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Monitoring phenol degrading *Candida* and bacterial pathogens in sewage treatment plant

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ABSTRACT

The fate and seasonal variation of several microbial pathogens (MPs), including *Salmonella* spp. (SS), *Escherichia coli* O157:H7 (EC), *Listeria monocytogenes* (LM), *Staphylococcus aureus* (SA), biomarker bacteria, and *Candida* spp. (CS) were investigated in a municipal sewage treatment plant (MSTP) located in Zagazig City, Egypt, employing an anaerobic/anoxic/oxic (A/A/O) process to monitor their incidences in both influent and effluent throughout the seasons of 2011. Enhancing the activity of *Candida* populations and the bacterial biodegradation activities in the anaerobic–anoxic–oxic process is an axial pathway for the removal of phenol. In summer season, phenol degradation in MSTP was about 85% which was higher than that in winter season (60%). The chemical treatments routinely used in MSTP can effectively reduce 70% of MPs in wastewater in summer and more than 80% in winter. The concentrations of microbial populations in the effluent were much higher in summer and spring than in winter and autumn, which was closely related to degradation of phenol. Therefore, this study may raise a particular concern regarding the removal of MP and phenol from wastewater in summer seasons.

Keywords: Bacterial pathogens; Phenol; *Candida*; Sewage treatment plant; A/A/O

1. Introduction

In most wastewater treatment systems, any pathogen removal that occurs is a fortuitous by-product of the principal design objective (usually organic carbon removal). A biological wastewater treatment system contains many types of micro-organisms, such as bacteria, protozoa, fungi, metazoan, viruses, and algae, while bacteria comprise approximately 95% of the total microbial population [1] and play a key role in the purification of water quality. Secondary treatment is one of the key components of a wastewater treatment plant. It involves the biological reduction of biochemical oxygen demand, suspended solids, and toxicity of industrial wastewaters and the production of low nutrient, environmentally benign outgoing effluent. Effluent also has been shown to reduce the incidence of toxic chemicals and pathogenic bacteria [2,3]. These functions are carried out by the resident microbial community which is considered the foundation of the secondary treatment process. Reclaiming

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and reusing wastewater before thorough treatment to reduce the concentrations of waterborne pathogens e.g. helminthes, protozoa, fungi bacteria, and viruses poses a health risk and great concern [4–9]. Anaerobic/anoxic/oxic (A/A/O) is the most common and widely spread strategy that eliminate pathogenic bacteria in wastewater through an A/A/O process for specific time intervals. It has the objective of minimizing health hazards and the risk arising from using untreated wastewater contaminated with endocrine-disrupting chemicals [3]. The concentrations of several typical endocrine-disrupting chemicals in the effluent and return sludge in a municipal treatment plant were much higher in winter and spring than in summer and autumn, which was closely related to the microbial activity [3].

Microbial indicators e.g. fecal coliforms are used to track contamination sources and the microbial quality of reclaimed water [5,10,11]. Coliforms and fecal coliforms concentrations in untreated municipal wastewater are generally 10^4–10^6 CFU/mL [11]. In particular, enterohemorrhagic *Escherichia coli* O157:H7, a pathogen known for causing hemolytic colitis and hemolytic uremic syndrome, has been isolated from both untreated and treated water [12–15]. *Salmonella* has been found in 55% of treated sewage sludge samples and *Listeria monocytogenes* in 12% of raw sludge samples from municipal sewage treatment process, whereas the survival of these pathogens is higher in conventional treatments such as sedimentation and mesophilic aerobic digestion than in thermophilic anaerobic digestion [2]. The presence of pathogens in untreated sewage suggests that it could be a reservoir for pathogens in the environment and a health risk since treatment does not eliminate all organisms [15]. Furthermore, new waterborne pathogens are continuing to emerge due to changing population demographics, globalization of world trade, and travel [16]. So, disinfection is necessary to control microbial health risks in reclaimed water. Among the many kinds of wastewater disinfection, chlorination has gained wide acceptance commercially, because of its simple application and moderate cost [17] and despite the potential problems associated with harmful disinfection by-products generated by this treatment [18,19].

Phenol is one of the most common pollutants found in various industrial wastewaters as it is a basic structural unit of a variety of synthetic organic compounds including agricultural chemicals as well as pesticides [20,21]. Thus, the use of micro-organisms that are able to metabolize phenol can be considered as one of the cheapest and safer alternative approach for the removal of phenol from wastewaters [22]. The conventional biological treatment methods using activated sludge or anaerobic cultures have been used for the removal of high concentration of phenolic compounds from wastewaters [23]. Micro-organisms such as *Aspergillus*, *Candida*, *Trichosporon*, *Paenibacillus thiaminolyticus*, and *Bacillus* [23–25] have the ability to degrade and use phenol as a source of carbon. In order to monitor the advantages of native microbial residents, it is important to better understand the biological agents associated with this system. Consequently, this study was to investigate the fate and seasonal variation of several microbial pathogens (MPs), e.g. *Salmonella* spp., *E. coli* O157:H7, *L. monocytogenes*, *Staphylococcus aureus*, biomarker bacteria (coliforms and faecal coliforms bacteria), and *Candida* spp., along different treatment processes of a municipal sewage treatment plant (MSTP) located in Zagazig City, Egypt, throughout the year 2011. The examined MSTP adopted an anaerobic/anoxic/oxic process as the core treatment unit.

2. Materials and methods

2.1. Sample collection

The sewage water samples were collected from a MSTP located in Zagazig City, Egypt, employing

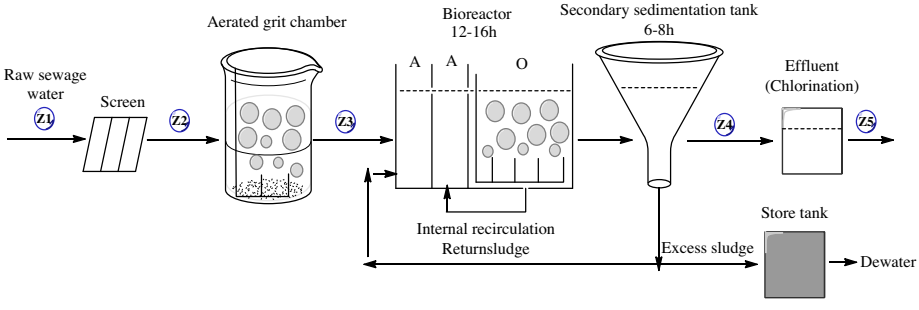

Fig. 1. Sampling points in each treatment stage from MSTP adopted an anaerobic/anoxic/oxic (A/A/O) process.

A/A/O system during seasons of 2011. Fig. 1 shows the sampling points at five sites (Z1–Z5) from untreated wastewater up to treated wastewater samples. At each sampling site, three samples were separately taken for microbiological and physicochemical analyses. After collection, all samples were stored in amber glass bottles (1 L) and then taken back to the laboratory for analysis. The sewage water samples were taken randomly to a depth of 5–10 cm. The samples were collected in four seasons on January and February (winter), April and May (spring), July and August (summer), and October and November (autumn) throughout 2011. The samples were placed in a container filled with ice, then transported to the microbiological laboratory, and stored at 4˚C prior to analysis.

2.2. Microbiological analysis

All water samples were diluted by serial tenfold dilutions in sterile buffered peptone water (peptone 1 g/L, pH 7.4). Total bacterial counts (TBC) were counted in nutrient agar (NA) after diluting 1.0 mL of sample into 10 mL NA (peptone: 10 g/L, beef extract: 3 g/L, NaCl: 5 g/L, agar: 15 g/L, pH 7.2) and incubated at 30˚C for 48 h [26].

For total coliforms counts (TCFC) enumerations, 1.0 mL from dilution sample was poured in sterile Petri dish then poured 10 mL of Violet Red Bile Dextrose Agar (Biolife 402188). After solidifying media, a 10 mL overlay of the same molten medium was added. The incubation was carried out at 37˚C for 24 h. For total fecal coliforms counts (TFCFC), the outline method 1680: Fecal Coliforms in sewage water by multiple-tube fermentation using Lauryl Tryptose Broth (LTB) and EC Medium was used. Total *Candida* counts (TCC) were counted on *Candida* Agar (Biolife, 4012802, Milano, Italy) by spreading 0.1 mL of sample onto media and incubated at 37˚C for 48 h. All plates were examined for typical colony types and morphological characteristics associated to each culture medium. Total staphylococci were counted onto Baird Parker agar (Biolife, 401116 supplemented with egg yolk) incubated at 37˚C for 48 h. Presumptive *S. aureus* colonies were further tested for positive coagulase reaction (Bactident Coagulase Biolife) and the colonies were confirmed genotypically, for instance *nuc* gen using PCR. *E. coli* were enumerated on Harlequin Tryptone Bile X-Glucuronide agar (TBX, LAB HAL003) incubated at 44˚C for 24 h. Particularly, presumptive *E. coli* O157:H7 on TBX agar was incubated at 37˚C for 4 h then incubated at 44˚C for 37 h [27]. *Listeria* spp. was enumerated on Polymyxin-Acriflavin-Lithium chloride-Ceftazidime-Aesculin-Mannitol agar (PALCAM, Biolife 401604) after incubation for 48 h at 35˚C. Confirmation of five presumptive *L. monocytogenes* colonies was further tested on horse blood agar. *Salmonella* was counted on Xylose Lysine Deoxycholate agar (XLD) (Merck, 1.05287) after incubation for 24 h at 37˚C. *Salmonella*, *L. monocytogenes*, and *E. coli* O157:H7 colonies were counted after incubation and confirmation biochemically then were verified genotypically according to the Kawasaki et al. [28] and AOAC International [29].

2.3. Physicochemical analysis

In order to determine phenol concentration in raw and treated sewage water, the samples were centrifuged at 5.000× g for 10 min at 4˚C using a JOUAN MR 1822 centrifuge (France) to remove microbial biomass and organic matter then, the supernatant collected was used for phenol quantification according to http://www.cpcb.nic.in/upload/Latest/Latest_67_guidemanualwandwwanalysis.pdf. In brief, phenol quantification was done by taking 50 mL of each sample followed by the addition of 0.3 mL of 4-aminoantipyrine (2%), 1 mL of ammonium hydroxide (2.0 N), and 1 mL of 2% potassium ferricyanide solution and mixed thoroughly. The developed reddish color was quantified by measuring the absorbance at 460 nm at every sampling point during all seasons using JENWAY 6405 UK, UV–vis spectrophotometer, and the phenol degradation was quantified by comparing results with the phenol standard curve. The sewage water quality parameters e.g. chemical oxygen demand (COD), total nitrogen (TN), and total phosphorus (TP) were determined with Hach test kits (Hach DR5000, USA) using JENWAY 6405 UK, UV–vis spectrophotometer.

2.4. Statistical analysis

All analyses were performed in three replicates. The results were expressed by the mean of the two experiments plus the standard error. Data were statistically analyzed using ANOVA through the general linear models procedure of the statistical analysis system software (SAS version 9.1, SAS Institute, Inc., 2003). Least significant differences were used to separate means at $p < 0.05$. The correlation coefficient (r) between the microbial counts (TBC and TCC) and some chemical variables (phenol, TN, and TP) was calculated using Microsoft Office Excel 2007.

3. Results and discussion

3.1. Bacterial pathogens and indicators in wastewater

Table 1 shows the pathogens e.g. *E. coli* O157:H7 (EC), *Salmonella* spp. (SS), *L. monocytogenes* (LM), and *S. aureus* (SA) found in a MSTP at Zagazig City, Egypt, during different seasons in 2011. The SS, LM, and SA were the most common pathogens in both influent and effluent amongst the pathogens. The EC was the lowest prevalence in treated effluent with incidence 11% in all seasons and 6% in winter. PCR resulted in a five different genotype out of 65 isolates of EC from the untreated and treated sewage (data will be published elsewhere). This indicated that this pathogen is able to pass sewage treatment processes and survive in the environment. The presence of this bacterium in untreated sewage could be a reservoir for pathogens in the environment and a health risk since treatment does not eliminate all pathogens [15]. The MSTP can effectively reduce about 78% and 80% from this pathogen in treated sewage in summer and winter, respectively. The SS was found in 22, 33, 17, and 17% of the samples from treated effluent in summer, autumn, spring, and winter, respectively. *Salmonella* was isolated from treated sludge in municipal sewage treatment by sedimentation system [2]. The finding of this bacterium in treated effluent should not be neglected because the survival of this pathogen in environments is long [30,31]. The MSTP can effectively reduce about 73% in summer and 78% in winter from SS in treated effluent. *Listeria* was found in 17, 38, 17, and 17% of the samples from treated effluent in summer, autumn, winter, and spring, respectively. The highest prevalence of this pathogen was in autumn compared with the other seasons. One explanation for the difference may be the higher number of population from this bacterium in raw sewage and also the processes of treatment were not efficient to eliminate this pathogen. Another explanation is that the concentration of chlorine was not enough to disinfect water. *L. monocytogenes* is omnipresent in various environments such as soil, decaying vegetation, water, pasture, and may be part of the normal intestinal microflora of man and animals [32]. The MSTP can effectively reduce 73 and 75% of LM from treated effluent in summer and spring seasons while reduced only 30 and 63% from this pathogen in autumn and winter seasons, respectively. The SA was found in 28, 22, 11, and 22% of the samples from treated effluent during summer, autumn, winter, and spring, respectively. These results indicated that the MSTP can effectively reduce about 72 and 83% from SA during summer and winter, respectively. Thus, the chemical treatments routinely used in MSTP can effectively reduce more than 70% of MPs in wastewater in summer and approximately 80% in winter. From this study, it is evident that sedimentation and chlorination employed by MSTP were insufficient to exclude the pathogens from the treated wastewater and the current system should be further improved and complemented. The results indicated that these pathogen levels in treated water, as presented in this study, are spread on river or land, increases the environmental load of pathogens and might be risky for food chain of humans and animals, in particular, enterohemorrhagic *E. coli* O157:H7, which has been isolated from both untreated and treated water [15]. The occurrence of pathogens in treated wastewater increase the risk of epidemics among animals and human beings handling such wastewater [9]. So, treated sewage water produced in Zagazig, Egypt, MSTP did not have a quality that fulfils criteria for unrestricted use in agriculture. These results support the suggestion that those pathogens might be transported to food chain through treated sewage [33].

Table 1
Incidence (%) of bacterial pathogens in MSTP during different seasons in 2011 from raw and treated wastewater

Pathogens	Raw sewage (18 samples at each season)				Treated water (18 samples at each season)			
	Summer	Autumn	Winter	Spring	Summer	Autumn	Winter	Spring
L. monocytogenes	11(61.1)	10(55.5)	8(44.4)	12(66.6)	3(16.6)	7(38.3)	3(16.7)	3(16.7)
RE%					72.7	30.0	62.5	75.0
Salmonella spp.	15(83.3)	12(66.6)	13(72.2)	13(72.2)	4(22.2)	6(33.3)	3(16.7)	3(16.7)
RE%					73.3	50.3	76.9	76.9
S. aureus	17(94.4)	11(61.1)	12(66.7)	14(77.7)	5(27.8)	4(22.2)	2(11.1)	4(22.2)
RE%					72.2	63.3	83.2	71.4
E.coli O157:H7	9(50.0)	8(44.4)	5(27.2)	8(44.4)	2(11.1)	2(11.1)	1(5.6)	2(11.1)
RE%					77.8	77.8	80.0	75.0

Notes: RE%, Removal efficiency, Number of sewage influent positive sample/Number of treated sewage effluent positive sample/Number of sewage influent positive sample × 100.

Fig. 2 shows that the concentration of the TBC, biomarker bacteria (TCFC and TFCFC), and TCC was much high in Z1, Z2, and Z3 compared with Z4 and Z5 points in all seasons during 2011. The level of TBC in influent wastewater during summer reached 8.32, 8.69, and 9.47 log CFU/mL at Z1, Z2, and Z3, respectively, which was two times higher than Z5. However, in winter, the level of TBC in influent wastewater reached 6.64, 6.82, and 6.88 log CFU/mL at Z1, Z2, and Z3, respectively, which was three times higher than Z5. In Egypt, the ambient temperature usually drops below 12˚C in winter, so the temperature of wastewater in municipal sewers is only about 18˚C. The low temperature could bring down the activity of microbial growth or reduces the bacterial populations. Under this condition, the bacterial groups could not multiply effectively during transport in bioreactor system. It was observed that the influent wastewater presented mean concentrations of TCFC and TFCFC higher than those usually reported in the literature for these bacteria in domestic wastewater [11]. While in winter and autumn, the levels of TCFC and TFCFC in sewage effluent decreased markedly and were detected by plating count in the originate samples without dilution. It shows clearly that the elimination of TCFC and TFCFC has a significant seasonal fluctuation in the MSTP. The chlorine and chlorine-based compounds are still the most widely used and effective to disinfect wastewater [17]. The populations of TCFC and TFCFC could be effectively reduced by the A/A/O process during the most seasons. Other correlations were notable between indicators (coliforms and fecal coliforms) and pathogens ($R = 0.79$) (Data not show).The reduced efficiency of TCFC and TFCFC from effluent varied between 70 and 80% in summer and winter, respectively. Enteric bacteria can remain viable in low temperature for prolonged periods [34]. Almost un-removal and inkling micro-organisms would be discharged into the receiving water body in summer and spring seasons thus, probably contaminating aquatic ecosystems by waterborne pathogens [16]. The *Candida* population in influent and effluent was less affected after conventional treatment during bioreactor system and showed slight reduction during the whole period of study (Fig. 2). *Candida* spp. was more resistant to chlorine and no significant differences ($p < 0.05$) were observed between Z1 and Z5 in most seasons except in summer because in summer the population of *Candida* reached 4–4.5 log CFU/mL in all sample points and decreased significantly to reach 1.85 log CFU/mL. So the higher population of *Candida* may be responsible for degrading phenol from wastewater in summer than in other seasons and *Candida* has the ability to use phenol as a source of carbon [23].

3.2. Degradation of phenol in wastewater

Table 2 shows the degradation of phenol, COD, TN, and TP in influent and effluent during 2011. The degradation was variable at each of the five sites (Z1, Z2, Z3, Z4, and Z5). No degradation was observed at Z1, Z2, and Z3 after aerated girt chamber. While after secondary sedimentation tank (Z4), the degradation of these parameters was significant compared with other points. Chemical parameters e.g. phenol, COD, TN, and TP were within the expected ranges in effluent wastewater. The overall average pH value was at near

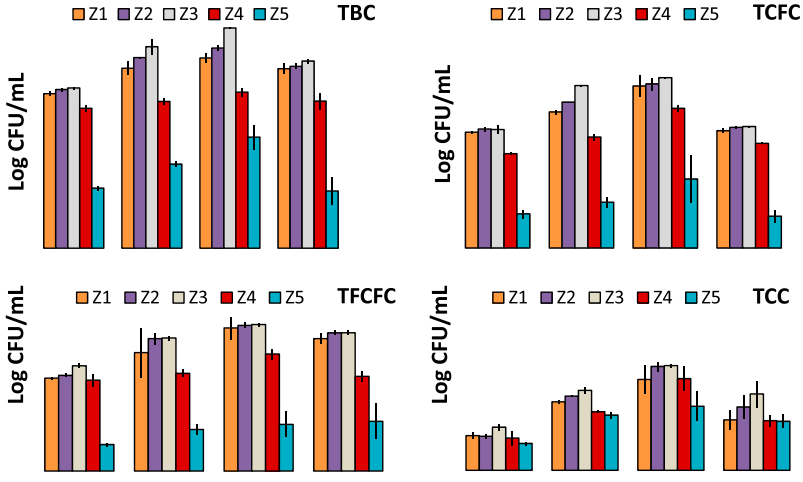

Fig. 2. Seasonal variation of TBC, TCFC, TFCFC, and total *Candida* spp. counts (TCC) in MSTP along different points from bioreactor process during different seasons in 2011.

Table 2
Concentrations of phenol, TN, TP, and COD through different sewage water treatment process along different seasons in 2011 (mean (SD) $n = 6$)

Season	Sampling points	Wastewater characteristics (mg/L)			
		Phenol	COD	TN	TP
Winter	Z1	5.69 ± 0.48a	209 ± 0.42a	56 ± 0.21a	2.77 ± 0.12a
	Z2	5.45 ± 0.26a	201 ± 0.33a	55 ± 0.23a	2.56 ± 0.41a
	Z3	5.30 ± 0.22a	200 ± 0.21a	55 ± 0.32a	2.23 ± 0.32a
	Z4	2.27 ± 0.21b	19 ± 0.37b	20 ± 0.32b	1.22 ± 0.32b
	Z5	ND	ND	ND	ND
Removal efficiency[a] (%)		60.01	90.90	64.28	56.32
Spring	Z1	6.79 ± 0.25a	246 ± 0.41a	59 ± 0.23a	2.88 ± 0.32a
	Z2	6.69 ± 0.17a	232 ± 0.21ab	53 ± 0.21ab	2.63 ± 0.11a
	Z3	6.36 ± 0.11a	219 ± 0.11ab	52 ± 0.11a	2.31 ± 0.23a
	Z4	2.17 ± 0.13b	17 ± 0.31b	16 ± 0.32b	1.31 ± 0.23b
	Z5	ND	ND	ND	ND
Removal efficiency[a] (%)		68.04	93.08	76.78	54.51
Summer	Z1	10.80 ± 0.20a	250 ± 0.34a	62 ± 0.17a	2.87 ± 0.12a
	Z2	9.59 ± 0.21ab	224 ± 0.33ab	64 ± 0.31a	2.67 ± 0.22a
	Z3	8.89 ± 0.05ab	220 ± 0.23ab	57 ± 0.21a	2.34 ± 0.27a
	Z4	1.60 ± 0.26c	16 ± 0.22b	12 ± 0.31b	1.12 ± 0.23b
	Z5	ND	ND	ND	ND
Removal efficiency[a] (%)		85.20	93.60	80.64	60.97
Autumn	Z1	9.27 ± 0.29a	231 ± 0.43a	61 ± 0.47a	2.85 ± 0.25a
	Z2	6.28 ± 0.15ab	218 ± 0.32a	62 ± 0.34a	2.68 ± 0.26a
	Z3	6.51 ± 0.37ab	218 ± 0.38a	56 ± 0.42a	2.32 ± 0.22a
	Z4	2.23 ± 0.17c	15 ± 0.41b	16 ± 0.42b	1.32 ± 0.22b
	Z5	ND	ND	ND	ND
Removal efficiency[a] (%)		75.94	93.50	73.77	53.68

[a]Removal efficiency = (Z1–Z4)/Z1 × 100, ND, not determined, a, b, c, means in the same row with different superscript differ significantly ($p < 0.05$).

neutral, 7.4 (data not shown). Water temperature (25.5˚C) decreased an average of 8˚C from summer to winter (data not shown). Likewise, the temperature during different seasons is supporting the conclusion that most of the chemical changes along fate of influent up to effluent originate from the activities of the microbial resident [1]. Concomitantly, the phenol, COD, TN, and TP decreased ($p < 0.05$) significantly from 10.80, 250, 62, and 2.87 mg/L at Z1 to 1.60, 16, 12, and 1.12 mg/L after passing secondary sedimentation tank. Degradation rate of phenol and other chemicals at MSTP showed a good performance leading to the removal of phenol and other chemicals. It was seam dependent on following the next order: summer > autumn > spring > winter. The efficient removal of phenol, COD, TP, and TN was higher in summer than in the other seasons when the resident microbiota was most active [23]. The increase in degradation of phenol in influent is mainly due to the use of phenol as a source of carbon and energy by a variety of bacteria and yeasts [23,25]. The removal efficiency by MSTP of phenol, COD, TN, and TP from treated water was 85, 94, 81, and 61% in summer, 76, 94, 74, and 54% in autumn, 68, 93, 77, and 55% in spring, and 60, 91, 64, and 56% in winter, respectively. The degradation efficiency of a number of different phenols had been shown to be correlated to the process temperature and higher degradation efficiency is observed at mesophilic process temperature than at thermophilic temperature [35]. However, it should be pointed out that when comparing microbial groups and phenol degradation, the strongest correlations were observed between total bacterial count and phenol removal ($R = 0.84$), and total *Candida* count and phenol degradation ($R = 0.77$) (Data not shown).

4. Conclusions

The present paper demonstrates that effluent wastewater produced in Zagazig (Egypt), MSTP did not have a quality to use in agriculture. The chemical treatments routinely used in MSTP can effectively reduce 70% of MPs in wastewater in summer and approximately 80% in winter. In order to typically

remove pathogens from MSTP by chemical agents (e.g. chlorination) and mechanical means (e.g. sedimentation), a better monitoring system for the critical control points is recommended. The wastewater degradation rate at MSTP had a good performance and beneficial removal of phenol and other chemicals based on season temperature.

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