigation concerning cadmium exposure. Hiroshima M. J. **28** : 625-632.

6. **Hiraoka, Y.** 1980d. Studies on the vertebral abnormalities of medaka as the biological index of water pollution. IV. Experimental investigation concerning zinc exposure. Hiroshima M. J. **28** : 633-642.
7. **Hiraoka, Y.** 1980e. Studies on the vertebral abnormalities of medaka as the biological index of water pollution. V. Experimental investigation concerning oxadiazon emulsion exposure. Hiroshima M. J. **28** : 643-650.
8. **Hiraoka, Y.** 1980f. Studies on the vertebral abnormalities of medaka as the biological index of water pollution. VI. Experimental investigation concerning MEP emulsion exposure. Hiroshima M. J. **28** : 651-660.
9. **Hiraoka, Y.** 1981. Studies on the vertebral abnormalities of medaka as the biological index of water pollution. VII. Vertebral abnormalities in the next generation of medaka treated by MEP. Hiroshima M. J. **29** : 303-308.
10. **Inouye, M.** 1976. Differential staining of cartilage and bone in fetal mouse skeleton by alcian blue and alizarin red S. Cong. Anom. **16** : 171-173.
11. **Kanazawa, J.** 1976. Vertebral abnormalities in fish and pesticides. Kagaku **46** : 108-115.
12. **Kanazawa, J. and Tanaka, J.** 1979. Aquatic organisms and pesticides. Theory and application, p. 167-188. Scientist Inc., Tokyo.
13. **Kolkwitz, R. und Marsson, M.** 1908. Ökologie der pflanzlichen Saprobien. Berdeutsch. bot. Ges. **26** : 505-519.
14. **Kolkwitz, R. und Marsson, M.** 1909. Ökologie der tierischen Saprobien. Beiträge zur Lehre von der biologischen Gewässerbeurteilung. Int. Rev. Hydrobiol. **2** : 126-152.
15. **Liebmann, H.** 1951. Handbuch der Frischwasser- und Abwasserbiologie. Bd. 1. Verlag Oldenbourg, München.
16. **Morishita, I.** 1981. Biological indicator of water environment. J. Environ. Pollut. Cont. **17** : 396-401.
17. **Morishita, I.** 1981. Biological indicator in brackish water and the trial idea for map biological water quality. J. Environ. Pollut. Cont. **17** : 413-418.
18. **Nakae, M.** 1981. A bibliographical list on biological indicators of environmental and water quality. J. Environ. Pollut. Cont. **17** : 563-574.
19. **Shirasu, Y. and Matsuoka, O.** 1975. Environmental toxicology method and safety evaluation. p. 605. Soft-science Inc., Tokyo.
20. **Tanaka, J.** 1978a. Aquatic organisms and pesticides. Acute toxicity of pesticides to aquatic organisms. p. 231. Scientist Inc., Tokyo.
21. **Tanaka, J.** 1980b. Aquatic organisms and pesdicides. Acute toxicity of pesticides to aquatic organisms. p. 119. Scientist Inc., Tokyo.
22. **Tsuda, M.** 1976. Biology of polluted waters. Hokuryukan Co., Ltd., Tokyo.
23. **Tsuda, M.** 1979. Ecology of water pollution. Kogai Taisake Gijutsu-Doyukai Co., Ltd., Tokyo.