Ecotoxicity testing of Tiber River water as compared to ClO_2, NaClO & PAA

L. Migliore [a*], L. Tancioni [a], D. Mattei [b], L. Mancini [b] and S. Cataudella [a]

[a] *Dipartimento di Biologia, Università "Tor Vergata", Via della Ricerca Scientifica, I-00133, Roma;*

[b] *Laboratorio di Igiene Ambientale, Istituto Superiore di Sanità, V.le Regina Elena 299 I-00161, Roma*

* *luciana.migliore@uniroma2.it*

Abstract: The Tiber River water quality, in a reach including a wastewater treatment plant, has been evaluated for toxicity on *Daphnia magna* by four seasonal samplings. Water tested at the outflow of the wastewater plant showed high acute toxicity only in summer. This effect can be attributed to disinfectants widely used in summer to lower the microbial count within bounds of Italian regulation [D.lgs. 152/99]. To charge the toxic effect to disinfectants, chlorine dioxide, sodium hypochlorite and peracetic acid were contemporary tested for toxicity on the same experimental system. The three disinfectants tested on *Daphnia* induced a) mortality in the range of concentrations allowed by Italian regulation for wastewaters, and b) reduction of survival and fecundity at NOEC (No Observed Effect Concentration in the acute toxicity tests); these results are in accordance with those on Tiber River water at the outflow of the wastewater plant in summer. The toxicity rank for the three disinfectants varies depending on the tests, so it is impossible to state which disinfectant is less harmful. The effects on natural system due to the presence of disinfectants in wastewater plant outfall is discussed.

Keywords: Acute toxicity test, reproduction toxicity test, chlorine dioxide, *Daphnia*, disinfectant, peracetic acid, sodium hypochlorite, Tiber River.

1. INTRODUCTION

Wastewater disinfection is an important process utilised in wastewater treatment plant to lower the microbial count of effluent before the introduction in natural systems. Chlorinate compounds, in particular sodium hypochlorite, held a very important position as reliable disinfectants because of its broad range biocidal effectiveness, its persistence in treated waters and its cost effectiveness. Unfortunately, in the presence of organic matter, chlorination results in the formation of undesiderable compounds; *i.e.* total halogenated compounds (TOX) and trihalomethanes (THM) [Christmas et al., 1983; Bruchet et al., 1984; Duguet et al 1984]. The effects of water chlorination on mutagenic activity have been demonstrated [Monarca et al. 2000]: the potential health risk of halogenated by-products formation promoted research of alternative disinfectants [Fiessinger et al., 1985], the most probable candidates being ozone, chlorine dioxide, ultraviolet light and peracetic acid [Caretti and Lubello, 2003; Veschetti et al., 2003].

The aim of this work was to compare toxicity of water Tiber river in a stretch including the outfall of the wastewater treatment plant, and toxicity of three disinfectants (sodium hypochlorite, chlorine dioxide and peracetic acid) in the lab. Both river water and disinfectants were tested for toxicity on *Daphnia* as acute toxicity (survival rate, 24-72 hrs) and reproduction toxicity (female and young survival rate and fecundity, 21 days).

2. MATERIAL AND METHODS

2.1. Test on field water

In 2001, Tiber River water was seasonally sampled (March, July, October and December) from 3 sites: a) 600 m upstream the Roma Sud wastewater treatment plant (U = upstream); b) outfall of the same plant (O = outfall); c) 200 m downstream the plant (D = downstream).

Waters were utilised within 4 hours from sampling: they were analysed for physical and chemical parameters (temperature, dissolved oxygen, conductivity and pH) and then utilised for ecotoxicological testing on *Daphnia*. Water was filtered and, if below 40%, oxygenated for 15 min, at room temperature. Reproduction test water, for the weekly renewals, was filtered and stocked at –20 °C; before the use it was oxygenated and kept at room temperature. All tests were made in triplicate. The evaluation of temperature, dissolved oxygen and pH were performed to check the compatibility of waters with ecotoxicological testing [Report EPS 1/RM/11, 1990].

Daphnia population

A genetically homogeneous population of *Daphnia magna* Straus (Crustacea) was obtained from a single female coming from a lab pond, outdoor. Females were cultured in 1 litre beakers, according to standard methods of IRSA [Guzzella, 1996]. Breeding water was ¾ of distilled water and ¼ dechlorinated and oxygen-saturated tap water. Water was renewed three times a week. *Daphnia* were fed daily with liquid commercial

feed; feeding rate was: 1 drop/100 ml/day. Temperature of the culture room was 20±2 °C, under continuous light. Beakers were covered with glass to avoid evaporation and placed on a white surface.
Both acute and reproduction toxicity tests were performed according to IRSA standard protocols [Guzzella, 1996].
ACUTE TEST. 30 newborn individuals, less than 24 h old, were exposed to filtered water, coming from each sampling site. Controls (30 newborn individuals) were kept in breeding water. Tests were static, lasting 72 h. 150 ml glass beakers were used for the test, containing 100 ml test solution or breeding water; neither food nor O_2 were administered during the test. Temperature of the culture room was 20±2 °C under continuous light and beakers were covered with glass to avoid evaporation and placed on a white surface. The number of alive and dead/immobile newborn individuals were checked every 24 hours, up to 72 hours. Data were analysed by χ^2 test, taking into account all individuals in the three replicates.
REPRODUCTION TEST. 24 ovigerous females were exposed to filtered water, coming from each sampling site; controls (24 females) were kept in breeding water. Test were semi-static, lasting 21 days. 500 ml glass beakers were used for the test, containing 400 ml test solution or breeding water (50 ml/female), renewed three times a week. Feed (1 drop/100 ml) was administered daily, O_2 continuously. Temperature of the culture room was 20±2 °C under continuous light. Beakers were covered with glass to avoid evaporation and placed on a white surface. The number of alive and dead/immobile females were checked daily; immobile females were removed. Offspring was counted and removed daily from the containers to obtain the total number. Data were analysed by the χ^2 test for death rate and by Student *t* test for fecundity, taking into account all individuals in the three replicates.

2.2. Test on disinfectants

Sodium hypochlorite, peracetic acid and chlorine dioxide toxicity was tested on *Daphnia magna* to determine, by acute toxicity tests: 1. EC_{50} values (*i.e.* Effective Concentration of the toxicant causing death/immobilisation in 50% of tested organisms); 2. NOEC values (No Observed Effect Concentration determined).
NOEC concentrations were checked in reproduction test (21 days). Both acute and reproduction toxicity test were performed as reported in § 2.1.
Disinfectant concentration ranges for acute toxicity tests were chosen according to bibliographic data on toxicity and mutagenicity of the three compounds [Buschini et al., 2001; Monarca et al., 2001]; they were, in geometric progression 2, Sodium hypochlorite (NaClO) 4 - 0.03 mg/l; Peracetic acid (PAA) 1.5 - 0.015 mg/l and Chlorine dioxide (ClO_2) 1 - 0.015 mg/l. Mortality data were analysed by the probit test, females survival data by χ^2 test, reproduction rate data by Student *t* test.

3. RESULTS

3.1. Test on field water

ACUTE TEST. Acute toxicity of the Tiber River water coming from the three sites to *Daphnia* newborn individuals is reported in Fig. 1 and 2, respectively as survival after 24 and 72 hours. After 24 h, water sampled at site U caused in spring and summer a survival of 44 and 53%, respectively; in autumn and winter survival was 100%. Water from site O only in summer caused a survival of 0%; this result was confirmed by subsequent weekly sampling and tests (during the entire month of July), with undiluted and 50% diluted water (as a total 180 newborn individuals were tested, data not shown); water from site D and lab control never reduced survival from 100%. After 72 h survival rate is lower. Water from site U in spring and summer caused a reduction of newborn individuals survival to 0% and 43%, respectively; in autumn and winter survival was 78% and 90%, respectively. Water from site D reduced survival to 70% and 90% in summer and autumn, respectively. Lab control water never reduced newborn individuals survival.
$\chi 2$ analyses (Tab. 1) evidenced significant differences in survival rate between site U and lab control, in summer and spring at both 24 and 72 hours, and in autumn at 72 hours. Significant differences between site O and lab control were found only in summer, while no significant differences were found for site D, neither at 24 nor at 72 hours.

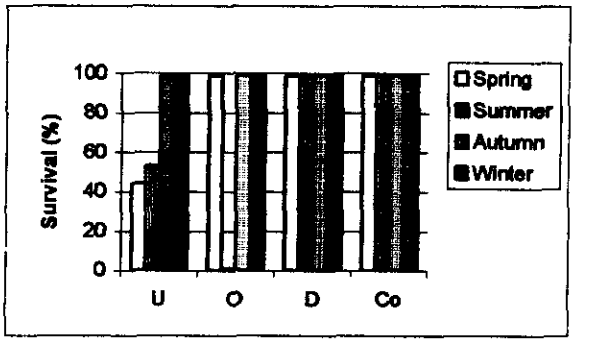

Figure 1. Tiber River: Acute toxicity (24h)

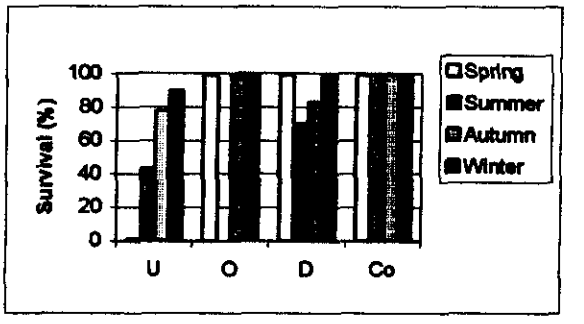

Figure 2. Tiber River: Acute toxicity (72h)

Table 1. χ^2 values. Comparison of survival rate between treated and control batches in acute toxicity tests at both 24 and 72 hours.

Site	Spring		Summer		Autumn		Winter	
	24h	*72h*	*24h*	*72h*	*24h*	*72h*	*24h*	*72h*
D	1.403 ns	0.873 ns	0.001 ns	1.422 ns	1.403 ns	2.588 ns	1.298 ns	0.523 ns
O	0.001 ns	0.185 ns	60.067 *	N.D.	0.517 ns	0.517 ns	0.120 ns	2.131 ns
U	24.631 *	52.795 *	19.247 **	9.697 **	2.411 ns	12.800 **	0.393 ns	1.422 ns

ns= not significant; * = p< 0.001; ** = p< 0.005; N.D. = Not done, because of the 0% survival at 24 hours.

REPRODUCTION TEST. Toxicity of the Tiber River water coming from the three above-mentioned sites to *Daphnia* ovigerous females was evaluated as females survival, offspring survival and females reproduction rates.

Spring. Water from site U caused very high decrease of females survival (to 25%), high decrease in young survival (to 50%) and high decrease in reproduction rate (from 36.68 to 25.65 young/female). Water from site O caused high decrease in females survival (to 50%) and very high decrease in young survival (to 33%). Water from site D never caused toxic effects. **Summer**. Water from site U caused maximum decrease in females survival (to 0%) and young survival (to 0%), and decrease in reproduction rate (from 72.67 to 9.0 young/female). No reproduction test has been possible for site O, due to the very high acute toxicity of the water causing the death of all individual exposed within 24 hours. Water from site D caused decrease in females survival (to 63%), light decrease in young survival (to 75%); and no wide differences in females reproduction (from 72.26 to 61.7 young/female). **Autumn**. Water from site U caused maximum decrease in females survival (to 0%), very high decrease in young survival (to 35%) and no wide differences were recorded in female reproduction rate (from 62.0 to 60.5 young/female) . Water from site O and D never caused toxic effects. **Winter**. Water from site U never caused toxic effects on female survival and caused a decrease in young survival (to 67%) and induced increase in females reproduction rate (95.1 young/treated female vs 71.0 young/control female). Water from site O caused decrease on females survival (to 38%) and light decrease in young survival (to 84%).

$\chi 2$ analyses on females survival rate (Tab. 2) evidenced significant differences in spring between sites U, O and lab control. At site D no significant differences were found; in summer and autumn significant differences were found only for site U; in winter only for site O.

Table 2. χ^2 values. Comparison of female survival rate between treated and control batches after 21 days.

Site	Spring	Summer	Autumn	Winter
U	9.143 ns	16.254 **	16.254 **	1.641 ns
O	4.654 ns	N.D.	1.641 ns	6.667 *
D	0.001 ns	3.00 ns	1.691 ns	3.00 ns

ns= not significant; * = p< 0.001; ** = p< 0.005; N.D. = Not done, because of the 0% survival at 24 hours.

Student *t* analyses (Tab.3) (reproduction rate) evidenced significant differences in spring between sites U, O and lab control; for site D no significant differences were found. In summer no significant differences were found between sites U, D and lab control, although a slightly higher value was found for site D. In autumn no significant differences were found between sites U, O, D and lab control; in winter significant differences were found only for site U, with higher values for wastewater.

Table 3. Student *t* values. Comparison of females reproduction rate between treated and control batches after 21 days.

Site	Spring	Summer	Autumn	Winter
U	2.833 *	2.225 ns	0.514 ns	-3.142 *
O	3.427 *	N.D.	0.524 ns	1.232 ns
D	0.854 ns	-0.821 ns	0.519 ns	1.011 ns

ns= not significant; * = p< 0.001; ** = p< 0.005; N.D. = Not done, because of the 0% survival at 24 hours.

χ^2 analysis on young survival rate (Tab. 4) evidenced significant differences with control in spring and summer at sites U and O.

Table 4. χ^2 values. Comparison of young survival rate between treated and control batches after 21 days.

Site	Spring	Summer	Autumn	Winter
U	7.724*s	21.426 *	11.357 **	2.230 ns
O	11.583 **	N.D.	1.565 ns	2.586 ns
D	0.233 ns	4.069 *	1.544 ns	1.950 ns

ns= not significant; * = p< 0.001; ** = p< 0.005; N.D. = Not done, because of the 0% survival at 24 hours.

3.2. Test on disinfectants

ACUTE TEST. Acute toxicity (after 24h) of chlorine dioxide (ClO_2), sodium hypochlorite (NaClO), peracetic acid (PAA) to *Daphnia* newborn individuals are reported in Figures 3, 4 and 5.
ClO_2 caused 0% survival from 1.5 to 0.125 mg/l, 17% at 0.062 mg/l, 37% at 0.031 mg/l. NaClO caused 0% survival from 4 to 0.062 mg/l. PAA causes 0% survival from 1.5 to 0.062 mg/l, 73% at 0.031 mg/l. Control (Co) showed 93% survival.

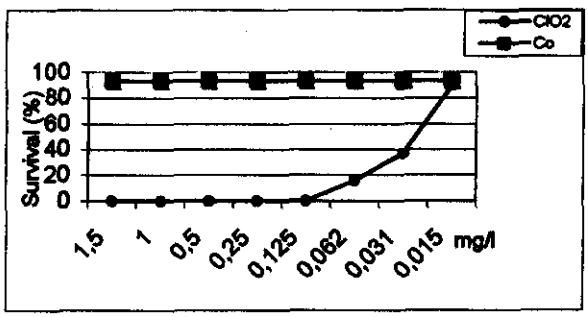

Figure 3. ClO_2 : Acute toxicity after 24h

On the base of these results NOEC has been determined as the concentrations causing death rate lower than 20%, showing not significant differences (χ^2 analyses) with control: for ClO_2 was 0.015 mg/l; for NaClO was 0.031 mg/l; for PAA was 0.015 mg/l.

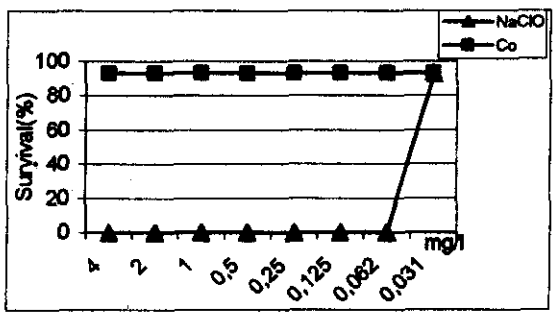

Figure 4. NaClO : Acute toxicity after 24h

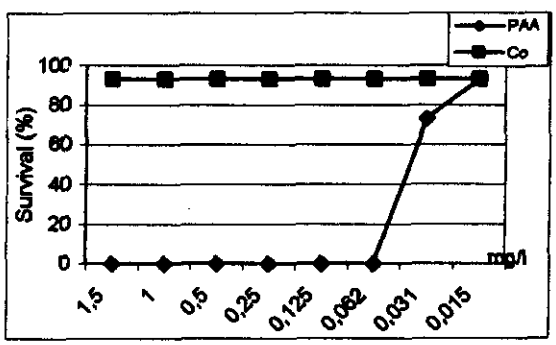

Figure 5. PAA : Acute toxicity after 24h

REPRODUCTION TEST. Toxicity of the three above-mentioned disinfectants was evaluated as death rate and fecundity after 21 days to *Daphnia* ovigerous females and as young survival after 21 days.
ClO_2 at NOEC concentration (0.015 mg/l) caused very high decrease in females survival (to 25%), very high decrease in young survival (to 25%) and high decrease in reproduction rate (from 53.8 to 6.4 young/female).
NaClO at NOEC concentration (0.031 mg/l) caused decrease in females survival (to 63%), maximum decrease in young survival (to 0%) and decrease in reproduction rate (from 36.9 to 28.4 young/female).
PAA at NOEC concentration (0.015 mg/l) caused decrease in females survival (to 12%), very high decrease in young survival (to 38%) and a very high decrease in reproduction rate (from 53.8 to 17.4 young/female).
χ^2 analysis on females survival rate evidenced significant differences with control (ClO_2 χ^2 =9.143, $p<0.05$; NaClO χ^2 =4.654, $p<0.05$; PAA χ^2 =12.250, $p<0.005$).
χ^2 analysis on young survival rate evidenced significant differences with control (ClO_2 χ^2 =6.853, $p<0.01$; NaClO χ^2=12.386, $p<0.05$; PAA χ^2=7.412, $p<0.01$).
Student t analysis on reproduction rate evidenced significant differences with control (ClO_2 t = 5.728, $p<0.05$; NaClO t = 2.429 $p<0.05$; PAA t = 4.173, $p<0.05$).

4. DISCUSSION

Tiber river water was sampled in three sites near a wastewater treatment plant (U = upstream, O = outfall, D = downstream). Water at sites U and O caused toxic effects (both acute and reproductive) on *Daphnia magna*. Site D, considered a sort of field control, confirmed this hypothesis: water from this site never caused strong toxic effects, neither in acute nor in reproduction tests on *Daphnia*. Instead, water at site U caused acute toxicity in spring and summer, and also reproduction tests showed a reduction in survival and fecundity. Water at site O showed very high acute toxicity only in summer (even at 50% diluted sample). In both sites the toxic effect got lost in few hundred meters, due to dilution of river water. The toxic effect found in summer at site O can be imputed to disinfectants widely used in that season to reduce microbial counts within the limits defined by the Italian regulation. This hypothesis was confirmed by mean of ecotoxicological tests performed on *Daphnia* with disinfectants: in fact EC_{50} and NOEC values are lower than limits imposed by Italian regulation. EC_{50} values for NaClO and ClO_2 were: 0.03 – 0.04 mg/l, lower than the limit for total residual chlorine (0.1-0.2 mg/l). EC_{50} value for PAA was 0.03 mg/l as for chlorinated compounds. NOEC values are also lower than limits imposed by Italian regulation: values for NaClO and ClO_2 were 0.031 mg/l and 0.015 mg/l respectively. NOEC value for PAA was 0.015 mg/l as for chlorinated compounds. The order of decreasing acute toxicity for three disinfectants is PAA>ClO_2>NaClO. Furthermore, at NOEC reproduction tests showed a chronic damage as

mortality and reproduction: the order of decreasing toxicity is PAA>ClO_2>NaClO and ClO_2>PAA>NaClO, respectively.

5. CONCLUSIONS

All the three disinfectants (ClO_2, NaClO and PAA) are toxic to *Daphnia*. Lab results indicate some possible effects of these compounds in nature and they are perfectly superimposed to Tiber river data in summer at the outfall of the wastewater treatment plant. As a consequence it is impossible to formulate a judgement of the 'less harmful' because of the different effects on different endpoints. Nevertheless, in the near future a more incisive assessment of the risks for natural aquatic ecosystems subjected to disinfectants contamination must be taken into consideration.

6. ACKNOWLEDGEMENTS

The authors wish to thank Dr.ssa Silvia Marchini and Dr.ssa Milena Bruno (ISS) for their significant contribution during all the phases of the work.

7. REFERENCES

Bruchet, A., Y. Tsutsumi, J.P. Duguet and J. Mallevialle, Characterization of total halogenated compounds along various water treatment processes, *5th Conference on Water Chlorination. Environmental Impact and Health Effect*, Williamsburg, Virginia, 1984.

Buschini, A., A. Martino, B. Gustavino, M. Monfrinotti, P. Poli, C. Rossi, M. Santoro and M. Rizzoni, Genotoxicity detected with comet assay and micronucleus test in *Cyprinus carpio* specimens exposed *in situ* to Trasimeno Lake waters treated with disinfectantsfor potabilisation, *30th Annual Meeting of European Environmental Mutagen Society*, Gant (Belgium), 2001.

Caretti, C. and C. Lubello, Wastewater disinfection with PAA and UV combined treatment: a pilot plant study, *Water Research* 37: 2365-2371, 2003.

Christmas, R.F., D.L. Norvood, D.S. Millington, J.D. Johnson and A.A. Stevens, Identification and yields of major halogenated products of aquatic fulvic acid chlorination, *Env. Sci. Tech.* 17: 625-628, 1983.

Duguet, J.P., A. Ellul, E. Brodard and J. Mallevialle, Evolution de la mutagenese au cours d'un traitement d'ozonation, IOA Congress, Brussels, 1984.

Fiessinger, F., J.J. Rook and J.P. Duguet, Alternative methods for chlorination, *Science Tot. Envir.*, 47: 299-315, 1985.

Guzzella, L., Saggio di tossicità acuta con *Daphnia magna*, Quad. Ist. Ric. Acque, 100 (suppl.), 1996.

Monarca, S., D. Feretti, C. Collivignarelli, L. Guzzella, I. Zerbini, G. Bertanza and R. Pedrazzani, The influence of different disinfectants on mutagenicity and toxicity of urban wastewater, *Water Res.* 34 (17): 4261-4269, 2000.

Monarca, S., L. Guzzella, I. Zerbini, C. Zani, M. Rizzoni, B. Gustavino, A. Alberti and D. Feretti, In situ monitoring of mutagenic by-products produced by disinfection of surface water using plant tests, IWA 2nd World Water Congress, Berlino, 2001.

Veschetti, E., D. Cutilli, L. Bonadonna, R. Briancesco, C. Martini, G. Cecchini, P. Inastasi, and M. Ottaviani, Pilot-plant comparative study of peracetic acid and sodium hypochlorite wastewater disinfection, *Water Res.* 37: 78-94.1, 2003.

Legislation frame

D. lgs 152/99. "Disposizioni sulla tutela delle acque dall'inquinamento e recepimento della Direttiva 91/271/CEE concernente il trattamento delle acque reflue urbane e della Direttiva 91/676/CEE relativa alla protezione delle acque dall'inquinamento provocato dai nitrati provenienti da fonti agricole". G. U. n. 124 del 29 maggio 1999, pp. 124.

Biological Test Method: Acute lethality Test Using *Daphnia* spp. Report EPS 1/RM/11, 1990.