Environ. Eng. Res. Vol. 8, No. 1, pp. 8~14, 2003
Korean Society of Environmental Engineers

IS INK HARMFUL TO WASTEWATER?

Jin-Hwa Park[†] **and Yong-Woo Yi**[*]

Department of Civil and Environmental Engineering, University of Wisconsin-Madison, 1415 Engineering Drive, Madison, WI 53706, U.S.A.

[*]Department of Electronic, Provincial College of Damyang, Chonnam 517-800, Korea

(received December 2001, accepted October 2002)

Abstract : In this study, we investigated what happens to wastewater after ink is discharged into the sewer. During oxygen uptake tests, the oxygen was depleted within 13 min for all the runs, indicating almost no inhibitory effect of the ink sample that was tested. It appears that it is not necessary to acclimate activated sludge for treatment of ink-containing wastewater. Furthermore, according to the RET assay result, the initial samples (5 min after injecting ink solution) had almost the same % inhibition values as the samples after 10 hr of biological treatment. However, the samples after 20 hr of biological treatment showed lower % inhibitions. The ink sample tested may not be readily biodegradable but can be treated when the detention time of activated sludge processes is long ($\geq$ 20 hr). The concentration of the sample that inhibits the SMP activity 50%, EC_{50}, was 312 mg/L. This value is much lower than those determined for dyes used in textile industries.

Key Words : concentration, harmfulness, ink, toxicity, wastewater

INTRODUCTION

The Village of Wrightstowns Wastewater Treatment Plant (WWTP) in Madison, Wisconsin, U.S. experienced operational problems leading to a decrease in biochemical oxygen demand (BOD) removal efficiency and an increase in suspended solids in the effluent. The wastewater discharged from Coating Excellence Inc. (CEI) was one of the potential suspects. The process upset at the WWTP may not be caused by the discharge of the ink sample tested. There may be a synergistic effect of various ink solutions used in CEI on the performance of the activated sludge process at the Village of Wrightstown. Furthermore, if the domestic sewage flow rate is low but the CEI's wastewater flow rate is high, it is possible that an upset may occur. The changes in the flow rates of both domestic and CEI wastewaters along with organic loading must be evaluated as well. Wastewater at CEI is generated in a shop sink when washing miscellaneous material (e.g. pails, rags, brushes, etc) contaminated with ink solution. The sample provided is the concentrated ink solution that CEI ships off site for disposal. They do not discharge this concentrated ink solution diluted 100 times with water from the shop sink. Estimated daily discharge of ink-contaminated wastewater would be 250 gpd. This CEI's wastewater flows into a lift station where it is held until it is pumped into the Village of Wrightstowns Public Own Treatment Wastewater (POTW). The POTW's daily flow averages around 125,000 gallons per day. The ink-contaminated wastewater is discharged into the lift station and into the POTW.

A sample of concentrated ink solution was received along with an influent sample and

[†]Corresponding author
E-mail: einsteinpark@hanmail.net
Tel: +82-62-530-3880, Fax: +82-62-530-3889

activated sludge from the Village of Wrightstown's WWTP and a toxicity screening test was performed. The test showed that the concentrated ink solution was not toxic to activated sludge processes at the worst-case concentration. So we began to do a toxicity screening test. For toxicity screening tests, one of the best methods is using the Reserve Electron Transfer (RET) method.[1,2] In our research, the toxicity screening tests for ink-contaminated wastewater were performed using the RET assay. Harkin and Alper explained that the new test should be useful in detecting toxicants in surface and groundwater, and in sewage treatment plant effluents.[3,4] Knobeloch et al., investigated new spectrophotometer bioassay procedures that evaluate chemical toxicity.[5] Blondin et al., has recognized that many surface and groundwater samples contain substances of natural or man-made origin, which in minute amounts can exert toxic effects on humans, domesticated animals, fish, and wildlife.[6]

Until recently, the information about ink toxicity and paper was not broad enough for an in-depth discussion. So during this paper, we would like to figure out if the ink wastewater is not toxic.

Therefore, the objective of this study was to determine whether an ink sample is inhibitory to activated sludge biological treatment. This study also discusses what happens to the wastewater after ink is discharged into the sewer. Our testing shows that CEI's wastewater is not toxic. We may want to confirm this by running an additional test using Wrightstown POTW's bio-mass.

MITOSCAN ASSAY BACKGROUND

The biochemical reactions that occur in the mitochondria are vital to the survival of all eukaryotes organisms. Mitochondria contain concerted enzyme systems which are practically identical across the range of evolutionary development from the higher mammals to the simplest eukaryotes. These enzymes mediate the reactions that supply vast majority of cellular energy. Many compounds are toxicants precisely because they block or damage one or more of these enzymes or enzyme systems in the mitochondria. However, the submitochondrial particles (SMP) are also sensitive to general cellular toxicants that act by a variety of non-specific mechanisms, such as, disruption of membrane, membrane-protein, and protein-protein interactions. Blondin et al. have described two complementary protocols used to evaluate general toxicity based on the effects on different subsets of the enzyme systems in SMP.[1,5,6]

MATERIALS AND METHODS

Experiment Methods

Oxygen Uptake Tests

A typical 315 mL BOD bottle was used for the oxygen uptake rate tests. The temperature was 22℃ and barometric pressure was 29.2 mm Hg. At Madison WI, U.S., the activated sludge obtained from Nine Springs WWTP aeration basins was used for the test. The dissolved oxygen (DO) was measured with a DO meter. The calibration of the DO meter was based on the room temperature and barometric pressure.

RET Assay

Toxicity screening tests were performed using the MitoScan® technology that utilizes SMP and is based on the membrane-linked enzymes associated with cellular electron transport and oxidative phosphorylation.

The replication of the Mitochondria key biochemical processes in a controlled in-vitro environment makes rapid low cost and reliable toxicity assessment possible. These biochemical reactions are virtually identical across the entire animal kingdom. MitoScan® tests which are usually only available from whole organism provide information about toxicant disruption that impacts higher levels of biological organization.[1]

The sub-cellular processes upon which Mitochondrial reactions depend include:

- Protein - Membrane Interactions
- Protein - Protein Interactions
- Protein and Membrane Integrity

These processes are integral to all biological systems not just to mitochondria. By tracking the ability of the SMP to carry out these reactions in the presence and absence of test samples, we can assess the degree to which the sample is toxic to living systems.

Because the MitoScan® SMP are fully functional biochemically and operate as a concerted complex of membrane-based enzymes, a variety of options are available to track different aspects of these vital processes. Two such bioassay protocols using MitoScan® SMP are widely used.

The Electron Transfer rervese assay (ETr) replicates the mitochondrial biochemistry as it occurs in normally functioning cells.[2] The RET assay uses only a part of the enzyme system by adding an alternative energy source to force the electron flow in the reverse direction of normal living systems. This assay allows the user to focus on a different set of functions than the ETr assay. ETr and RET assays are summarized in Table 1. In this study, RET assay was used since this assay gave more sensitive results than ETr assay in similar previous studies. Figure 1 Shows that the RET assay reaction scheme.

The RET protocol is more biochemicallly complex, but once set up it is as simple to run as the ETr protocol. This assay monitors the rate of appearance of NADH as NAD^+ is reduced. Because this conversion is not energetically favorable, the reaction is known to be uncompleted in this direction in vivo. To run this test, three components must be provided: a source of electrons, a source of energy, and

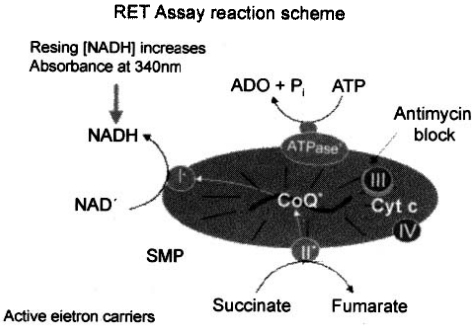

Figure 1. RET assay reaction scheme.

NAD^+. Normally, succinate is used to provide a source of electrons, while ATP is used as an activator to provide a source of energy. Upon the addition of ATP, the ATP assay enzyme (complex V) couples energy from the oxidation of ATP to ADP and uses it to drive the reduction of NAD^+ to NADH. Antimycin is used to block the pathway to Complex III, Cytochrome c, and Complex IV, thus preventing the NADH produced from being oxidized back to NAD^+.[7~10] Additionally, antimycin prevents electrons from siphoning to oxygen, thus reducing it to H_2O. As in the case of the ETr protocol, the rate of reaction is measured spectrophotometrically by taking absorbance readings at 340 nm, at timed intervals to obtain the rate of reaction.[8~10]

The RET protocol is sensitive to toxicants that inhibit Complex I, Complex II, Coenzyme Q, and ATP assay (Complex V). It is also sensitive to specific inhibitors of complexes II and V and to "uncouplers", such as 2,4-dinitrophenol.

Analytical Methods

All the analysis were performed following Standard Methods (1999)[11] and / or Hack Wastewater and Biosolids Analysis Manual (1999).[12]

Table 1. Summary of ETr and RET assays

Assay	Process monitored	Absorbance at 340 nm	Effect of reaction
Electron Transfer (ETr)	Electron transfer I > CoQ > III > Cyt C > IV	Loss of NADH decreases absorbance	Oxidation of NADH to NAD^+
Reverse Electron Transfer (RET)	Energy coupling/transfer II > CoQ > I, ATPase	Rising NADH increases absorbance	Reduction of NAD^+ to NADH

RESUTS AND DISCUSSION

Oxygen Uptake Tests

Experimental Conditions

The experimented started once the sample from Sigma Environmental Services was received, who states that the worst-case discharge would be the concentrated ink solution diluted 100 times with water from the shop sink. The Wrightstown's WWTP daily flow averages around 125,000 gpd. Since the ratio of the ink wastewater flow to average WWTP flow is 0.002 and the dilution rate is 100, the ratio of the concentrated ink solution to the total wastewater flow is 0.00002.

In order to evaluate the effect of ink on the oxygen uptake rate, a series of oxygen uptake tests were performed. The tests were performed at room temperature. The experimental conditions are shown in Table 2.

Experimental Results

The experimental results are shown in Figure 2. Run 1~4 (1,000 μL of ink) had the lowest oxygen uptake rate, followed by Run 5 (500 μL). Surprisingly, Run 1~2, which received the greatest amount of ink (2,000 μL), and Run 1~3 (10 μL) had greater oxygen uptake rate than the control. Oxygen was depleted within 13 min for all the runs, indicating almost no inhibitory effect of the ink sample tested. Since the presence of wastewater lead to high oxygen uptake rates, additional testing will be performed without wastewater. This will allow us to evaluate the effect of the ink sample on activated sludge processes more clearly.

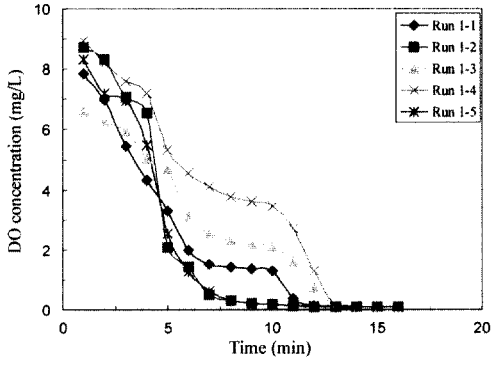

Figure 2. Oxygen uptake over time at different ink concentrations.

The United States (U.S.) Environmental Protection Agency (EPA) suggested that water-based inks are safer than solvent-based or UV-cured inks. There is greater concern related to flammability and worker health with solvent-based and UV-cured inks. Water-based inks are not toxic. However, the Clean Water Act and local water treatment permits regulate only a few of these chemicals with high aquatic toxicity. Solvent-based inks are less energy efficient than water-based or UV-cured ink systems.[13,14]

The Minnesota Pollution Control Agency (MPCA) considers paint and ink wastes to be hazardous until properly evaluated and shown to be not hazardous. Generally, ink wastes are assumed to be Toxicity Characteristic (T.C.).[15]

RET Assays

The objective of this study was to determine whether the ink sample is inhibitory to activated sludge after treated with activated sludge biomass for 10 hr.

Experimental Conditions

The operation temperature was 18±3℃. The total volume of both activated sludge biomass

Table 2. Oxygen uptake test conditions

Run No.	Activated sludge (mL)	Wastewater (mL)	Ink volume (μL)	Dilution ratio by volume (Ink/wastewater)
1-1	100	100	0 (control)	0
1-2	100	100	10	0.0001
1-3	100	100	500	0.005
1-4	100	100	1,000	0.01
1-5	100	100	2000	0.02

and wastewater in each reactor was 200 mL. The activated sludge was obtained from Nine Springs WWTP in Madison, Wisconsin in March. The wastewater sample was obtained at the Village of Wrightstown's WWTP on February 23, 2001. The wastewater was stored at 4℃ until testing. The biological treatment lasted for 20 hr. Raw wastewater samples and samples after biological treatment were filtered with 0.45 μm filters and the filtered samples stored in a 4℃ cold room for the RET assays next day. In order to avoid any possible microbial activity that may occur during the storage in a cold room, the RET assay was performed within one day. The experimental conditions of the RET assay are summarized in Table 3. For the control, Wrightstown's wastewater was used. Most lithographic inks are not classified as hazardous wastes under state and federal regulations.[16] The exception is if ink contains pigments with heavy metals (for example, cadmium, lead or chromium), or if the ink is mixed with solvents classified as hazardous wastes. Proper disposal of ink wastes can be expensive, but is necessary to meet regulatory compliance requirements and is important to minimize liabilities faced by the printer. When ink is disposed of in a landfill, waste ink must be in a non-liquid state or otherwise stabilized.[17] Whether ink can be reduced or recycled is dependent upon the quality of the waste ink that is generated. Waste ink can typically be classified in one of the following two categories: uncontaminated, excess ink or contaminated, combined ink.[17]

Experimental Results

Figure 3 shows the change of inhibitions depending on ink concentrations and duration of biological treatment. The raw data were collected and recorded by the micro plate software over time. A least-squares fit of the portion of each data series following the NADH (for the ETr assay) or ATP (for the RET assay) was conducted to determine the rate of absorbance change (slope) of each well. The % inhibition of each dilution or sample is obtained by comparing the sample's reaction rate to the control's using following formula:

$$\%Inhibition = \left(1 - \frac{Sample\ \ slope}{Control\ \ slope}\right) \times 100 \qquad (1)$$

If the slope of a sample is steeper than the control's slope or the % inhibition is negative, this indicates that the sample is less toxic than the control. The control slope is the standard

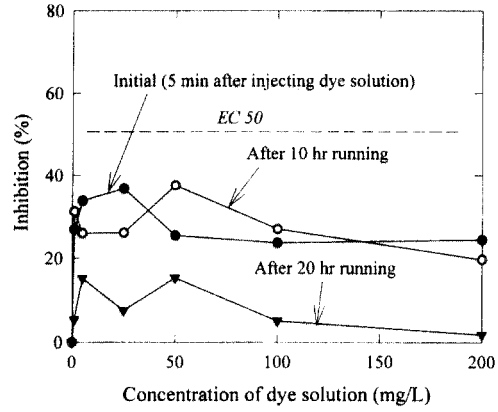

Figure 3. Inhibition of ink solution for Nine Springs WWTP activated sludge.

Table 3. Experimental conditions of the RET assay

Run No.	Activated sludge (mL)	Wastewater (mL)	Ink (mg/L)	Dilution ratio by volume (ink/wastewater)	Remarks
2-1	100	100	0	0	Control
2-2	100	100	1	0.0001	-
2-3	100	100	5	0.0005	-
2-4	100	100	25	0.0025	-
2-5	100	100	50	0.050	-
2-6	100	100	100	0.1	-
2-7	100	100	200	0.2	-

sample, which is activated sludge that is almost non toxic.

The % inhibitions of all samples were below 40%. The initial samples (5 min after injecting ink solution) had almost the same % inhibition values as the samples after 10 hr of biological treatment. However, the samples after 20 hr of biological treatment showed lower % inhibitions. Furthermore, the % inhibition values were higher at lower concentrations between 1 to 50 mg/L than at or above 100 mg/L. The % inhibitions of samples after 20 hr of biological treatment were reduced by 20% compared with samples after 10 hr. Therefore, it can be said that the ink sample tested is not readily biodegradable but can be treated when the detention time in activated sludge processes is long ($\geq$ 20 hr).

Using the RET assay, EC_{50}* values of the ink solution were obtained. Reaction rates from a series of toxicant or sample dilutions are used to calculate EC_{50}. The EC_{50} value for the MitoScan toxicity test is the concentration of the toxicant or sample that results in 50% inhibition for the enzyme activity in the MitoScan preparation. The ink solution was diluted with distilled water to achieve the concentrations of 0.98, 1.95, 3.91, 7.81, 15.63, 31.25, 62.5, 125, 250, 500, and 1,000 mg/L, respectively. The control was the distilled water. The slope of RET assay was obtained using data points between 6 to 14 min. Figure 4 shows the change of absorbance at 340 nm due to the rate of appearance of NADH when NAD^+ is reduced. At 1,000 mg/L (the highest concentration), the % inhibition was 93%. When the ink was further diluted to 500 mg/L, the % inhibition decreased sharply to 67%. When the ink solution was diluted further, the % inhibition decreased continuously.

Figure 5 showed that after calculating the inhibition of each concentration, the EC_{50} was obtained by interpolating data points. The EC_{50} of the ink solution was found to be 312 mg/L. Most dyes used in textile industries had the

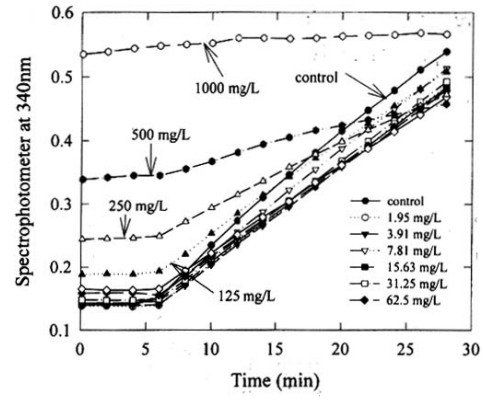

Figure 4. RET results for EC_{50} analysis of ink solution.

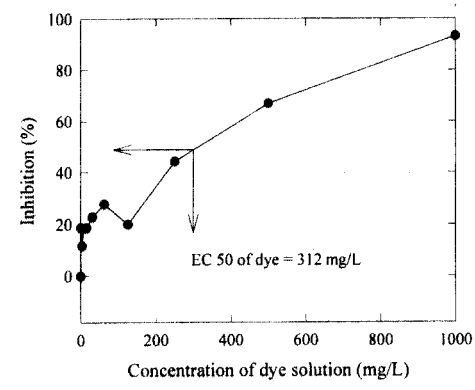

Figure 5. EC_{50} of ink solution.

EC_{50} values ranging from 3.3 mg/L for Rubine 5BLF to 34.8 mg/L for Blue LGGL.

CONCLUSIONS

From oxygen uptake tests and RET assays, the following conclusions can be drawn : According to DO results, oxygen was depleted within 13 min for all the runs, indicating almost no inhibitory effect of the ink sample tested. Run 1~4 (1,000 μL of ink) had the lowest oxygen uptake rate, followed by Run 5 (500 μL). Run 1~2, which received the greatest amount of ink (2,000 μL), and Run 1~3 (10 μL) had greater oxygen uptake rate than the control. It is not necessary to acclimate activated sludge for the

*Concentration of sample that inhibits the SMP activity 50% ; used as a benchmark for comparing the toxicity of different samples.

treatment of ink-containing wastewater. The samples after 10 hr of biological treatment had almost identical % inhibition values as the initial samples. However, the samples after 20 hr of biological treatment showed lower % inhibitions. Although the ink sample tested may not be readily biodegradable it can be treated when the detention time in activated sludge processes is long ($\geq$ 20 hr). The EC_{50} value of the sample was much lower than those found for the dyes utilized in textile industries since the SMP activity 50% was 312 mg/L.

The process upset at the Village of WWTP may not be caused by the discharge of the ink sample tested. There may be a synergistic effect of various ink solutions used in CEI on the performance of the activated sludge process at the Village of Wrightstown. Furthermore, if the domestic sewage flow rate is low but the CEI's wastewater flow rate is high, it is possible that an upset may occur. The changes in the flow rates of both domestic and CEI wastewaters along with organic loading must be evaluated as well.

REFERENCES

1. http://www.mitoscan.com
2. Chance B., and Hollunger G., "Succinate-Linked PuriDine Nucleotide Reduction in Mitochondria," *Fed. Proc.* **16**, 163~172 (1957).
3. Harkin, J. M., "Cell's Energy Factory Detects Toxic Substances," *Chemical week*, August 27, 37~38 (1986).
4. Harkin, J. M. and Joseph, A., "A Faster, Cheaper Assay for Toxins," *Chemical Week*, October 28, 40~41 (1987).
5. Knobeloch, L. M., Blondin, G. A., Read, H. W., and Harkin, J. M., "Assessment of Chemical Toxicity Using Mammalian Mitochondrial Electron Transport Particles," *Arch. Environ. Contam. Toxicol.* **19**, 828~835 (1990).
6. Blondin, G. A., Knobeloch, L., and Harkin, J. M., "Bioassay of Toxic Substances in Water," *Wisconsin Academy Review*, **32**(1), 31~33 (1985).
7. Knobeloch, L. M., Blondin, G. A., and Harkin, J. M., "Use of Submitochondrial Particles for Prediction of Chemical Toxicity in Man," *Bull. Environ. Contam. Toxicol.* **44**, 661~668 (1990).
8. Blondin, G. A., Knobeloch, L. M., Read, H. W., and Harkin, J. M., "Mammalian Mitochondria as in Vitro Monitors of Water Quality," *Bull. Environ. Contam. Toxicol.* **38**, 467~474 (1987).
9. Blondin, G. A., Knobeloch, L. M., Read, H. W., and Harkin, J. M., "Rapid in Vitro Evaluation of Chemical Toxicity using Mammalian Submitochondrial Particles," Abstracts, Envirotech Vienna (1989a).
10. Blondin, G. A., Knobeloch, L. M., Read, H. W., and Harkin, J. M., "An in Vitro-mitochondrial Bioassay for Predicting Acute Toxicity in Fish," Aquatic Toxicology and Environmental Fate. ASTM-STP-1007, **11**, 551~563 (1989b).
11. Standard Methods, For the Examination of Water and Wastewater, 20th ed., pp. 8-14~8-19 (1998).
12. Hack Water Analysis Handbook, 3rd ed., pp. 897~898 (1997).
13. http://www.flexography.org/flexo/0200/022000a.cfm
14. http://www.sgia.org/memino/regula.html
15. http://www.pca.state.mn.us/waste/pubs/4-40.pdf
16. http://www.flintink.com/domino/envirotk.nsf/a26ee3fb95e18886852564d600, Summer (1998).
17. http://www.uwex.edu./shwec/pubs/pdf/lithoink.pdf