Bioassays and biomarkers for ecotoxicological assessment of reclaimed municipal wastewater
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ABSTRACT

The aim of this work was to examine the ecotoxicity of reclaimed wastewater by the use of bioassays and the determination of immunological parameters. Secondary and tertiary municipal wastewater samples were examined for their physicochemical and microbiological characteristics as well as for their endotoxin concentrations. The ecotoxicological characteristics were assessed by a battery of bioassays, using Vibrio fischeri, Daphnia magna and Tetrahymena thermophila as test species and phytotoxicity. The mitogenic responses of mouse splenocytes were as well used as bioassay. The cytokines of IL-1, IL-2, IL-10, IFNγ and TNFα, were also determined in the supernatant of splenocyte cultures and served as molecular biomarkers. All bioassays exhibited decrease of the ecotoxicological responses after tertiary treatment. However, mitogenic responses were proved to be more sensitive. IL-1 increased, while IL-2 production was unaffected. The fact that IL-10 production increased in response to secondary treated effluents in conjunction with the increased endotoxin levels, suggest Th2 type immune responses. Although results obtained from the toxicity bioassays after the tertiary treatment showed comparable results to those of controls, cytokine levels indicated the induction of immune response even after tertiary treatment. Consequently, cytokine production could be used as a sensitive biomarker for the evaluation of treatment efficiency of the reclaimed wastewaters intended for reuse.

Key words | bioassays, mitogenic response, molecular biomarkers, reclamation, reuse, tertiary treatment

INTRODUCTION

The increased demand of water consumption, in association with the scarcity of the available sources, imposes the need for wastewater reclamation and reuse. Wastewater reclamation and reuse is an environmental friendly and cost efficient process in the management of water resources (Angelakis et al. 1999). The potential use of reclaimed wastewater determines the level of treatment required and thus the application of the respective treatment technology. The current practices of tertiary treatment usually include one or a combination of processes such as coagulation, disinfection and adsorption on activated carbon, prior to reuse (Samaras et al. 1995). Frequently, coagulation followed by sedimentation or sand filtration, consists a conventional stage of a wastewater reclamation process (Duan & Gregory 2003).

Tertiary treatment of secondary treated effluents is assessed by the obtained effluent quality, as determined by the physical and chemical parameters. However, the quality of reclaimed water should be well established prior to its reuse, in order to anticipate potential long-term health and ecological risk hazards. In particular, emerging contaminants are previously unknown or unrecognized pollutants that could comprise compounds such as pharmaceutical products, steroids, xenoestrogens, surfactants, sulfophenyl car-
boxylates, gasoline additives, microorganism-derived free molecules, algal toxins, pesticide degradation products, etc. (Rodriguez-Mozaz 2007). Most of them have been present in the environment for a long time, but their significance and presence are only now being elucidated and, therefore, they are generally not included in the legislation. Current legislation on wastewater reuse is based solely on physicochemical and microbiological parameters, which are not sufficient in the evaluation of biological effects. Chemical contaminants are generally detected by chemical analysis focusing on contaminants known or suspected to be present. Single chemical analyses confront certain limitations; organic micro-pollutants detection is laborious and difficult to be accomplished (Aguayo et al. 2004); the interactive effects, synergistic or antagonistic, between the components of a mixture and the bioavailability of the compounds cannot be predicted by single chemical measurements (Kungolos et al. 2004).

On the other hand, analysis of microbial contamination rarely considers microorganism-derived free molecules, such as endotoxins, which are cell wall components of Gram-negative bacteria, known to cause adverse health effects (Wichmann et al. 2004). Many of the above contaminants may affect directly or indirectly cells of the immune system. It is well known that bacterial endotoxins induce polyclonal activation of B lymphocytes (Bucala 1991). Activation of lymphocytes is mediated by well characterized growth or differentiation factors called cytokines such as interleukin-1 (IL-1), interleukin-2 (IL-2), interleukin-10 (IL-10) and interferon-γ IFNγ (Romagnani 1992). Thus, the incorporation of biomonitoring in regulatory schemes is necessary, since it could be able to determine the effect of various pollutants, on the indicator species used, such as growth rate, reproduction, survival, stress (De Coen et al. 2000). Therefore, bioassays as well as determination of sensitive biomarkers should be performed, supplementary to chemical analyses for the assessment of the environmental impact of effluents. Ecotoxicological testing provides an overall direct estimation of the environmental hazard of effluents, by the exposure of selected test species on samples and determination of certain end-points, such as lethal effect, growth ability, etc.

The aims of this work included (i) the examination of coagulation and activated carbon efficiency, both combined with chlorination on the physicochemical and microbiological characteristics of the secondary treated effluents, (ii) the study of effluent ecotoxicity by using Vibrio fischeri, Daphnia magna, protozoa and 3 plant species as test organisms and immunological responses by using in vitro systems, including spleen cell proliferation as well as cytokine production.

METHODS

Wastewater samples collection

Secondary municipal wastewaters were collected from a sewage treatment plant within the area of Thessaloniki (North Greece). A sample of 8 L was collected from the overflow of the secondary sedimentation tank.

Jar test, granular activated carbon and chlorination

Chlorination was conducted by using sodium hypochloride solution of 11.5% Cl, and utilizing 4 ppm of chloride under continuous rapid agitation for 10 min followed by slow agitation in order to remove residual chloride. The examination of coagulation of municipal effluents was implemented by conducting laboratory-scale coagulation tests (jar tests) in 6 1l glass beakers. The applied coagulant was ferric chloride 0.1 M (FeCl₃ ·6H₂O, Fluka). The coagulation process consisted of a rapid agitation stage at 100 rpm for 2 min, followed by a second stage of slow agitation for 15 min at 50 rpm and a third step of sedimentation for 30 min. Activated carbon treatment was conducted through a Granular Activated Carbon (GAC, 830 NORIT) column of 30 cm high and 10 cm internal diameter. The contact time was 10 min. Sterilization of water samples was performed by passing them through 0.22 μm pore filters and autoclaving for 30 min.

Physicochemical and microbiological parameters, endotoxin quantification

Secondary and tertiary treated wastewater samples were subjected to analyses, for the measurement of chemical oxygen demand (COD), nitrogen–ammonia, pH, orthophosphates and suspended solids, according to Standard
Methods for the Examination of Water and Wastewater (APHA-AWWA-WEF 1995).

Bacteriological examination was carried out using the multiple tube technique in a series of 10-fold dilutions, with five tubes per dilution, and quantified using the Most Probable Number (MPN). Lauryl sulphate broth (Merck KGaA, Germany) was used for the detection and enumeration of total coliforms (T.C.). *E. coli* confirmation was done in EC broth (Merck KGaA, Germany) in 44.5°C in water bath with the addition of Kovac’s reagent. Azide dextrose broth was used as a presumptive test following by the confirmation test in kanamycin azide (KA) agar (Merck KGaA, Germany) for the detection and enumeration of enterococci.

The endotoxin concentrations were measured using the limulus amoebocyte lysate (LAL) from the certified European Endotoxin Testing Service CAMPREX, in the above samples, before and after sterilisation.

Toxicity testing

The toxicity of wastewater samples was evaluated using the marine luminescence bacteria *V. fischeri* originally in freeze-dried form and activated prior to use by a reconstitution solution. The changes in light emission of test organisms, obtained by their direct contact with the samples, were measured after exposure time of 30 min using the Microtox 500 Analyzer (SDI). For the determination of the toxic effects to *Daphnia magna* the Daphtoxkit F test by Microbiotests was used. The organisms were obtained in the form of ephipia. Ephipia were hatched for 3–4 days in an incubator under adequate light and standard temperature. Once the organisms were hatched, five organisms were transferred to each plastic test well containing control medium and the samples. The toxic effect was evaluated as the percentage of nonviable/immobilized organisms after 24 h of exposure to the samples in the absence of light. In the microbiotest with the ciliate protozoan *Tetrahymena thermophila*, the growth inhibition of the ciliate protozoan was evaluated, after 24 h exposure in the undiluted wastewater sample, at 30°C in darkness. The test is based on the optical density measurement of the food substrate provided to the ciliates, in 1 cm disposable spectrophotometric cells.

Samples were tested for their phytotoxic properties, by using three plant organisms, i.e. *Alba sinapis, Shorgum saccharatum* and *Lepidium sativum* (Phytotoxkit® supplied by Microbiotests) as test species. Ten seeds from each species were placed in flat shallow transparent test plates, composed of two compartments, the lower one able to maintain the reference OECD soil saturated to the water holding capacity. A control sample was also prepared using deionized water. All samples were prepared in triplicates and the average values are presented in the results. Test plates with the seeds were incubated for 3 d at 25°C. The inhibition of seed germination and of root growth was calculated by the following equation:

\[ I = \frac{A - B}{A} \times 100 \]

where:

- \( I \) = Inhibition (%)
- \( A \) = mean seed germination or root length for the control soil
- \( B \) = mean seed germination or root length for the examined mixture

Cell cultures

Mouse spleen cells were cultured in RPMI-1640 medium (complete with 2-mercaptoethanol 5 × 10⁻⁵ M, 2 mM L-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin and 24 mM NaHCO₃), supplemented with 5% foetal bovine serum (FBS), prepared using autoclaved secondary and tertiary treated wastewater. Mitogenic responses of spleen cells were performed in serial two fold dilutions of the above medium, in triplicate, in 96-well microtiter plates, at a final cell density of 1 × 10⁶ cells/mL at 37°C in 5% CO₂ atmosphere for 72 h. In all cultures 20 μL of 0.4 μCi tritiated thymidine (³H-TdR) were added 18 h before cell harvesting and total ³H-TdR incorporation was determined by liquid scintillation spectrophotometry.

Spleen cell culture supernatants for cytokine production were prepared by culturing 5 × 10⁶ cells/mL in the appropriate medium for 48 h at 37°C and 5% CO₂ atmosphere. Thereafter, cell culture supernatants were harvested by centrifugation and passed through a 0.22 μm filter. The levels of IL-1 and IL-2 in the supernatants were
assessed with bioassays as described from Paetkau et al. (1976) and Watson (1979) respectively. IL-1 activity was measured by the standard thymocyte proliferation assay, were thymocytes from 1–2 month old rats were used. In particular 100 μL of serially diluted supernatant were added to triplicate wells of a 96-well culture dish. Equal volumes of 1 × 10⁵ rat thymocytes in 100 μL RPMI supplemented with 10% FBS were distributed into each well together with 20 μL RPMI containing Con-A 1.5 μg/mL. After 54h incubation at 37°C and 5% CO₂ air, the cells were pulsed with 0.4 μCi of ³H-TdR. Total ³H-TdR incorporation was measured as previously. Augmentation of Con-A induced thymocyte proliferation indicating IL-1 activity.

Supernatant fluid IL-2 activity was determined by measuring its effect on the proliferation of rat spleen cells which had been activated with Con-A and had expressed receptors for IL-2. Briefly, 50 mL of 2 × 10⁶ cells/mL spleen cell suspension in complete RPMI with 5% FBS, and 2.5 μg/mL Con-A were distributed in tissue culture flasks. Cultures were incubated at 37°C, in 5% CO₂ and 48 h later the cells were collected, washed with complete RPMI with 10% FBS, containing 25 mM methyl-mannoside (to inactivate Con-A) and then resuspended in the above medium to concentration 0.5 × 10⁶ cells/mL. The cells were cultured in microtiter plates for 24 h in the presence of serial dilutions of the previously isolated supernatants. Then, 20 μL of 0.4 μCi of ³H-TdR were added to each well and cultured for another 18 h.

The concentrations of the cytokines IL-10, IFNγ and TNFα in culture supernatants of splenocytes were determined using ELISA kits from eBioscience.

RESULTS AND DISCUSSION

The evaluation of the efficiency of tertiary treatment was performed by the analysis of physicochemical and microbiological characteristics; furthermore endotoxin content as well as toxicity bioassays and immunological parameters were determined. In total 7 samples were assessed: secondary treated effluent (ST), chlorination after secondary treatment (Chl.), chlorination-coagulation (Chl.-Coag.), coagulation-chlorination (Coag.-Chl.), coagulation-GAC-chlorination (Coag.-GAC-Chl.), GAC-coagulation-chlorination (GAC-Coag-Chl.) and GAC-chlorination (GAC-Chl.).

Physicochemical and microbiological parameters, endotoxin quantification

The results obtained from the physicochemical, microbiological parameters and endotoxin levels are shown in Table 1.

Chlorination and tertiary treatment resulted in the reduction of all microbiological and physicochemical parameters. However, endotoxin levels were not affected by chlorination and tertiary treatment of wastewater.

| Table 1 | Physicochemical and microbiological characteristics from the secondary and tertiary treated wastewater samples |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Total Coliforms/dl | 1500           | 25             | 54              | –               | –               | –               |
| E. coli/dl         | 1020           | 3              | 32              | –               | –               | –               |
| Enterococci/dl     | 675            | 3              | 23              | –               | –               | –               |
| Endotoxin (Eu/mL)  |                 |                |                 |                 |                 |                 |
| Without steriliz.  | 201            | 180            | 172             | –               | –               | –               |
| Steriliz.          | 150            | 78             | 75              | –               | 160             | 56              |
| pH                | 8.2            | 6.8            | 8.1             | 6.7             | 6.9             | 6.9             |
| BOD (mg/L)         | 30             | –              | –               | –               | –               | –               |
| COD (mg/L)         | 131            | 129            | 5               | 9               | 8               | 3               |
| NH₃-N (mg/L)       | 10             | 9              | –               | 6               | –               | –               |
| PO₄ (mg/L)         | 23             | 23             | 2               | 2               | 5               | 2               |
| SS (mg/L)          | 67             | 67             | 1               | 2               | 2               | 0.5             |

The concentrations of the cytokines IL-10, IFNγ and TNFα in culture supernatants of splenocytes were determined using ELISA kits from eBioscience.
More specifically, samples treated with Coag.-Chl, GAC-Coag-Chl, Coag-GAC-Chl, GAC-Chl, eliminated the microbial load and decreased the nutrient and suspended solids concentrations. Sterilization of the above samples by autoclave resulted in a significant decrease of endotoxin levels. Advanced treatment of secondary effluents by GAC-Chl. reduced the endotoxin levels by about 75% of the initial level in the S.T. sample.

Toxicity testing

The results obtained from the battery of bioassays and phytotoxicity tests (root length growth) are shown in Tables 2 and 3 respectively.

Secondary treated effluent had greater effect on the survival of *D. magna* reaching up to 50% immobilization/mortality of the organisms, the food uptake of *T. thermophila* least effected, while the bioluminescence of *V. fischeri* was inhibited up to 30% (Table 2). As far phytotoxicity concerns (Table 3), all the seeds were germinated in all samples tested, while the root length was slightly inhibited up to 20% in the cases of secondary treated effluent, Coag.-Chl. and Ch.-Coag. treated samples. Treatment of the secondary effluents with Coag.-GAC-Chl., GAC-Coag.-Chl. and GAC-Chl., decreased further the toxic responses of all organisms and plant species used, reaching up to negligible effect. However for the case of *D. magna* immobilization/mortality, inhibition effects were observed that were persisted even after the advanced treatment of the samples, while in the case of *V. fischeri*, negative bioluminescence inhibition values were detected, suggesting hormesis effects.

Cell cultures

Culture of mouse spleen cells in RPMI-1640 medium, prepared with S.T. effluent, resulted in increased incorporation of $^3$H-TdR by the spleen cells which means increased mitogenic response. Tertiary treatment and chlorination (apart from the sample Chl.-Coag.) of the S.T. resulted in

![Figure 1](https://iwaponline.com/wst/article-pdf/57/6/947/438831/947.pdf)
reduction of the mitogenic responses. Maximum reduction was observed in the case of GAC-Chl. samples, where the mitogenic responses were similar to the respective of controls (Figure 1a). The increased mitogenic responses using S.T. medium is due to certain constituents present in wastewaters, since serial dilutions of S.T. medium resulted in gradual reduction of mitogenic responses of splenocytes (Figure 1b). Chlorination in combination with tertiary treatment of S.T. reduced the amount of these constituents from wastewater. However, at least 30 times dilution of tertiary treated wastewater was required to reduce mitogenic responses to control levels (Figure 1b).

For the determination of the cytokines responsible for spleen cell proliferation several bioassays were performed aiming to the detection of the IL-1 and IL-2 activity in supernatants obtained from cell cultures in medium prepared with the S.T. and the GAC-Chl sample. IL-1 activity was increased in S.T. supernatants (Figure 2) while IL-2 activity was not affected (Table 4), suggesting that wastewater constituents activated in the spleen the B lymphocytes rather than the T lymphocytes. Such a wastewater constituent could be the microbial derived endotoxin, already found to be in high levels (Table 1). Furthermore, the S.T. supernatants contained high levels of IL-10 and TNFα (Table 4), suggesting that endotoxin stimulated spleen macrophages to produce IL-1, IL-10 and TNFα (De Waal Malefyt et al. 1991). Culture of spleen cells in medium prepared with GAC-Chl. sample resulted in the detection of low levels of IL-1 in the supernatant, while the levels of IL-10 and TNFα remained increased (Figure 2, Table 4). In both cases, as shown in Table 4, the cytokines IL-2 as well as IFNγ were not affected in the supernatants suggesting that S.T. wastewaters, used in these experiments, activated the Th2 immune response.

CONCLUSIONS

In all cases the responses of the organisms used in bioassays decreased after the application of tertiary treatment to the samples, reaching almost control values. However, no significant changes were observed in D. magna responses, generally indicating the increased sensitivity of the organism. In addition, both spleen cell mitogenic responses bioassay and cytokines as biomarkers were proved to be very sensitive tools for the evaluation of the effectiveness of tertiary wastewater treatment.

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