International Journal of Science and Research (IJSR)
ISSN: 2319-7064
Index Copernicus Value (2016): 79.57 | Impact Factor (2017): 7.296

Toxicity and Behavioural Changes in Freshwater Fish *Rasbora daniconius* Exposed to Tributyltin Oxide

Pradeep B. Mohate[1], Jitendra T. Jagtap[2]

[1]Department of Zoology. Vivekanand Shikshan Sanstha Samarth Nagar, Aurangabad. Pin Code-431004. (M.S.) India

[2] Department of Zoology. Swami Vivekanand Sr. College, Mantha. Dist. Jalna, Pin Code-431504. (M.S.) India

Abstract: ***The active substance TBT is highly toxic and also damaging to a multitude of non target species. The fishes have been used for many years to determine the pollution status of water. Static bioassays were performed on fish, Rasbora daniconius to evaluate the median lethal concentrations of TBTO (tributyltin oxide) for 24, 48, 72 and 96 hrs. The LC_{50} values were 0.56 ppm, 0.48 ppm, 0.36 ppm and 0.23 ppm respectively. The results show that the LC_{50} values decreased with increase in exposure period. At this concentration, erratic swimming, jerky movement, thick mucous covering over the whole body surface and rapid opercular movement leaping out of water were observed during experiments***.

Keywords: *Rasbora daniconius, Toxicity, Behavioural changes,* tributyltin oxide

1. Introduction

The release of organotins into terrestrial and aquatic environments has decreased recently, but inputs still occur and previously contaminated sites continue to act as sources., Ritsema, 1994. Much of the attention on the release of organotin compounds into the environment has focused on tributyltin (TBT), which has been widely used as a biocide in paints and coatings in ship antifouling applications. Antifouling products play an important role in the shipping industry and are of significant economic importance. It is estimated that, on average, fuel consumption increases 6% for every 100 ml increase in the average hull roughness caused by fouling organisms (Townsin, 1987., Liu *et al*., 1997). Several reviews on the tributyltin compound, which cover the production, use, chemistry, toxicity, fate and hazards of TBT in the aquatic environment (Laughlin *et al.,* 1996; Maguire 1996; 1996; WHO 1990). Toxicity to aquatic organisms generally increases as the number of organic components increases from one to three and decreases with the incorporation of a fourth, making triorganotins more toxic than other forms. Considerable work has been carried on effect of TBT on aquatic organism, Alzieu et al., (1980) found 100% mortality in pacific oyster, Crassostrea gigas exposed to TBT. Newton et al., (1985) observed significantly enhanced growth and hatching success in *Leuresthes tenuis* after effect of TBTO in the duration of 10 days. Reproductive abnormalities have been observed by toxic effect of TBT in the European flat oyster, *Ostrea edulis*, Thain, (1986). Salazar and Salazar, (1996) observed accumulation of TBT in blue mussel, Mytilus species. Meador, (1997) reported that tributyltin chloride strongly affect on amphipod, *Rhepoxynius abronius*. Tim Verslyce et al., (2003) revealed that the cellular energy allocation in the estuarine mysid shrimp Neomysis integer to different TBT exposure, Richard Louis and Emilien, (2004) reported that the lost of TBT to atmosphere by volatisation and its effect on biota. Rabbito, (2005) have been studied the effect of TBT on fish, *Hoplias malabaricus*. The effects of organotin compounds have been extensively studied on experimental animals (Wada et al., 1982; Merkord and Henninghausen 1989; Takagi et al., 1992).

The evaluation of acute toxicity is essential for determination of sensitivity of animals to the toxicants and also useful for evaluating the degree of damage to the target organs and the consequent physiological and behavioral disorders, Mary (1984). Freshwater fish, *Rasbora daniconius* selected for the present study, fulfils most of the criteria listed for a standard test fish. Fishes have been used for many years to determine the pollution status of water, and are thus regarded as excellent biological marker of metals in aquatic ecosystem. Godavari river is considered one of the most important water bodies in Maharashtra state, large, shallow, and exposed to high levels of pollutants from industrial, domestic and agricultural resources.

The aim of this study was to investigate and gather information on the toxicological potential and behavioral changes in Rasbora daniconius exposed to lethal concentration tributyltin oxide.

2. Materials and Methods

Toxicity evaluation

Healthy adult fish *R. daniconius* were collected from a Godavari river at Kayagaon village Tq. Gangapur, Dist. Aurangabad. This village is 45 Km away from Aurangabad City. Animals were brought to the laboratory within plastic bags with sufficient air. The plastic bags were placed into the aquarium for 30 to35 minutes for acclimatization. During the period of acclimatization the water was changed for every 24 hours, and the fishes were fed thrice a day. Feeding was stopped 24 hours before the toxicity tests. The used water was clear aged and dechlorinated which was used to maintain the fishes as well as for the tests concentrations. The aging of the water is necessary before it is used for maintaining the fishes as it helps to stabilize its composition and moreover so as to eliminate residual chlorine which is otherwise considered highly toxic to fishes. The fishes were maintained in sufficiently large aquaria so as to avoid overcrowding. The fishes were exposed to diffused day light during the daytime, where the daily photoperiod was about 10-12 hrs.

The stock fish in which the mortality exceeds 5% the complete batch was discharged. Pilot experiments were conducted to find out the range of the toxicity of the particular toxicant. The chosen range of concentration was such that it resulted in 0 to 100% mortality. The fishes used, were washed with very light $KmnO_4$ solution before they were transferred from the acclimatization aquarium to the experimental container one by one with the help of a small hand net. Similarly controlled groups of fishes were also maintained with 0 toxicant concentrations under similar conditions.

The stock solution of Tributyltin oxide (1-ppm) was prepared by dissolving a known quantity of glass distilled water and various concentrations were made from this stock solution for toxicity evaluation. The Series of statistic bioassay were conducted under laboratory condition as described by Finney (1971).

Acute toxicity tests were conducted separately for tributyltin over 96 hrs. The experimental troughs containing 5 litres dechlorinated water were used to keep the animals. For each experiment ten fishes, *R. daniconius* of approximately similar size were exposed to different concentrations of tributyltin oxide. After every 12 hours the polluted water was changed by the fresh solution of the same concentration. The behavior and mortality of the fishes were recorded before each change of water. The resulting mortality was noted in the range of 10 to 90% for each concentration for the duration of 24 h, 48 h, 72 h and 96 hrs. Each experiment was repeated thrice to obtain constant results. The data collected was analyzed statically by means of probit method on transforming toxicity curve (% mortality vs. concentration), which allows the average median lethal concentration of LC_{50} to be calculated for 24 h, 48 h, 72 h and 96 hrs. Dead fishes were counted individually.

3. Results

LC_{50} values were calculated for 24 h, 48 h, 72 h and 96 h by Finney's method (1971). The results of acute toxicity are summarized in (Table 1). The LC_{50} values obtained for tributyltin oxide exposed for 24 h, 48 h, 72 h and 96 hrs exposures were 0.56 ppm, 0.48 ppm, 0.36 ppm and 0.23 ppm respectively. *R. daniconius* showed behavioral observation before die when exposed for 24 h 48 h 72 h and 96 hrs exposure of tributyltin oxide. They tried to avoid the toxicant by irregular erratic swimming, jerky movements, rapid opercular movements, restlessness, frequent surfacing, gulping of air, upside down surface movement, revolving, convulsions and extension of fins. At 96 hrs of exposure an important effect was the discharge of mucus at the gills and on the skin.

Concentration and Safe concentration were calculated and are shown in Table 1. From the above results it appears that the freshwater fish Rasbora daniconius is highly sensitive to organotin Tributyltin oxide.

Table 1: Relative toxicity of TBTO to the freshwater fish, *Rasbora daniconius*

exposure Time of (Hrs.)	Regression equation $Y=\bar{y}+(X-\bar{x})$	LC50 Values in ppm.	Variance V	Chi-square	Fiducial limit		Lethal dose	concentration Safe (ppm)
					m1	m2		
24	Y=16.7288X-7.5055	0.5593	0.0001188	0.0026114	0.7262	0.7689	13.4232	0.07044
48	Y=14.4035X-4.8037	0.4794	0.0001707	0.002287	0.6363	0.6875	23.0112	
72	Y=10.7270X-0.9525	0.3589	0.000289	0.005170	0.5216	0.5882	25.8408	
96	Y=8.4076X+1.9297	0.2319	0.0004687	0.138553	0.3159	0.4008	22.2624	

4. Discussion

Mortality of *R. daniconius* is a more sensitive measure of toxicant. The percent survival rate of the fish decreased with increasing concentration and period of exposure. The evaluation of LC_{50} concentration of pollutants is an important step before carrying out further studies on physiological and biological changes in *R. daniconius*. In the present study the *R. daniconius* exposed to tributyltin oxide for the study of acute toxicity. It was expressed in terms of LC_{50} values. The LC_{50} values for 24 h, 48 h, 72 h and 96 hrs for organotin tributyltin oxide were recorded 0.56 ppm, 0.48 ppm, 0.36 ppm and 0.23 ppm respectively.

In the present study, the LC_{50} values for tributyltin oxide exposed to test animal *R. daniconius* for 24, 48, 72 and 96 hrs showed significant difference. The measured values is attained their lowest values indicated high

toxicity response. Variations of the lethal concentration may be due to changes of the organism's tissues weight rather than to any variability in the absolute metal content of the organism. There are many factors which may affect the bioavailability and intake of heavy metals by the organisms, such as variations in the physicochemical parameters in the surrounding water like, temperature, pH, total suspended solids, dissolved organic carbon.

Among others (Van Hattum et al., 1996); variations in water flow, which may cause dilution of the concentrations of pollutants in water (Camusso et al., 1994); and variations in the physiology of organisms (Naimo et al., 1992). These factor remains in constant interaction in the environment and these interactions could cause of different intake patterns of heavy metals by organisms.

During present study, Rasbora daniconius showed irregular erratic swimming, jerky movements, rapid opercular movements, restlessness, frequent surfacing, gulping of air, upside down surface movement and thick mucous covering over the whole body surface. Similar results were observed by Lokhande M.V., (2017)

Secretion of mucus was regarded as a defense and excretory response (Benett and Dooley, 1982) which might help in protecting gills and skin from heavy metal toxicity. Suffocation of fish exposed to heavy metals was discernible in the form of air bubbles on the water surface when the fish had been directed towards the water surface. Finally they lost their equilibrium and settled at the bottom before death. Similar abnormal behavioural pattern was also observed when the animals were exposed to Zinc and Cadmium separately (Benoit et al., 1976; Spehar, 1976)

The increase in mortality response of the test fish species with increased exposure and time could be because of the accumulation of metals in different tissues of body especially in the gills which are important sites for the entry of metals, therefore causing lesions and gill damage and failure of metabolic activities (Bols et al., 2001; James et al., 2003). So it is possible that the cumulative action of copper and organotin at various metabolic sites is responsible for the death of the fish (Basa and Rani, 2003).The main reason of death in fish exposed to pollutants is the hypoxia because the metals act on the gill function and structure causing damage of the gill epithelia, disturbances in osmo-regulation process, decrease of oxygen consumption and then death (Peuranen et al., 1994, V.R.Chavan* and D.V.Muley, 2014, Mercy and Dhanalakshm , (2017).

The behavioural changes of *R. daniconius* were found to be different on tributyltin oxide. Sprague and Drury (1969) reported that organisms have exhibited an avoidance response at 24 hrs concentrations of pollutants. The lethal toxicity of TBTO for fish varies considerably depending on species and age of target individual (Triebskorn et al., 1994). The route of uptake for dissolved TBTO is mainly over the gills, but intake via food may also be of significance (Holm et al., 1991).

Robert, (1987) maintained adult oysters, *Craffostrea virginica* in TBT solutions containing 0.05, 0.1, 0.5 and 1 gm / lit for upto eight weeks. He observed that 20% and 30% mortality occurred between second and fourth week of exposure. Meador et al., (1993) have reported acute toxicity (LD $_{50}$ s) for *Rhepoxynius abronius, Eohaustorius washingtonianus and Armandia brevis* at concentrations ranging from 34 – 89 mg TBT / kg body weight. Fargasova, (1997) reported long-term toxicity value (21-day NOEC) for *Daphnia magna* is 0.19: g/liter; the 96-h LC_{50} for *Tubifex tubifex* is 0.1: g/liter. Davidson et al., (1986) calculated the 96 h LC_{50} to be 0.42 µg / lit, after exposing the mysid shrimp, *Acanthomysis sculpta* to a leachate of TBT. Effect on common oyster lavae to 0.02-100 g/L tributyltin acetate were studied by His and Robert (1985), as a result , in the group of larvae exposed to tributyltin acetate at 0.05 g/L (0.05 g/L L in terms of tributyltin chloride) or over, growth was inhibited and deaths were observed within 10 days. None observed effect concentration on growth was reported to be 0.02 g/L (0.02 g/L in terms of tributyltin chloride). Waldock and Thain (1983) exposed *C. gigas* to TBT oxide (TBTO) for 56 d; they reported that exposed to 0.15 µg/L TBTO did not grow as well as controls and had pronounced thickening of the upper shell, and that spat exposed to 1.6 µg/L TBTO were severly inhibited in terms of growth. Roberts (1987) conducted 48-h acute toxicity tests of the hard clam (*Mercenaria mercenaria*) and the oyster (*Crassostrea gigas*), using tributyltin chloride (TBTCl). The maximum exposure concentration was 1.3µg/L TBTCl. The 48-h LC_{50} values were 1.13µg/L for clams and 1.30µg/L for oysters. For the larvae of both species, the 48-h LC_{50} values were 1.65µg/L for clams and 3.96µg/L for oysters. Shejule et al., (2006) reported LC_{50} values of the organotin tributyltin chloride exposed to freshwater prawn, Macrobrachium kistnensis; to 24, 48, 72 and 96 hours, LC 50 values were found to be 0.33 ppm, 0.26 ppm, 0.17 ppm and 0.09 ppm respectively. They showed the LC_{50} values decreased with increase in exposure period.

5. Conclusion

Thus it is concluded that the Tributyltin oxide is not safe to non-target organisms like fishes. This type of study can be useful to compare the sensitivity of various species of aquatic animals and potency of effluent using LC_{50} values and to derive safe concentration. Behavioural effects of TBTO-exposure are poorly documented. When beheaviour response is considered in the present investigation R.daniconius found more sensitive to tributyl tin oxide It has been suggested that the variation in behaviour responses between individuals and pollutant may act as an indicator of pollution.

6. Acknowledgements

The authors thankful to Dr. K.B. Shejule, Dept. of Zoology Dr. Babasaheb Ambedkar Marathwada University, Aurangabad providing necessary facilities and kind cooperation during the work.

References

[1] Alzieu, C., Y. Thibaud, M. Heral and B. Boutier. (1980): Evaluation of the risks of using antifouling paints near oyster zones. *Rev. Trav. Inst. Peches Marit.* 44:301-348.
[2] Basa, Siraj, P, Usha Rani, A, (2003):Cadmium induced antioxidant defense mechanism in freshwater teleost Oreochromis mossambicus (tilapia). EcoToxicology Environment Safety, 2(56), pp 218- 221.
[3] Bennett, R. O. and J. K. Dooley. (1982): Copper uptake by two sympatric species of killifish Fundulus heteroditus (L.) and F. majalis (Walbaum). J. Fish Biol. 21:381-398.
[4] Benoit. D.A. E.N. Leonard G.M. Christensen and J.T. Fiandt (1976): Toxic effects of cadmium on three generations of brook trout (salvelinus fontinalis). Trans. Am. Fish. Soc. 105:550-560.
[5] Bols NC, Brubacher JL, Ganassin RC, Lee LE. (2001): Ecotoxicology and innate immunity in fish. Dev Comp Immunol;25:(8) 53–873.
[6] Camusso, M.; Balestrini, R.; Muriano, F. and Mariani, M. (1994): Use of freshwater mussel *Dreissena polymorpha* to assess trace metal pollution in the lower river Po (Italy). *Chemosphere,* 29: (4), 729-745.
[7] Davidson, B.M., A.O. Valkirs and P.F. Seligman. (1986): Acute and chronic effects of tributyltin on the

mysid *Acanthomysis sculpta* (Crustacea, Mysidacea). In: Oceans 86, Vol. 4. *Proceeding International Organotin Symposium. Marine Technology Society,* Washington, DC. pp. 1219-1225.

[8] Fargasova, A. (1997): Comparative study of ecotoxicological effect of triorganotin compounds on various biological subjects. *Ecotoxicology and environmental safety*, 36:38–42.

[9] Finney, D. J. (1971): Statistical methods in biological assay. *3rd edition. -Cambridge University Press*, London, England. Pp. 1-333.

[10] His, E. and Robert, R. (1985): Developpement des veligeres de Crassostrea gigas dansle Bassin d'Arcachon, etudessurles mortalities larvairres.*Rev.Trav.Inst.Peches Marit*., 47,63-88.

[11] Holm, G., Norrgren, L. and Lindén, O. (1991): Reproductive and histopathological effects of long-term experimental exposure to bis(tributyltin)oxide (TBTO) on the three-spined stickleback Gasterosteus aculeatus Linnaeus. Journal of Fish Biology 38, 373-386.

[12] James TC, et al. (2003) Transcription profile of brewery yeast under fermentation conditions. *J Appl Microbiol* 94(3):432-48

[13] Laughlin, Jr., R.B., J. Thain, B. Davidson, A.O. Valkirs and F.C. Newton, III. (1996): Experimental studies of chronic toxicity of tributyltin compounds. In: Organotin: *Environmental Fate and Effects.* Champ M.A. and P.F. Seligman (*Eds*.). Chapman and Hall, London. pp. 191-217.

[14] Liu D., Maguire R.J., Lau Y.L., Pacepavicius G.J., Okamura H., Aoyama I.. (1997): Transformation of the new antifouling compound Irgarol 1051 by Phanerochaete chrysosporium, *Wat. Res.,* 31: 2363-69.

[15] Lokhande MV (2017) Oxygen consumption and behaviour surveillance in the freshwater fish *Rasbora daniconius* exposed to dimethoate, International Journal of Fisheries and Aquatic Studies 2017; 5(2): 712-716.

[16] Maguire, R.J. (1996): The occurrence, fate and toxicity of tributyltin and its degradation products in fresh water environments. In: *Tributyltin: Case study of an environmental contaminant*. S.J. de Mora (Ed.). *Cambridge University Press*, UK. pp. 94-138.

[17] Mary Sr. Avelin (1984): Effect of pesticides on some aspects of physiology of freshwater prawn *Macrobrachium lamerrii*. Ph. D. thesis. Marathawada University, Aurangabad.

[18] Meador, J. P. (1993): The effect of laboratory holding on the toxicity response of marine infaunal amphipods to cadmium and tributyltin. *Journal of Experimental Marine Biology and Ecol- ogy* 174:227-242.

[19] Meador, J.P. (1997): Comparative toxicokinetics of tributyltin in five marine species and its utility in predicting bioaccumulation and acute toxicity. *Aquat. Toxicol.* 37:307-326.

[20] Mercy M , B. Dhanalakshmi (2017): toxicological evaluation of heavy metals in tissues of freshwater fish *oreochromis niloticus* collected from lakes of coimbatore district, tamilnadu, india, *mercy m & b. Dhanalakshmi. Int. Res. J. Pharm. 2017, 8 (1)*

[21] Merkord J, Hennighausen G. (1989): Acute pancreatitis and bile duct lesions in rat induced by dibutyltin dichloride. *Exp Pathol* 36:59-62.

[22] Naimo, T. J.; Waller, D. L. and Holland-Bartels, L. E. (1992): Heavy metals in the threeridge mussel *Amblema plicata plicata* (Say, 1817) in the Upper Mississipi river. *J. Fresh. Ecol.,* 7: (2), 209-218.

[23] Newton F, Athum, B david son A. Valkirs and B. Scligman. (1985): Effects on the growth and survival of eggs and embryos of the *California grunion* (Leuresthes tenuis) exposed to stress level of tributyltin NOSC- TI-1040or AD-AL162-445-1. *Nat. Tech.. Inform. Sevice. Spring field, VA*. 17 pp.

[24] Peuranen S., Vuorinen P.J., Vuorinen M. & Hollender A. (1994): The effects of iron, humic acids and low pH on the gills and physiology of brown trout (Salmo trutta).

[25] Richard Saint Louis and Emilien Pelletier (2004): Sea – to-air flax of contaminants via bubbles bursting. *An experimental approach for tributyltin. Mar. Chem*. 84. 211-224.

[26] Ritsema R. (1994): Dissolved butyltins in marine waters of the Netherlands three years after the ban, *Appl. Organomet. Chem.,* 8: 5-10.

[27] Roberts Jr., M.H. (1987): Acute toxicity of tributyltin chloride to embryos and larvae of two bivalves mollusks, *Crassostrea virginica* and *Mercenaria mercenaria*. *Bull. Environ. Contam. Toxicol.* 39:1012-1019.

[28] Roberts Jr., M.H. (1987): Acute toxicity of tributyltin chloride to embryos and larvae of two bivalves mollusks, *Crassostrea virginica* and *Mercenaria mercenaria*. *Bull. Environ. Contam. Toxicol.* 39:1012-1019.

[29] Salazar, M.H. and S.M. Salazar. (1996): Mussels as bioindicators: Effects of TBT on survival, bioaccumulation, and growth under natural conditions. In: Organotins*: Environmental Fate and Effects. Champ,* M.A., and P.F. Seligman (Eds.). Chapman and Hall, London. pp. 305-330.

[30] Shejule, K.B., Kharat P.S., Kale, R.S. (2006): Toxicity of organotin tributyltin chloride to freshwater prawn, *Macrobrachium kistensis, Jon. Aquacult*. Vol. 7. (1). 141-144.

[31] Spehar R.L. (1976): cadmium and Zinc toxicity to Jordanella floridae. Journal of fisheries research board of Canada . 33:1939-1945.

[32] Sprague, J.B. and D.E. Drury: (1969): avoidance reactions of salmonid fish to representative pollutants . advances in water pollution research, proceddings of the 4th international conference held in prague, 1969, pergaaon, oxford, England. Vol. 1. Pp-169-179.

[33] Sreenivasula Reddy, Narasimha Murthy, M.B. and K.V. Ramanrao, (1985): Toxicity of phoshamidon palaemonid shrimp, Macrobrachium Molcomonii Environ. Ecol., 3: 278-279.

[34] T.S. Pathan, D.L. Sonawane and Y.K. Khillare (2009): Toxicity and Behavioural Changes in Freshwater Fish *Rasbora daniconius* Exposed to Paper Mill Effluent. Botany Research International 2 (4): 263-266,

[35] Takagi S, Mano H, Tsunoda M, (1992): Acute toxicity of tri-n-butyltin chloride (TBTC) in the Syrian golden hamster. *J Exp Med* 166(3):309-319.

[36] Thain J. E. (1986): Toxicity of TBT to bivalves; effectgs on reproduction growth and survival in: Oceans

86, vol. *4 Proc. Internat. Symp.. Mar. Technol. Soc.* Washington, D. C. Pp 1306-1313.

[37] Tim Verslycke, Jordy Vercauteren, Christophe Devos, Luc Moens, Pat Sandra, Colin R. Janssen (2003): Cellular energy allocation in the estuarine mysid shrimp *Neomysis integer* (Crustacea: Mysidacea) following tributyltin exposure*. J. Exper, Mar. Biol Ecol.* 288 (2003) 167– 179.

[38] Townsin R.L. (1987): Development in the calculation of rough underwater surface power penalties, Celena 25th Anniversary Symposium, Genoa.

[39] Triebskorn, R. Kohler, H.D. Flemming J., Braunbeck T., Negele, R.D. Rahmann. H., (1994): Evaluation of bis (tri-n butyltin) oxide (TBTO) neurotoxicity in rainbow trout (oncorhynclus mykiss). I. Behavior weight increase, and tin content. Aquatic Toxicology. 30, 189-197.

[40] V.R.Chavan* and D.V.Muley (2014) :Effect of heavy metals on liver and gill of fish *Cirrhinus mrigala, Int.J.Curr.Microbiol.App.Sci* ,3(5): 277-288.

[41] Van Hattum, B.; Van Straalen, N. M. and Govers, A. J. (1996): Trace metals in populations of freshwater isopods: influence of biotic and abiotic variables. *Arch. Environ. Contam. Toxicol.,* 31, 303-318.

[42] Wada, O. Manabe, S. and Iwai, H. (1982): [Recent progress in the study of analytical methods, toxicity, metabolism and health effects of organotin compounds.] *Sangyo Igaku* 24:24-54. (Japanese)

[43] Waldock, M.J. and J.E. Thain. (1983): Shell thickening in *Crassostrea gigas:* organotin antifouling or sediment induced. *Mar. Pollut. Bull.* 14:411-415.

[44] WHO. (1990): International programme on chemical safety environmental health criteria for tributyltin. *World Health Organization* (ICS/EHC/89.29), *Geneva, Switzerland.* 229 pp.

.